Studies on the glucose family phosphotransferases of *Clostridium beijerinckii*

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Abstract

Revival of the ABE fermentation will be enhanced by the ability of bacterial strains to utilise cheap, renewable substrates containing a range of fermentable carbohydrates. Development of an effective process will, however, depend on a detailed understanding of the mechanisms of uptake and metabolism of the available sugars. The predominant mechanism for uptake of sugars and sugar derivatives in the clostridia is the phosphoenolpyruvate (PEP) - dependent phosphotransferase system (PTS), which not only catalyses the concurrent uptake and phosphorylation of its substrate but also plays a central role in regulation of carbohydrate metabolism.

Complete characterization of the PTS in the solventogenic clostridia will therefore be instrumental in developing strategies for constructing effective fermentation strains. The *Clostridium beijerinckii* 8052 genome encodes 43 complete phosphotransferase systems, including sixteen belonging to the glucose-glucoside family. Three of the PTSs are members of the glucose subgroup in a phylogenetic branch, and might therefore transport glucose. Since glucose has been shown to repress utilization of other sugars by *Clostridium beijerinckii,* these systems could also potentially be involved in glucose sensing and carbon catabolite repression (CCR).

The *cbei* 0751 gene encoding a IICBA PTS permease was amplified by PCR, and cloned into *Escherichia coli* ZSC113, a mutant which cannot take up and phosphorylate glucose and mannose. Transformants showed a positive fermentation phenotype for glucose and mannose. Extracts showed glucose PTS activity, and *cbei* 0751 was therefore shown to be a functional glucose PTS. The activity was inhibited by mannose confirming that the system also recognises mannose as a substrate. The expression of this gene appeared to be constitutive although quantitative expression was not performed. Similar experiments were used to investigate the function of a second system encoded by *cbei* 4983 (IICB) and *cbei* 4982 (IIA). Although these genes were successfully cloned, their function could not be identified. Since the *cbei* 4984 gene encodes a putative glycoside hydrolase, this suggests that the primary function of this PTS may be to transport and phosphorylate a disaccharide, but further experimental analysis is required to identify the substrate of this system. Attempts to inactivate the two phosphotransferases to examine the effect on the cells were not successful.

With my love and humility

I dedicate this work to my children

Khadija, Ahmed, Al-mostafa, Aisha and beloved my wife

for their patience, support, and encouragement.

Mohemed, 2014

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List of abbreviations

A	adenine
ABC	ATP binding cassette
ATP	adenosine 5´-triphosphate
BLAST	Basic Local Alignment Search Tool
bp	base pair
C	cytosine
°C	degree celsius
cm	centimetre
CRP	cAMP receptor protein
DEPC	diethylepyrocarbonate
DTT	dithiothreitol
dH_2O	distilled water
dNTP	deoxyribonucleoside 5- triphosphate
EDTA	ethylene diamine tetraacetic acid
g	gravitational force
G	guanine
KD	kilodalton
KF	potassium fluoride
M	molar
mg	milligram
ml	millilitre
μl	microliter
mM	millimolar

mm	millimeter
mRNA	messenger RNA
ng	nanogramm
nm	nanometre
OD	optical density
RNase	ribonuclease
rpm	revolutions per minute
T	thymine
TAE	Tris-acetate-EDTA
Tris	2-amino-2 hydroxymethylpropane-1.3diol
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume

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CHAPTER 1

GENERAL INTRODUCTION

1. Introduction

The acetone, butanol, ethanol (ABE) fermentation catalysed by the solventogenic clostridia will be enhanced by the ability of bacterial strains to utilise cheap, renewable substrates containing a range of fermentable carbohydrates. Development of an effective process will, however, depend on a detailed understanding of the mechanisms of uptake and metabolism of the available sugars, which also play an important role in regulation of carbohydrate metabolism. In general glucose is the dominant sugar which represses the metabolism of alternative carbon sources. The clostridial phosphoenolpyruvate(PEP) dependent phosphotransferase system (PTS) is a widespread mechanism for uptake of substrates, which involves a group translocation in which the carbohydrate is phosphorylated as it crosses the membrane. The PTS consists of a multi-protein phosphoryl transfer chain that passes phosphate from the PEP to the carbohydrate which is phosphorylated as it enters the cell. The PTS also plays an important role in bacteria carbon catabolite repression (CCR). The solventogenic clostridia exhibit the phenomenon of CCR in which expression of genes concerned with sugar uptake and metabolism is repressed by glucose. Understanding the mechanism of CCR will be useful in formulating strategies for manipulation of the bacteria to maximise the rate of conversion of carbohydrate mixtures to solvent. A range of sugars and polymers are important as potential substrates for the ABE fermentation, and so uptake and metabolism should be investigated at genetic and biochemical levels. Waste materials from different sources containing different fermentable substrates including glucose, mannose, sucrose and maltose can potentially be exploited as renewable raw materials substrates for the industrial solventogenic process.

1.1. Carbohydrates

Carbohydrates are organic compounds composed of carbon, hydrogen and oxygen (in the ratio 1:2:1, CH_2O) and are divided into groups such as monosaccharides, disaccharides, oligosaccharides and high molecular weight polysaccharides. Carbohydrates are considered a very important energy source for humans, animals and microorganisms and also are used to store energy in the form of polysaccharides. Carbohydrates are important in food science particularly as complex carbohydrates found in bread and pasta, or simple carbohydrates such as glucose found in jams or desserts (Bockov *et al*., 1991).

1.1.1. Monosaccharides

The most important natural monosaccharides are simple sugars which most often contain five or six carbon atoms carrying hydroxyls and also contain an aldehyde or ketone group. Examples are glucose, mannose, galactose, and sorbose. In addition there are amino sugars considered as monosaccharides, in which a hydroxyl group is replaced by an amino group such as in N-acetylglucosamine, N-acetylgalactosamine, D-glucosamine, D-mannosamine, D-galactosamine and fucosamine (Robyt, 1998, Macek *et al.*, 2011). Also the sugar alcohols mannitol, glucitol and galactitol are considered to be monosaccharides (Stoker, 2012, Cummings and Stephen, 2007). Metabolism of these sugars is very important in the cell metabolism to release energy (Miljkovic, 2010, Hames and Hooper, 2006).

1.1.2. Disaccharides

Disaccharides are formed by two units of simple sugar which are linked by a covalent glycosidic bond. Sucrose is a common disaccharide composed of glucose and fructose joined by an $\alpha(1-2)$ glycosidic bond, and is found in all plants. Another important disaccharide is maltose, consisting of two glucose molecules linked by an $\alpha(1-4)$ glycosidic bond. Maltose occurs as a product of starch degradation by amylase. Lactose is the main sugar component in milk and consists of glucose and galactose joined by a $\beta(1-4)$ glycosidic bond. Cellobiose is plant sugar belong to the group of carbohydrates called β-glucosides and is composed of two $\beta(1-4)$ linked glucose residues and is the most abundant β-glucoside (Rajvaidya and Markandey, 2005).

1.1.3. Oligosaccharides

Oligosaccharides are composed of several units (from 3-10) of simple sugars joined together by covalent glycosidic bonds. They are commonly found in food, mammalian tissues, and vegetables such as legumes, wheat, and have various applications in the food and medical industry. Examples are trisaccharides like raffinose, tetrasaccharides such as stachyose, pentasaccharides like verbascose, and hexasaccharides such as ajugose (Hames and Hooper, 2006).

1.1.4. Polysaccharides

Polysaccharides are long chains of monosaccharides which may contain hundreds or thousands of units linked together by covalent bonds. Polysaccharides found in nature are classified into three groups. Water-binding polysaccharides prevent tissues and cells from drying, structural polysaccharides provide a mechanism of stability to an organism, and reserve polysaccharides are storage compounds which release simple sugars as required. Polysaccharides include starch, glycogen, cellulose, and chitin.

Starch is a polysaccharide plant storage compound found in the chloroplasts in leaves and in seeds, tubers, and fruits. Cereal grains contain approximately 75 % starch (Koolman and Röehm, 2005). Starch consists of two polysaccharides, amylopectin and amylose. Amylose consists of glucose units linked α(1- 4), it may have a molecular weight from thousands to half a million. Amylopectin is a branched polymer, found in starches from for example potato, rice, and wheat and often makes up between 70- 80 % of the starch content (BeMiller and Whistler, 2009).

The main storage polysaccharide in animals is glycogen. Glycogen is a highly branched polymer composed of glucose chains linked by α(1- 4) bonds, similar to starch, and it's function is energy storage by muscle and liver.

Cellulose is the main component in plant cell walls (Garrett and Grisham, 2010). It is a linear polymer of glucose molecules up to 10^4 units long, and can reach lengths of 6-8 μm. The glucose residues are joined by β(1-4) glycoside bonds.

Chitin is a polymer of N-acetylglucosamine linked by β(1-4) bonds. It is the second most abundant carbohydrate in the environment after cellulose, being present in the cell walls of fungi and in the exoskeletons of insects, crustaceans, and spiders (Young *et al*., 2005).

A full range of carbohydrates is available in different renewable and waste materials that could support a solventogenic fermentation process. The ability of the fermenting bacteria to utilise these materials should therefore be examined and optimised in order to develop efficient industrial processes.

1.2. Clostridia and the history of acetone – butanol – ethanol (ABE) fermentation

The genus *Clostridium,* was proposed by Prazmowski more than 100 years ago. Many bacterial species were assigned to the genus. Andreesen *et al*., (1989) considered that there were 83 species of *Clostridium* Some of them are able to degrade polysaccharide into monosaccharide (Minton and Clarke, 1989) and some are pathogenic and excrete proteinaceous toxins, although they are predominantly anaerobic, some such as *C. histolyticum, C. durum*, *C. aerotolerans, C. carnis* are moderately aerotolerant (Shone and Hambleton, 1989). The acetone-butanol-ethanol fermentation (ABE fermentation) is not new but dates back to the beginning of the 20 century (Jones and Woods, 1986). In 1862, Louis Pasteur was the first person who observed that fermentation by a species of *Bacillus* named *Bacillus granulobacter* was able to produce butanol (Dürre, 2008). Acetone fermentation dates back to 1905 (Schardinger, 1905). Between 1912-1914 during World War I, the process was scaled up considerably to produce acetone to support the manufacture of cordite (Jones and Woods, 1986). The demand for acetone decreased after the World War I; however, butanol was needed for production of lacquers in the automobile industry which was growing in the USA (Dürre, 2007, Dürre, 2008). In 1927 solvent production by fermentation in the USA reached 50,000 gallons (Gabriel, 1928). In 1936 a new strain, *Clostridium saccharo-acetobutylicum* was isolated which fermented molasses refined from sugar cane or sugar beet. In 1938 other new strains were named *Clostridium saccharobutylacetonicum liquefaciens,* and these strains have given higher yields of about 30-33 % conversion to ABE from 6.5 % (w/v) sugar in the medium. These and other strains, including *Clostridium beijerinckii* were used to produce acetone during World War II (Claassen *et al*., 1999, Woods, 1995, Jones and Keis, 1995). After World War II the commercially important ABE fermentation catalysed by clostridia was the second largest biological industrial process in the world, before being supplanted by the petrochemical industry (Jones and Woods, 1986, Lee *et al*., 2008a). The molasses price increased, and the petroleum industry was able to produce a larger quantity of solvents at lower price. Also, the fermentation process was rejected in the USA in the 1950s because starch and molasses could be used for animal feed (Morris, 1993). ABE production plants soon closed down in some countries, particularly in the western world, although the process still continued in the former Soviet Union, the People's Republic of China, South Africa, and Egypt (Jones and Woods, 1986).

Several factors contributed to the decline of the ABE fermentation (Claassen *et al*., 1999). Firstly high cost of materials; the major factor in ABE fermentation is substrate cost, and using biomass from food crops or wastes material would significantly lower the cost of the ABE fermentation process. Secondly, low final concentration of solvent due to high butanol toxicity to the bacteria. Butanol disrupts the cell membrane and associated functions such as carbohydrate transport (Evans and Wang, 1988), and it is expensive to recover it from a dilute solution. However, since the 1970s oil crisis, researchers have focused on other energy alternatives, hence the desire to improve the ABE fermentation (Ezeji *et al*., 2004), by understanding the principles of genetics, biochemistry and physiological pathways in the bacteria (Gheshlaghi *et al*., 2009). This renewed interest has been strengthened by a number of factors including the finite nature of the supply of oil, environmental concerns relating to burning fossil fuels and the fact that butanol can be used as a biofuel. Reintroduction of the fermentation will however depend on a number of things including use of cheap, renewable substrates, full understanding of genetics and metabolism, and improved butanol tolerance, production and recovery from fermentations.

1.3. Biofuels

Liquid biofuels are fuels produced from solid biomass and used for energy production (Peri and Baldi, 2012). Biofuels have particular importance as transportation fuels (Demirbas, 2009). Biomass refers generally to matter including crops, waste residues and wood produced by farming and other agricultural processes. Currently, biofuels are produced mostly from sugar or starch. However, lignocellulose is an abundant biomass composed mainly of cellulose, hemicellulose, and lignin. Cellulose has a high molecular weight of hemicellulose, which is composed of pentoses such as arabinose, xylose and hexoses (glucose and mannose). Lignin is a polymer of aromatic alcohols and provides rigidity to the cell wall, and may be converted into biofuels by physical treatment followed by microbial metabolism (Brodeur *et al*., 2011). Biofuels can contribute to reducing carbon dioxide emissions (Ali *et al*., 2013). In   2008-2011, 13% of vegetable oil, 11% of coarse grain and 21% sugarcane global production were used to produce fuel (Serra and Zilberman, 2013). In the European Union (EU), production of biofuel increased by 395% from 2005 to 2009. The biggest producers in the EU are Spain, Germany, Italy and France because of the economic incentives available (Peri and Baldi, 2012). There are variety of potential fuel products such as biodiesel, bioethanol, and biobutanol.

1.3.1. Biodiesel

Biodiesel is a fuel for diesel engines. There are different biological sources for biodiesel production such as animal fats (beef tallow, poultry oil) or vegetable oil (sunflower oil, soybean oil, peanut oil) and long chain fatty acids (Atabani *et al*., 2013). Use of vegetable oil as a fuel began in 1978 in the United States and in 1981 in South Africa. In Europe approximately 2.7 million tones of biodiesel were produced in 2003. Petroleum diesel is considered less safe than biodiesel, especially environmentally, and burning it is considered more toxic compared with biodiesel (Demirbas, 2009). The advantages of biodiesel are reducing engine emissions such as sulfur oxide, unburned hydrocarbons and carbon monoxide (Kiakalaieh *et al*., 2013). There are however some problems with biodiesel, for example high viscosity, high nitrogen oxide emission, low energy content, and lower power and engine speed, which may damage the diesel engine (Balat, 2011, Ramadhas *et al*., 2004).

1.3.2. Bioethanol

Bioethanol is produced from renewable agriculture products such as sugar cane in Brazil and corn in the USA (Mathew *et al*., 2013). These feedstocks can also be utilised for human and animal food, which causes these materials to be subject to price fluctuations and then food scarcity, caused by biofuel production. Sugar and starch are converted to ethanol by yeast (Hägerdal *et al*., 2006). There is a lot of interest in producing bioethanol from lignocelluose, however, lignocelluose is not easily broken down, it requires to be pre-treated chemically or physically or enzymatically in order to release the fermentable sugar. Bioethanol used for transport fuel has some advantages. Firstly, to increase the impact of ethanol, it can be blended with gasoline and will reduce gasoline utilization. Secondly, it improves the efficiency of an engine cycle, and also provides a greenhouse benefit, as it is releases less SO_2 and CO emissions than gasoline and also diesel fuel (Dodić *et al*., 2009). As an environmentally friendly renewable liquid biofuel (John *et al*., 2011, Ranalli, 2007), bioethanol is already being used on a large scale in some European countries, USA, Canada, China and Brazil (Prabhakar and Elder, 2009, Kecebas and Alkan, 2009).

1.3.3. Biobutanol

As previously described butanol has been produced on an industrial scale from sugar and starch by the solventogenic clostridia such as *Clostridum beijerinckii* and *Clostridum acetobutylicum* under anaerobic conditions (García *et al*., 2011). Although butanol has been mainly used as solvent and as an important industrial chemical, its potential as a commercially attractive biofuel for transportation purposes has been recognised recently (Lee *et al*., 2008b). Butanol is a flammable liquid and is colourless, and it has a number of advantages as transport fuel which make it more attractive than ethanol. For example, it is less miscible with water and is not corrosive, meaning that it can be distributed by pipelines. It also has a higher energy content, and can be used without any modification in current car engines, at any concentration blend with gasoline up to pure butanol (Shapovalov and Ashkinazi, 2008, Dürre, 2007). At the present time 350 million gallons per year of butanol production enter the World market, and 220 million gallons per year are consumed just by the USA (Shapovalov and Ashkinazi, 2008). By 2020 in the biofuel market butanol is predicted to be worth \$ 247 billion and also has the potential to substitute for both ethanol and diesel (Green, 2011).

1.4. Metabolic production of acetone and butanol

The revival of the ABE fermentation process will require the use of cheap renewable substrates as the raw material, for example agriculture wastes containing complex polysaccharides that must be degraded to monosaccharides, disaccharides, and oligosaccharides that can be taken up and metabolised by the bacteria. Most clostridia are strictly anaerobic bacteria, and two distinct metabolic phases during growth are found with the majority of solventogenic species. The first phase is the acidogenic phase in which acetate, butyrate, carbon dioxide, and hydrogen are produced as metabolic end products (Figure 1.1). Acid formation causes a decrease of the pH of the culture medium, and metabolism shifts to solventogenesis (Ballongue *et al*., 1985). The second phase, or solventogenic phase, is accompanied by an increase in pH and some of the acetate and butyrate is converted into acetone and butanol. A small amount of ethanol is also produced (Ross, 1961, Davies and Stephenson, 1941, Johnson *et al*., 1931, Reilly *et al*., 1920).

The ABE fermentation begins in clostridia with the transfer of the carbohydrate substrate across the cytoplasmic membrane. Most sugars can be taken up by a specific mechanism known the phosphoenolpyruvate (PEP) dependent phosphotransferase system (PTS) which both transports and phosphorylates the sugar (see section 1.6) (Mitchell *et al*., 1991, Hutkins and Kashket, 1986). Some strains like *C. beijerinckii* and *C. acetobutylicum* then utilize the glycolytic route as shown in Figure 1.1. After glycolysis, acetyl-CoA, acetoacetyl-CoA and butyryl-CoA are considered to be branch points in the metabolic pathway. During the acidogenic phase, acetyl-CoA and butyryl-CoA are converted to acetate and butyrate, respectively, which results in production of one molecule of ATP for each molecule of product formed.

Four major enzymes, phosphotrans-butyrylase, butyrate kinase, phosphotrans-acetylase, acetate kinase, are involved in formation of acids. The highest amount of hydrogen production also occurs in the acidogenic phase. After the switch to solvent production the activity of these enzymes rapidly decreases (Andersch *et al*., 1983, Hartmanis and Gatenbeck, 1984), associated with a reduction in the cell growth and metabolic activity (Byung and Zeikus, 1985).

When the cells enter stationary phase to begin to produce solvent, the first enzyme required is an acetoacetyl–CoA; acetate/butyrate CoA transferase that converts reinternalized acetate and butyrate into acetyl CoA and butyryl CoA. Acetyl CoA is then converted into acetaldehyde to produce ethanol or is recycled to acetoacetyl –CoA via thiolase. Acetoacetate is converted into acetone by acetoacetate decarboxylase, white butyryl CoA is reduced to butyraldehyde and the butanol. These reactions are catalysed by AdhE, which is a bifunctional butanol dehydrogenase and butyraldehyde-CoA dehydrogenase, whereas, butanol dehydrogenase is responsible for the production of butanol (Dürre, 2007, Palosaari and Rogers, 1988).

The synthesis of major solvent-forming enzymes is induced, perhaps partly in response in pH. The influence of pH has been recognized in transition from acid to solvent production (Monot *et al*., 1984, Fond *et al*., 1985). This results in changes in gene expression and enzyme synthesis, so that acetate and butyrate are now converted into solvents including acetone, butanol and a small amount of ethanol. There is some evidence that Spo0A which is a master regulator of stationary phase development in *Bacillus subtilis* (Errington, 1993), plays a role in regulation of the switch from acidogenic phase to solvent production in clostridia (Ravagnani *et al*., 2000). Although

control of solvent formation is important, another principal control point in metabolism is uptake of metabolisable sugar substrates. Understanding the mechanism and control of sugar uptake is vital for optimisation of the ABE fermentation process.
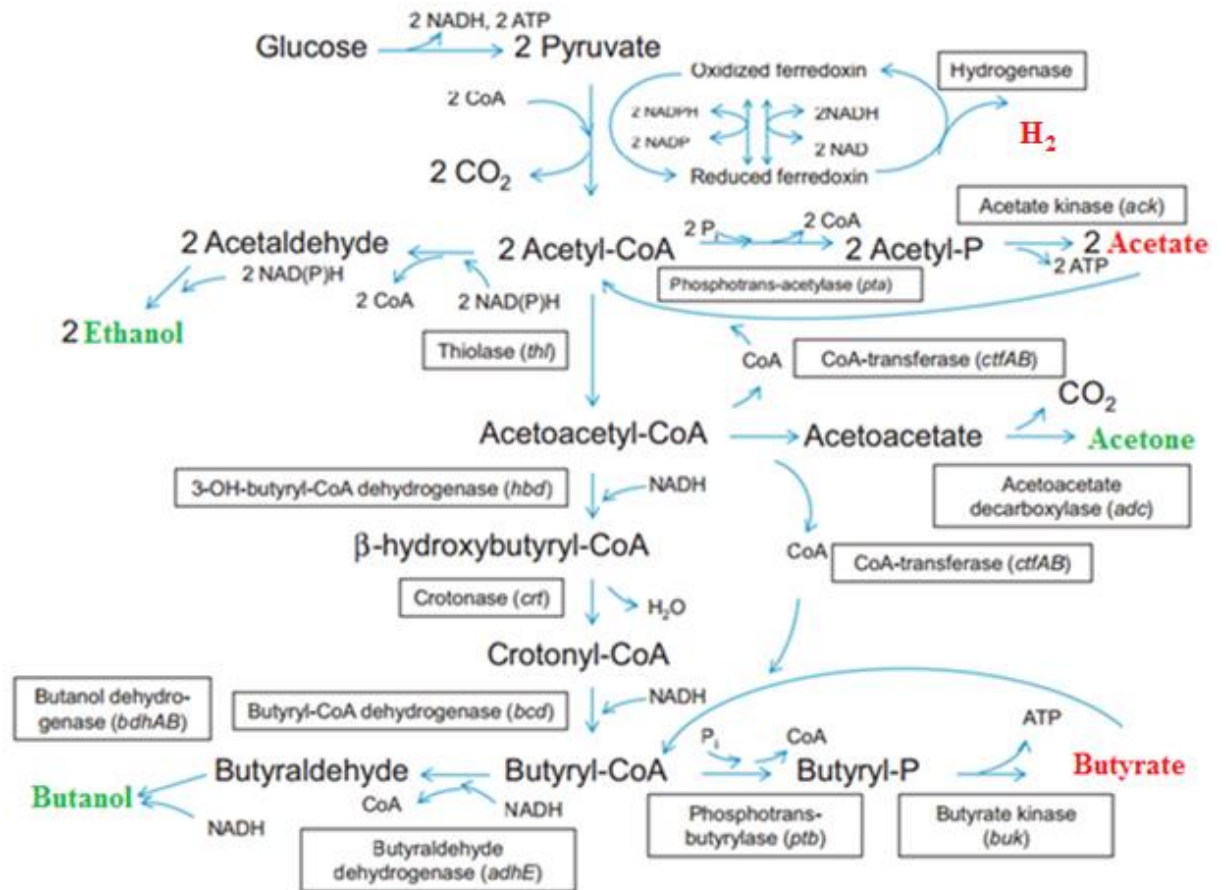
Figure 1.1: Metabolic pathways of acid and solvent formation by clostridia during the fermentation. Products of acidogenic phase and solventogenic phase are shown by red and green colours, respectively. Enzymes are indicated by boxes (from Garcia *et al.*, 2011).

1.5. Transport systems in bacteria

Understanding of sugar uptake in the clostridia is required for the economic formation of butanol. The substrate is an expensive component of the fermentation, therefore the sugar uptake process must support the highest productivity and economic production. Sugars are transported across the cytoplasmic membrane, and metabolised to generate energy. The bacterial cytoplasmic membrane is a selectively permeable structure which consists of a phospholipid bilayer which is impervious to carbohydrates, amino acids, and other polar or ionic molecules (Saier, 2000)**.** Transport mechanisms which allow translocation of these molecules are an integral part of metabolism, but also act as an apparatus for sensing in the cell, that allows the bacteria to monitor their extracellular environment and communicate with it, they are classified on the basis of the form of energy used to support substrate translocation and accumulation (Figure 1.2). The transport mechanisms are generally divided into groups dependent on the source of energy input.

Passive transport, also called facilitated diffusion, is a process which occurs when the substrate concentration is high in the medium and lower in the cytoplasm. Molecules are transported from the area of high concentration to the area of low concentration until equilibrium is achieved (Kell *et al*., 1981). No energy is coupled to this process, and so the concentration of the substrate within the cells will never exceed that of the surroundings.

Bacterial cells usually live in famine conditions in which the concentration of metabolisable molecules is low. Therefore, molecules must be usually transported against concentration gradients, and energy must be consumed (Nikaido and Saier, 1992). Mechanisms by which concentration of substrates is achieved are referred to as active transport. These transport processes are classified on the basis of their dependence on different forms of energy. There are different kinds of active transport. Electrochemical ion gradient driven transporters such as uniporters, symporters and antiporters, are described as secondary transporters and they derive energy in the form of an ion gradient. ABC binding cassette transporters derive energy from hydrolysis of ATP. They generally consist of two proteins which form the translocation pore, and two peripheral nucleotide-binding domains (ATPases). Among prokaryotes, ABC importers may require a periplasmic substrate-binding protein or domain (Rees *et al*., 2009, Davidson *et al*., 2008, Higgins, 1992). Tripartite ATP-independent periplasmic (TRAP)

transporters, have properties related to both secondary transporters and ABC systems, since they are dependent on ion gradient but include a periplasmic binding protein.

A final type of transport process is typified by the PEP dependent phosphotransferase system (PTS) (Figure 1.2). Unlike other transport systems, the PTS both transports and phosphorylates its substrates. The PTS thus initiates the metabolism of the substrate, and its products are readily metabolised further by intracellular enzymes. There are some advantages of this type of transport system in the cell. Firstly, these systems are responsible for accumulation and conversion of substrate and are considered part of metabolism. Secondly, extensive studies of the phosphotransferase system have shown that it plays a central role both in sugar uptake and sensing of small molecules in the environment (Reizer and Peterkofsky, 1987, Saier, 1985).
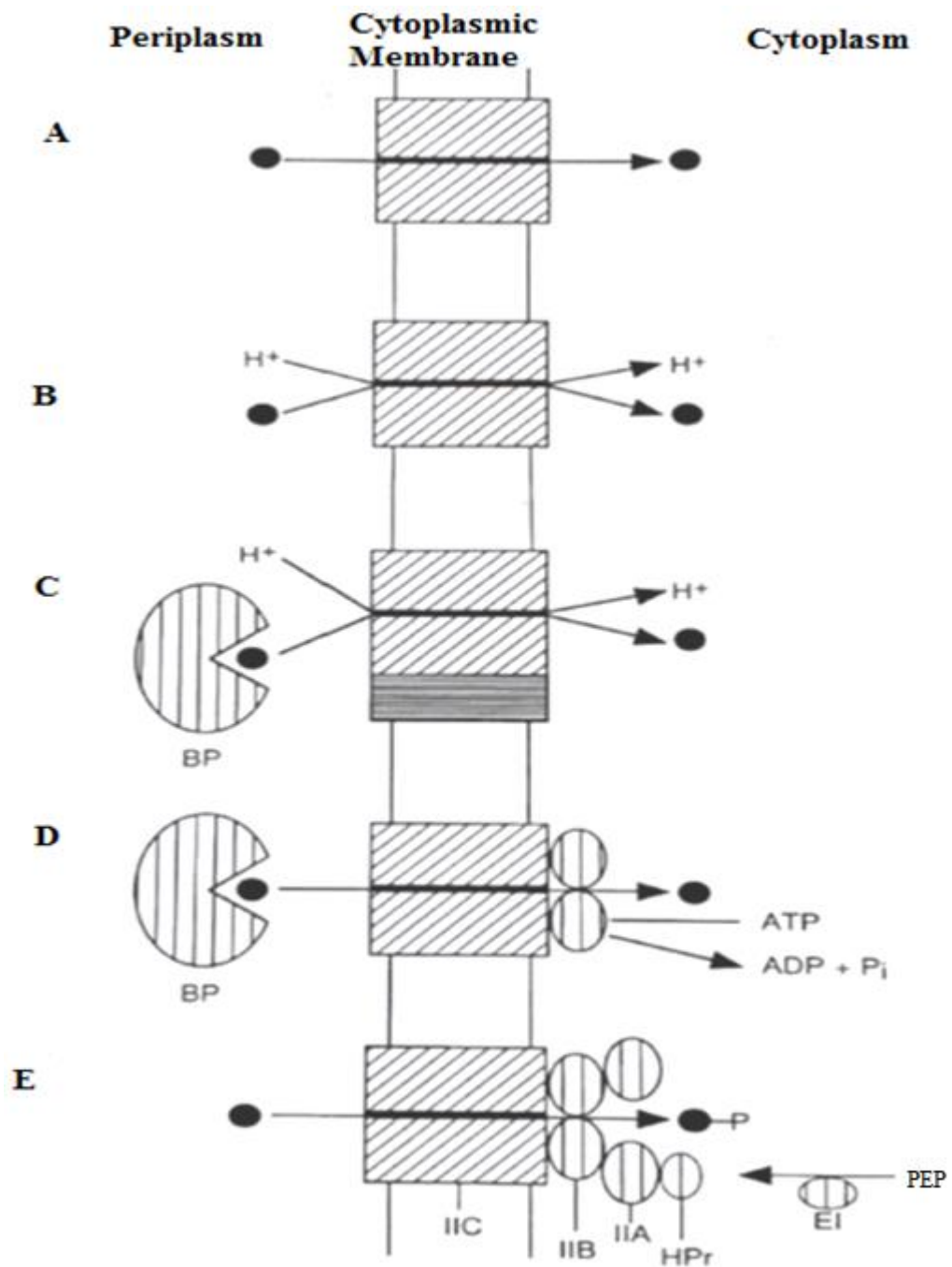
Figure 1.2: Model of classes of transport system in bacteria, (A) facilitated diffusion, (B) ion-symporter, (C) TRAP transporter, (D) ABC type transporter, (E) phosphotransferase system transporter (Adapted from Nikaido and Saier, 1992, Rabus *et al*., 1999).

1.6. The carbohydrate phosphotransferase system (PTS) in bacteria

As described above this system is the first step in sugar or carbohydrate metabolism, and constitutes a process known as group translocation. The PTS was discovered in *Escherichia coli* by Kundig *et al.*, (1964), and is now known to transport many carbohydrates such as glucose, maltose, mannose, sucrose, mannitol and fructose, (Deutscher *et al.*, 2006a, Postma *et al.*, 1993). The PTS is a complex system found widespread in facultative and obligately anaerobic bacteria (Romano and Saier, 1992). In addition to achieving both translocation and phosphorylation of its substrates, the PTS is known to be involved in regulation of carbohydrate metabolism (Deutscher *et al.*, 2006a, Postma *et al.*, 1993). There are some advantages of sugar(s) uptake by the PTS. Phosphorylation of the substrate during entry into the cells alters the substrate configuration, meaning that the sugar(s) are unable to leave the cells. Also, both uptake and metabolism are accomplished by a single metabolic step, meaning less energy input (Tangney *et al.*, 2001). The PTS carries phosphate between PEP and the substrate through a multiprotein phosphoryl transfer chain. There are three major components of the PTS which are Enzyme I, histidine- phosphorylatable protein HPr and Enzyme II (respectively EI, HPr and EII) (Kotrba *et al.*, 2001) (see Figure 1.3).

1.6.1. Enzyme I (EI) and HPr

This first two proteins in the PTS, EI and HPr, are cytoplasmic proteins that participate in the phosphorylation of all PTS carbohydrates in a given organism and thus have been called the general PTS proteins. They are found in the soluble fraction of cell extracts. EI is phosphorylated by PEP in the presence of Mg^{2+} (Weigel *et al.*, 1982). Mg^{2+} is required for EI phosphorylation, but is not required to transfer the phosphate from EI to the general phosphocarrier protein HPr (Weigel *et al.*, 1982, Chauvin *et al.*, 1996). EI consists of approximately 570 amino acids, with a size around 63 kDa (Kuang-Yu and Saier, 2002). EI is considered the first step in the phosphotransferase system (Postma *et al.*, 1993), and has been purified from many species of bacteria for example *E.coli* (Waygood and Steeves, 1980, Dooijewaard *et al.*, 1979) *Streptococcus faecalis* (Alpert *et al.*, 1985), *Mycoplasma* (Ullah and Cirillo, 1976) and *Salmonella typhimurium* (Weigel *et al.*, 1982).
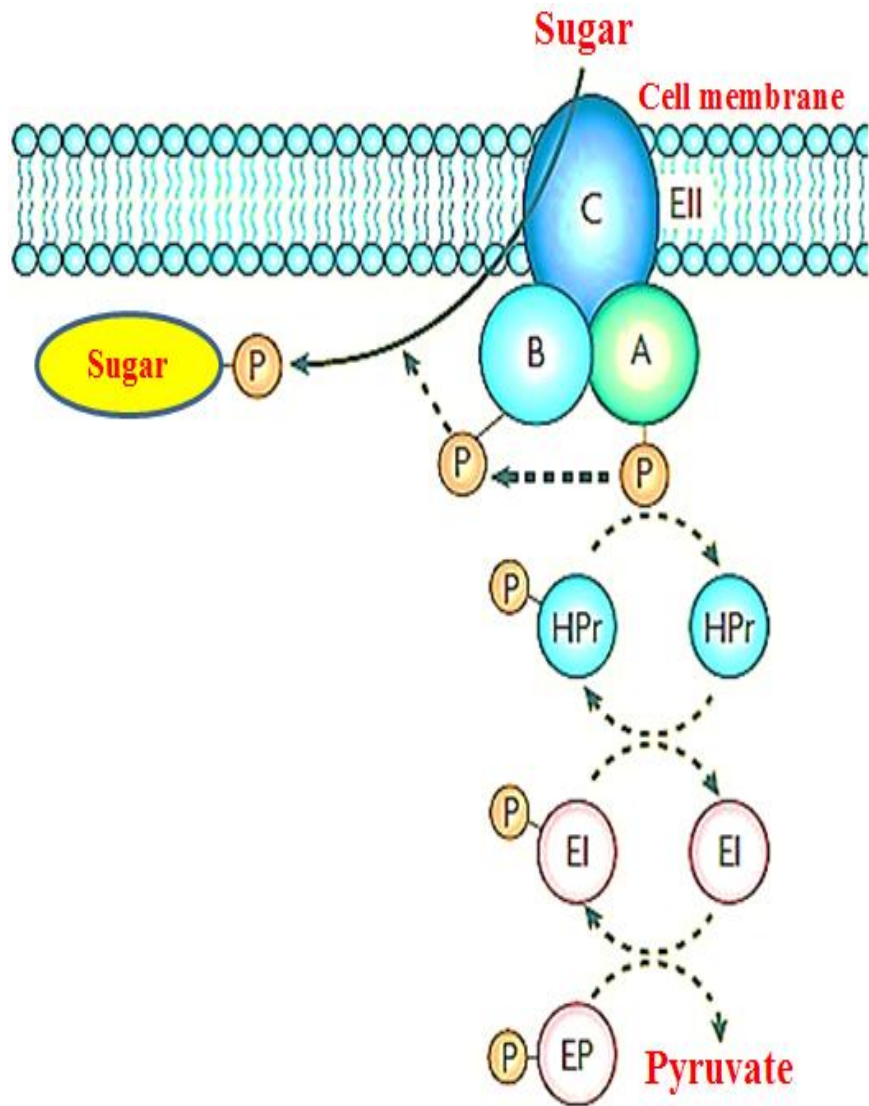
Figure 1.3: Phosphotransferase system (PTS) of bacteria. The PTS is composed of Enzyme I, HPr, and an Enzyme II complex which form a phosphorylation chain reaction that transfers the phosphoryl group from PEP to the substrate. The sugar substrate is transported into the cell by EIIC (from Gorke and Stulke, 2008).

HPr is the second protein in the PTS, which receives a phosphate from EI, and passes it to the EII (Kotrba *et al.*, 2001). This protein consists of around 90 amino acids with a molecular mass of about 10 kDa, and is phosphorylated on a conserved His-15 (Postma *et al.*, 1993). In the presence of a variety of carbohydrates, the expression of the genes *ptsI* and *ptsH* encoding EI and HPr is increased (Tanaka *et al.*, 2008). The *ptsI* and *ptsH* genes are usually linked in an operon *ptsHI* (Postma, 1987).

1.6.2. Enzyme II (EII)

EII is complex and usually consists of three domains or components IIA, IIB and IIC (Martin-Verstraete *et al.*, 1990, Reizer *et al.*, 1992). Some systems also have a fourth domain IID (Saier Jr and Reizer, 1992). The EII domains may be present on different proteins, or they may be fused together in different combinations depending on the system. The IIA and IIB domains are hydrophilic. The IIA domain is the receiver of the phophoryl group from HPr, and it is then transferred to the IIB domain (Kotrba *et al.*, 2001). The IIC and IID domains are found in the cell membrane, these domains are hydrophobic. The IIC domain has a high hydrophobicity compared with IID (Mitchell and Tangney, 2005). The IIC (and IID) domains consist of 6 - 8 transmembrane helices and form a channel across the membrane which is required for translocation of the substrate into the cell (Saier Jr and Reizer, 1992, Postma *et al.*, 1993). In the majority of systems the sugars are transported by the IIC domain alone (Postma *et al.*, 1993). IIB and IIC domains in many cases are linked together, presumably to facilitate the transfer of phosphate from the IIB domain to the substrate as it enters the cell. There is no evidence that the IIC (or IID) domains are phosphorylated during this transfer. All domains IIA, IIB and IIC are required in EII to complete a functional PTS, although the EIIA domain is absent in some systems and has to be provided by an alternative system to complete the phosphorylation transfer chain (Reizer *et al.*, 1999).

Seven PTS families have been identified on the basis of sequence relationships, and these families are characteristically involved in transport and phosphorylation of a range of identified substrates.

(1). The glucose/glucoside(Glc) family translocates a variety of α- and β-glucosides, and also includes permeases for glucose, glucosamine and N-acetylglucosamine. In this family, several of the PTS permeases lack their own IIA domain and use the glucose IIA protein (Nguyen *et al*., 2006).

(2). The mannose/fructose/sorbose(Man) family, is the only family in which members possess a IID domain, and only in the Man family is the domain IIB phosphorylated on a histidine residue rather than a cysteyl residue. The Man family exhibits a wide specificity for a range of carbohydrates, for example in *E.coli* systems can transport and phosphorylate a broad range of sugars including glucose, fructose, mannose, glucosamine, N-acetylmannosamine, and N-acetylglucosamine (Plumbridge and Vimr, 1999).

(3). The fructose/mannitol (Fru) family is a complex and a large family and includes many sequenced fructose and mannitol permeases. Systems belonging to this family phosphorylate fructose on the 1-position compared with Man family systems which phosphorylate fructose on the 6- position (Nguyen *et al*., 2006).

(4). The lactose/diacetylchitobiose/β-glucoside(Lac) family includes many sequenced lactose permeases from Gram-positive bacteria, while in *Borrelia burgdorferi* and *E.coli* systems can transport cellobiose and β-glucosides (Nguyen *et al.*, 2006).

(5). The glucitol(Gut) family is a small family consisting of glucitol specific permeases that occur in Gram- positive and Gram- negative bacteria. Unusually the IIB domain is flanked by two parts of the IIC domain (Nguyen *et al*., 2006).

(6). The galactitol(Gat) family, is a very small family including the galactitol permease of *E.coli* (Nobelmann and Lengeler, 1995).

(7). Finally the ascorbate(Asc), family is represented by the SgaTBA permease of *E. coli* (Zhang *et al*., 2003).

1.7. Carbon catabolite repression (CCR)

CCR is an important mechanism responsible for control of bacterial metabolism. In 1940, *B. subtilis* and *E. coli* were studied by Jacques Monod who demonstrated that, these strains showed a preference for selected carbon sources within a mixture of different carbohydrates (Monod, 1942). This phenomenon has come to be known as carbon catabolite repression (CCR) (Deutscher *et al*., 2006b). In some bacteria CCR is the paradigm regulatory mechanism to achieve both metabolic capacity and catabolic balance of the organism and it is found to occur in most bacteria (Deutscher, 2008, Görke and Stülke, 2008).

For most bacteria, glucose is the preferred carbon source which blocks the utilization of other substrates, by preventing the synthesis of enzymes for their uptake and metabolism. CCR helps bacteria become adjusted to a physiological condition that does not overtax the metabolism of the organism (Brückner and Titgemeyer, 2002). CCR in many bacteria is exerted through the PTS (Cases *et al*., 2007, Deutscher *et al*., 2006b, Postma *et al*., 1993). CCR has been observed in both Gram-negative and Gram positive bacteria, and the PTS is involved in both cases, although the mechanisms have been shown to be very different (Deutscher, 2008).

1.7.1. CCR in Gram negative bacteria

The major player in *E. coli* is the cyclic AMP receptor protein (CRP), sometimes known as catabolite activator protein (CAP) which is activated by binding cAMP in the absence of a carbon source such as glucose (Fic *et al*., 2008, Gama-Castro *et al*., 2008). CRP activates transcription in the presence of cAMP at many promoters by binding upstream of the promoter (Lawson *et al*., 2004, Busby and Ebright, 1999).

cAMP was observed to be linked with catabolite repression and controlled by the activity of the enzyme for cAMP synthesis, adenylate cyclase (Epstein *et al*., 1975). cAMP was observed at a high concentration when glucose was absent, therefore stimulating CRP binding to the DNA to activate transcription. However, in the presence of glucose, cAMP is at a low level, that means no transcriptional activation occurs (Lawson *et al*., 2004, Crasnier-Mednansky *et al*., 1997). cAMP synthesis by adenylate cyclase is stimulated by the phosphorylated form of EIIA^{glc}, a glucose-specific component of the PTS(Postma *et al*., 1993). In the presence of glucose, because glucose is coming into the cell and being phosphorylated, this results in dephosphorylation of

EIIA^{glc} which means it can no longer stimulate adenylate cyclase activity, therefore cAMP concentration goes down (Deutscher *et al*., 2006a, Postma *et al*., 1993).

Another important transcription factor besides CRP is the catabolite repressor activator (Cra) protein which plays a key role in *E.coli* in controlling metabolic pathways (Chavarría *et al*., 2011). The Cra protein was first discovered as the fructose repressor of the genes involved in fructose metabolism. It consists of two domains, a DNA-binding N-terminal domain and a C-terminal domain which binds effectors. Cra is regulated by fructose 1-phosphate via stimulating removal of Cra from its DNA binding sites, in the presence of glucose the concentration of fructose 1-phosphate increases which interact with Cra to prevent binding to the target promoter (Shimada *et al*., 2011).

1.7.2. CCR in Gram positive bacteria

The catabolite control protein CcpA plays a central role in regulation of metabolic pathways in low G-C Gram positive bacteria which are known as firmicutes including endospore forming bacteria such as the *Clostridia* (Singh *et al*., 2008). The protein belongs to the LacI-GalR family of transcriptional regulators (Shimada *et al*., 2011, Deutscher, 2008), and in addition to controlling carbohydrate metabolism is also involved in regulation of toxin production and sporulation in some pathogenic species (Antunes *et al*., 2011, Varga *et al*., 2008, Varga *et al*., 2004). The CcpA protein binds to *cis*-acting catabolite repression elements (*cre*) located between the upstream activating sequence and the promoter (Martin-Verstraete *et al*., 1995).

In firmicutes the PTS protein HPr is the key in the regulation of carbon metabolism (Figure 1.4). HPr of these organisms has two phosphorylation sites. In addition to the histidine that is phosphorylated by Enzyme I and PEP, the HPr protein can be phosphorylated on residue serine-46. There are two main effects of this serine phosphorylation. Firstly, it strongly interferes with phosphorylation by EI on His-15, resulting in a decrease of PTS dependent transport of carbohydrate. Secondly, CcpA forms a complex with (P-Ser-HPr) in the presence of fructose 1,6-bisphosphate (FBP) that stimulates binding to *cre* sites (Deutscher *et al*., 1995).

The protein which catalyses phosphorylation of HPr on serine 46 is a HPr kinase/phosphorylase (HPrK/P), a sensor enzyme for catabolite repression in low G-C Gram positive bacteria (Nessler *et al*., 2003). The activity of the phosphorylation and dephosphorylation reactions is controlled by the concentration of metabolic

intermediates. In particular, ATP, FBP and other glycolytic intermediates stimulate the kinase activity, on the other hand the phosphorylase activity is inhibited by ATP but stimulated by inorganic phosphate (Deutscher and Saier, 1983). Therefore, under conditions of high nutrient status (in the presence glucose), when the kinase activity of HPrK/P is stimulated, HPr is phosphorylated at Ser46. This results in inhibition of carbohydrate transport via the PTS and repression of catabolic genes via the (P-Ser-HPr)-CcpA-FBP complex. On the other hand, under condition of low nutrient status the phosphorylase activity of HPr K/P is favoured, and the effects of glucose are reversed. HPrK/P has been observed in a range of low G-C Gram positive bacteria, including *B. subtilis* (Jault *et al*., 2000, Reizer *et al*., 1998, Galinier *et al*., 1998). *Enterococcus faecalis* and *C. acetobuylicum* (Tangney *et al*., 2003). It is also present in *Mycoplasma pneumoniae* (Steinhauer *et al*., 2002), but has not been found in Gram negative bacteria such as *E. coli.*
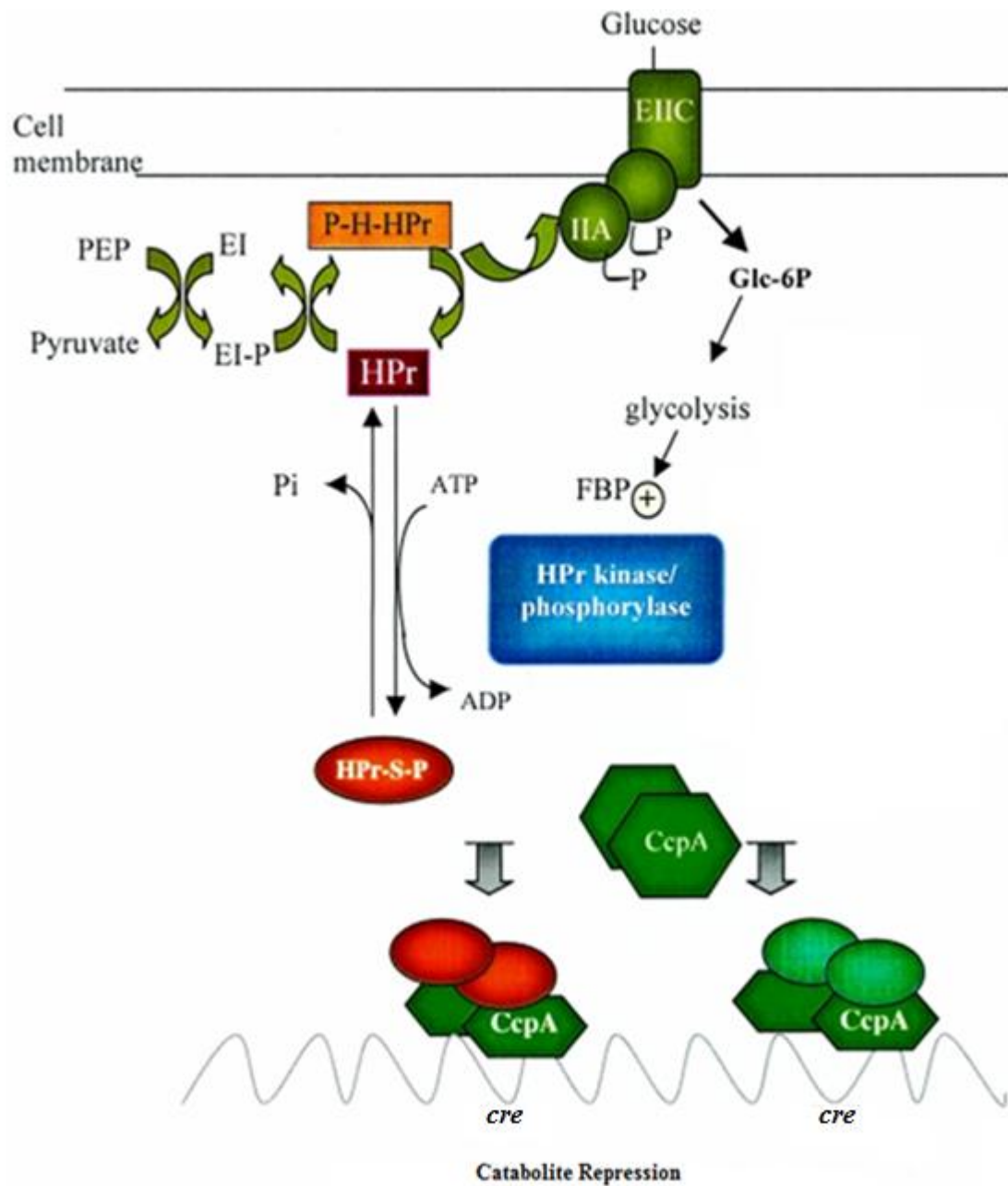
Figure 1.4. Model of catabolite repression in low G-C Gram- positive bacteria. Proposed sensory transduction pathway by which exogenous glucose is believed to activate the CcpA transcription factor to promote catabolite repression (CCR) by binding to a catabolite responsive element (*cre*) in the control region of a target gene. Proteins primarily involved are (i) the glucose phosphotransferase system including EI, HPr and EIIBCA^{Glc}, (ii) glycolytic enzymes, (iii) HPr/kinese/ phosphorylese and HPr, and (iv) CcpA (from Lorca *et al*., 2005).

1.8. Sugar uptake by the clostridia

The transport and metabolism of sugars is a key requirement of clostridia for growth and development (Mitchell and Tangney, 2005). As for other anaerobes, the predominant mechanism for sugar uptake in these bacteria is the phosphoenolpyruvate (PEP) dependent phosphotransferase system (PTS), which characterized by transport and metabolism of its sugars as it enters the cell (Mitchell, 1998). It has been demonstrated that the PEP dependent PTS plays an important role in the uptake of a wide range of sugars in the clostridia.

As shown in Table 1.1, PTS activity has been recorded for a variety of sugars including lactose, fructose, cellobiose, glucitol, sucrose, and glucose (Yu *et al*., 2007, Mitchell and Tangney, 2005). In addition has recently been shown that N- acetylglucosamine is phosphorylated by both *C. beijerinckii* and *C. acetobutylicum* (Al Makishah and Mitchell, 2013).

Table 1.1. Phosphotransferase activities that have been demonstrated by activity assays in some species of clostridia (Mitchell and Tangney, 2005).

Clostridium	PTS system carbohydrate
C. acetobutylicum	glucose, lactose, maltose, mannitol, sucrose, cellobiose, fructose
C. beijerinckii	glucose, glucitol, lactose, mannitol, fructose, sucrose
C. difficile	mannitol
C. pasteurianum	glucose, fructose, mannitol, sucrose, glucitol
C. saccharobutylicum	glucose, lactose, galactose
C. thermocellum	fructose, mannitol
C. perfringens	glucose, mannose
C. butyricum	fructose
C. rubrum	fructose

The four major solvent producing species, *C. beijerinckii, C. acetobutylicum, C. saccharobutylicum, C. saccharoperbutylacelonicum,* were distinguished by genetic techniques including 16S rRNA sequence and genomic DNA (Keis *et al*., 2001) and these are able to ferment a wide variety of sugars including disaccharides hexoses and pentoses. The most detailed studies of the PTS and carbohydrate utilisation have been done using *C. beijerinckii* and *C. acetobutylicum.* In this context, it should be noted that before 1995 the strain *C. beijerinckii* NCIMB 8052 was classified as *C. acetobutylicum* and this organism was considered to be the same organism as *C. acetobutylicum* ATCC 824. Glucose has been shown to repress the metabolism of several alternative substrates by clostridia. Several operons in clostridia including the glucitol operon of *C. beijerinckii* (Tangney *et al*., 1998), the sucrose operon of *C. beijerinckii* (Reid *et al*., 1999), the lactose operon of *C. acetobutylicum* (Yu *et al*., 2007), and the mannitol operon of *C. acetobutylicum* (Behrens *et al*., 2001), have been shown to be repressed by glucose. Although the mechanisms have not been completely described, *C. acetobutylicum* extracts were shown to have HPrK/P activity (Tangney *et al*., 2003), and the mechanism is believed to be similar to the CcpA- dependent mechanism shown in Figure 1.4.

Full redevelopment of the ABE fermentation will require a more complete characterization of the PTS and its metabolic function in clostridia. The *C. acetobutylicum* genome encodes 13 complete PTSs. As a result of transcriptomic study and experimental analysis, most of their substrates have been identified (Servinsky *et al*., 2010, Tangney and Mitchell, 2007, Yu *et al*., 2007, Tangney *et al*., 2003). Therefore, strategies for strain improvement can be tested.

Xiao *et al*., (2011), knocked out *glcG* which encodes the glucose phosphotransferase system in *C. acetobutylicum* (Tangney and Mitchell, 2007). When the gene was knocked out, the glucose metabolic rate did not decrease, however, they did observe a less severe CCR of xylose and arabinose metabolism compared to the wild type cells. This suggested that the GlcG PTS is in involved in sensing glucose in the medium and it might be important for CCR. Therefore, it was demonstrated that it is possible to interfere with CCR in the clostridia.

1.9. *Clostridium beijerinckii*

C. beijerinckii was first isolated by Martinus Beijerinck from soil (Durre, 2008) and as a member of the solvent producing group (Lee and Blaschek, 2001), it is able to convert a range of carbon sources to acetone, butanol and ethanol (ABE) (Ezeji *et al*., 2007). As described earlier *C. beijerinckii* strains were isolated and used in the ABE fermentation process (Jones and Woods, 1986). There are number of factors which favour *C. beijerinckii* as an organism for solvent production. Firstly, it is more tolerant to organic acids, for example formic acid and acetic acid (Cho *et al*., 2012), and also solventogenic inhibitors such as hydroxymethylfurfural and furfural (Ezeji *et al*., 2007), compared with other strains such as *C. acetobutylicum.* Secondly, the genes for solvent production are located on the chromosome in *C. beijerinckii,* unlike *C. acetobutylicum* which carries then on a plasmid, *C. beijerinckii* is therefore not so likely to show degeneration, that is a loss of the ability to make solvent. Thirdly, the genome size of *C. beijerinckii* NCIMB 8052 is around 6.0 Mbp, which is 50 % bigger than the genome of *C. acetobutylicum* (Wang *et al*., 2011). This suggests that the bacterium has greater metabolic capacity than *C. acetobutylicum* and this may be useful with respect to exploiting a number of different carbon sources for fermentation.

There are 43 complete PTSs encoded in the genome. Shi *et al*., (2010) listed 47, but five were incomplete and the system encoded by *cbei* 3367 was not included. A phylogenetic tree (Figure 1.5), of these systems shows that all seven PTS families are represented. Only two systems were characterised experimentally before the genome sequence was available. Their IIC domains are encoded by *cbei* 0336 and 0337 (glucitol PTS; Tangney *et al*., 1998) and by *cbei* 5012 (sucrose PTS; Reid *et al*., 1999). Recently a third system has been characterized encoded by *cbei* 4532 and has been shown to phosphorylate N- acetylglucosamine and glucose (Al Makishah and Mitchell, 2013). Therefore, overall the metabolic capacity of the bacterium associated with the phosphotransferase systems which are encoded by the genome is not well understood.
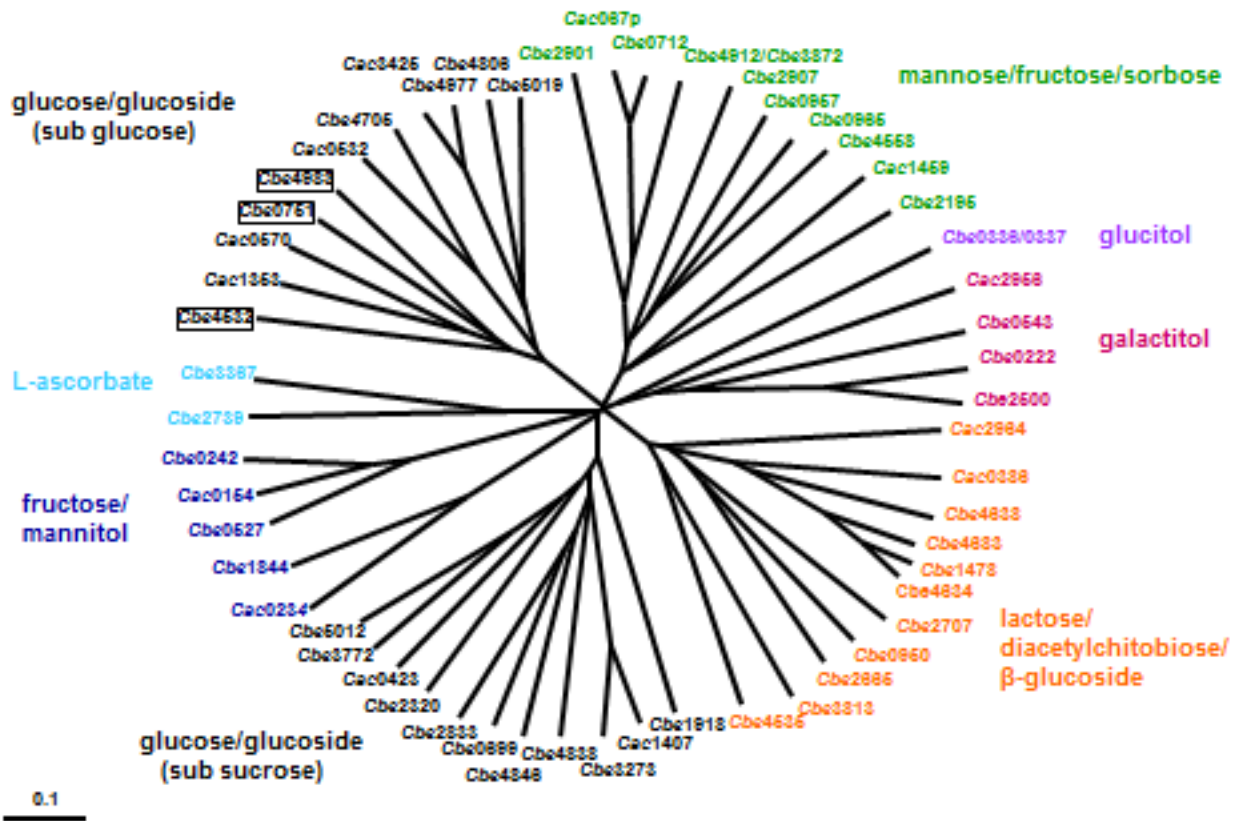
Figure 1.5. Phylogenetic tree of IIC domains of PTS of *Clostridium beijerinckii.* (Courtesy of W.J. Mitchell). The tree shows different PTS families and their relationships. *C. beijerinckii* systems are highlighted in colour and belong to * black, glucose family (sucrose subfamily),* black, glucose family (glucose subfamily),*green, mannose-fructose-sorbose,*red, galactitol,*boring blue, L-ascorbate,*purple, glucitol,*blue, fructose-mannitol, *gold, lactose-diacetylchitobiose-β-glucoside.

1.10. The ClosTron

Clostridium is the second largest bacterial genus and encompasses bacteria of both industrial and medical importance which are described as anaerobic Gram positive, and rod shaped bacteria (Tangney and Mitchell, 2007). Numerous genome sequences are now available, but the ability to exploit the information has been limited by the lack of reliable genetic manipulation techniques. A common approach to studying functionality of clostridial genes is to clone them into *E.coli* and try to demonstrate the function. This has been done successfully for a β-glucoside PTS in *Clostridium longisporum* (Brown and Thomson, 1998), and has also recently been done for the N-acetylglucosamine PTS in *C. beijerinckii* (Al Makishah and Mitchell, 2013). This approach is therefore an important part of this particular project, which is aimed at characterization of two additional phosphotransferase systems in *C. beijerinckii* that belong to the glucose family. However, a few years ago Heap *et al*., (2007), reported a system called the ClosTron which appears to be a reliable system for gene inactivation in clostridia as shown in Figure 1.6. This system opens up additional possibilities for studying gene function, and it seems to work in a variety of different clostridia such as *C. acetobutylicum, C. difficile* and also *C. beijerinckii* (Jia *et al*., 2011, Kuehne *et al*., 2011a, Heap *et al*., 2010). The ClosTron is an intron- based integration system which can target the inactivation of a specific gene, leaving behind a selectable antibiotic resistance marker to enable isolation of mutants. It has been used successfully for isolation of mutants affected in solvent formation by *C. acetobutylicum* (Cooksley *et al*., 2012, Wietzke and Bahl, 2012, Heap *et al*., 2010) and toxin production in *C. difficile* (Baban *et al*., 2013, Dingle *et al*., 2011, Antunes *et al*., 2011, Heap *et al*., 2007).
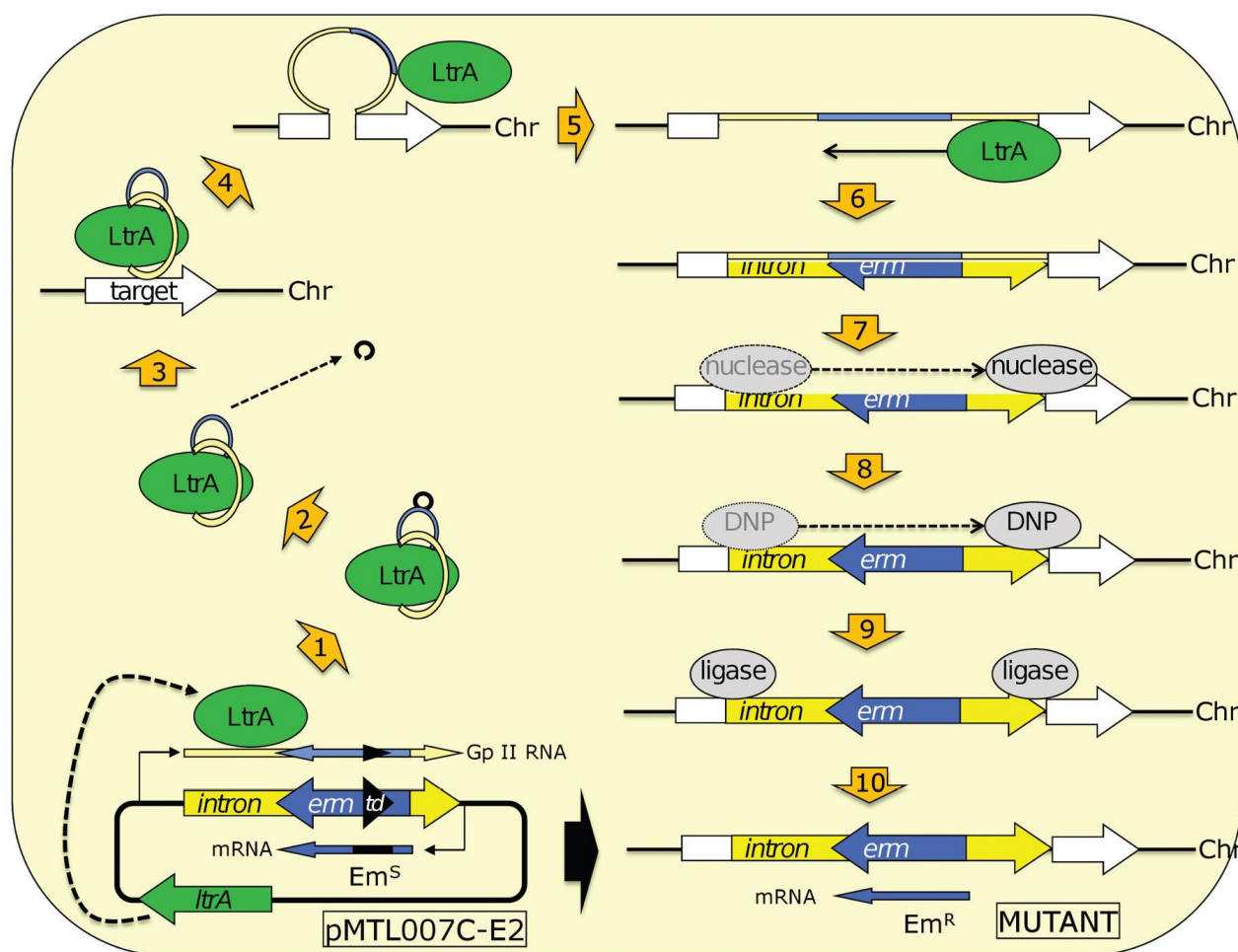
Figure 1.6. Schematic of ClosTron insertion using pMTL007C-E2 (from Kuehne SA and Minton NP, 2011). The ClosTron plasmid pMTL007C-E2 encompasses a Group II intron containg the *ermB* gene encoding resistance to erythromycin (blue colour is insert). The *ermB* gene has been inactivated by the phage (*td*) intron (black region). The *td* intron can self-catalytically splice from mRNA, but the process of splicing is orientation dependent. When the *ermB* is transcribed the *td* intron is in the incorrect orientation, and the ClosTron plasmid does not confer Em resistance on the clostridial cells. The LtrA protein (green region) binds to the intron transcript mRNA, and *td* is allowing to splice out restoring functionality to *ermB* gene. The RNA is then directed towards it is DNA target, and is inserted within it. A series of events involving synthesis of cDNA by LtrA, degradation of RNA by nuclease, DNA synthesis by DNA polymerase (DNP) and ligation result in insertion of a functional *ermB* gene within the target.

1.11. Aim of the study

The aim of this project was to characterize potential glucose phosphotransferase systems in *C. beijerinckii*. Glucose is the preferred substrate for clostridia and enters the cell via one or more phosphotransferase systems. Any PTSs involved in glucose uptake may also be involved in glucose sensing and CCR. Therefore, identifying these systems may lead to development of strategies to construct strains showing altered metabolic control and fermentation performance.

The first aim of the study was to use a functional approach to identify the function of the *cbei* 0751 gene. The gene was amplified by PCR, and then cloned and transformed into a mutant *E.coli* ZSC113 unable to phosphorylate glucose. The characteristics of selected recombinants were then studied with respect to uptake and phosphorylation of glucose. Attempts were also made to knock out the *cbei* 0751 gene using the ClosTron, in order to examine the effect on glucose utilization and CCR.

The second aim of the project was to analyse the genes *cbei* 4982/ *cbei 4983* which also code for a PTS belonging to the glucose subfamily. The experimental approach involving PCR, cloning, and transformation was similar to that used for *cbei* 0751.

CHAPTER 2

MATERIALS AND METHODS

2. Materials and Methods

2.1. Bacterial strains used in this study

C. beijerinckii NCIMB 8052 was the main strain used in this study and was obtained from National Collection of Industrial and Marine Bacteria (NCIMB). Stock culture was maintained at 4 °C as a spore suspension in distilled water. *Escherichia coli* strains were used in complementation studies and stored in glycerol containing media at -70 °C. Details of *E.coli* strains are shown in Table 2.1.

2.2. Buffers and solutions

The composition of buffers and solutions used in this study are either described in the relevant sections or listed in Appendix V.

2.3. Growth media composition

All media were autoclaved at 121 °C for 15 minutes.

Reinforced Clostridial Medium (RCM) was obtained from Oxoid LTD, England. Synthetic Reinforced Clostridial Medium (sRCM) was prepared according to the composition of RCM, but did not contain glucose, soluble starch or agar. A carbon source was added as necessary.

Clostridial Basal Medium (CBM) contained per litre: casein hydrolysate, 4 g; $FeSO_4$ $7H_2O$, 10 mg; $MgSO_4.7H_2O$, 0.2 g; $MnSO_4.4H_2O$, 10 mg; thiamine HCl, 1 mg; p-aminobenzoic acid, 1 mg; d- biotin, 20 µg; 10 g sugar. 10 x phosphate solution was autoclaved separately from the other medium components [K_2HPO_4, 0.5 g; KH_2PO_4, 0.5 g in 100 ml] at 121 °C for 15 minutes and then the separate solutions were cooled in the anaerobic cabinet. Phosphates (10 % v/v) were added to the medium before use.

Luria-Bertani (LB broth) medium contained per litre: tryptone 10 g; sodium chloride 5 g; yeast extract 5 g. (Sambrook *et al*., 2001, Gerhardt *et al*., 1994).

When solid media were required, agar technical (Agar No.3) (Oxoid) was added to concentration of 12.0 g per 1000 ml.

Difco™ MacConkey agar base used for examination of fermentation phenotype contained per litre: peptone 17 g; proteose peptone 3 g; sodium chloride 5 g; bile salts 1.5 g; crystal violet 0.001 g; neutral red 0.03 g; and agar 13.5 g. Carbohydrates were added into the medium as required.

Antibiotics were added to the media as required after cooling to 55 °C (Table 2.2).

Table 2.1: *E. coli* strains used in this study

E.coli strain	Genotype	Source
ZSC 113	*gpt-2 mpt-2glk-7strA*	Lab collection (Curtis and Epstein, 1975)
TOP10F	F-*mcrAΔ(mrrhsdRMSmcrBC) Φ80lacZΔM15ΔlacX74 recA1araD139Δ(araleu)7697gal UgalKrpsL(StrR) end A1nupG*	Invitrogen
TOP10	F-*mcrAΔ(mrrhsdRMSmcrBC) Φ80lacZΔM15ΔlacX74 recA1araD139Δ(araleu)7697gal UgalKrpsL(StrR) end A1nupG*	Invitrogen
JW3381-4	F-, *Δ(araD-araB)567, ΔlacZ4787(::rrnB-3),λ-, ΔmalT 752::kan,rph-1, Δ(rhaD-rhaB)568, hsdR514*	CGSC *
JW3701-2	F-,*Δ(araD-araB)567,ΔlacZ4787(::rrnB-3),λ,rph1,ΔbglG 754::kan, Δ(rha-DrhaB)568, hsdR514*	CGSC
JW4199-1	F-,*Δ(araD-araB)567,ΔlacZ4787(::rrnB-3),λ-,rph1,Δ (rhaD-rhaB) 568, ΔtreB787::kan,hsdR514*	CGSC
JW0665-1	F-,*Δ(araD-araB)567, ΔlacZ4787(::rrnB-3),ΔnagE728:: kan, λ-,rph-1, Δ(rhaD-rhaB) 568, hsdR514*	CGSC
JC10279	*srl 301:: Tn10 srlD50 gnt C300 ? metB1 mtl2 gat C300 gatA50 malAl xyl 7 rpsL 104 sup⁻*	Lab collection
C600RK2	*thr1,leuB6(Am), fhuA21, cyn101, lacY1, glnV44(AS), λ-, e14-, rfbC1, glpR200(glpᶜ), thiE1*	S. Mastrangelo, University of Nottingham

* CGSC, Coli Genetic Stock Center

Table 2.2: Antibiotics used in this project

Antibiotic name	Abbreviation	Final concentration
Ampicillin	Amp	50 or 100 µg/ml
Kanamycin	Kan	30 µg/ml
Spectinomycin	Spc	750 µg/ml or 250 µg/ml
Erythromycin	Ery	10 µg/ml
Cycloserine	Cyl	250 µg/ml

2.4. Growth of *C. beijerinckii* NCIMB 8052

0.8 ml spore stock was heat–shocked for 10 minutes at 80 °C, and then transferred into 20 ml of Reinforced Clostridial Medium (RCM,Oxoid), and incubated overnight at 37 °C under N_2-H_2-CO_2 (80:10:10) mixed gas in an anaerobic cabinet. 2 ml of starter culture was transferred to 100 ml Clostridial Basal Medium (CBM) supplemented with the required sugar(s) and incubated overnight at 37 °C. Cells were harvested by centrifuging for 10 minutes at 4 °C and 4,000 xg using tubes sealed with a rubber stopper sub-a-seal. The pellets were resuspended and then washed two times in Clostridial Basal Medium (CBM) without a carbon source before being resuspended in 10 ml of the same medium. The cells were inoculated into 100 ml CBM containing the required sugar(s) to an OD_{600} of 0.05-0.1. The working culture was incubated in the anaerobic cabinet at 37 °C.

A sample of cultures was routinely checked for contamination. Gram stains were used to confirm purity of culture. Growth test were also set up using RCM agar medium. A loopful of culture was spread around the RCM plate and incubated aerobically at 37 °C.

2.5. Preparation of *C. beijerinckii* NCIMB 8052 spores

0.8 ml of *C. beijerinckii* NCIMB 8052 spores was inoculated into 20 ml of RCM and incubated overnight. 1 ml of overnight culture was transferred into 500 ml of synthetic Reinforced Clostridial Medium (sRCM) and then incubated for two weeks at 37 °C in the anaerobic cabinet. The culture was transferred into 250 ml centrifuge bottles and cells were harvested by centrifuging for 15 minutes at 12,000 xg and 4 °C. The pellets were washed three times in 200 ml sterile dH_2O. After the final spin the pellets were combined and resuspended in 100 ml sterile dH_2O and then stored at 4 °C. The pellets

were washed three times in 200 ml sterile dH_2O. After the final spin the pellets were combined and resuspended in 100 ml sterile dH_2O and then stored at 4 °C.

2.6. Genomic deoxyribonucleic acid (DNA) extraction

2.6.1. DNA extraction from *C. beijerinckii* NCIMB 8052

DNA isolation was carried out using PUREGENE® DNA ISOLATION kit (Gentra®, Cat# D-6000A) for Gram-positive bacteria.

0.8 ml of heat-treated spores was add to 20 ml starter culture and incubated under anaerobic conditions at 37 °C overnight. One ml of overnight culture was transferred into 100 ml of CBM medium. 5 ml of the cells were harvested by centrifuging for 10 minutes at 4 °C and 4,000 xg. 3 ml of cell suspension solution was added to the cell pellet and gently pipetted up and down. 15 µl of lytic enzyme solution was added to the tube and incubated at 37 °C for 30 minutes, then centrifuged at 13,000 rpm for 10 minutes and the supernatant was removed. 3 ml of cell lysis solution was added to the cell pellet and mixed gently, then 15 µl of RNase A solution was add to the cell lysate and the sample was mixed by inverting the tube several times and then incubated at 37 °C for 25-30 minutes. 1 ml of protein precipitation solution was added to the cell lysate and vortexed for 20 seconds and centrifuged at 13,000 rpm for 10 minutes. The supernatant containing the DNA was added into a clean 15 ml polypropylene tube containing 3 ml of 100 % isopropanol and mixed several times, and centrifuged at 13,000 rpm for 3 minutes, after which the DNA pellet was visible. 3 ml of 70 % (v/v) ethanol was added to the pellet and inverted several times to wash the DNA, and centrifuged at 13,000 rpm for 1 minute. The tube was drained on clean absorbent paper, and allowed to air dry for 15-20 minutes. 500 µl DNA hydration solution was added to the tube, and incubated for 1 hour at 65 °C and then overnight at room temperature. DNA was stored at -20 °C.

The DNA concentration was determined by taking 2 µl of DNA and adding 48 µl of 1x TE buffer in a sterile PCR tube. The absorbance of DNA was measured at 260 nm in an Eppendorf uvette® using a BioPhotometer, with TE buffer as a blank.

2.7. Cloning and screening for genes

2.7.1. Design of primers

To amplify fragments of DNA, the primers were designed to be around 18-22 bp long to make the melting temperature (Tm) in the range from 50–60 °C. The primers were synthesised by Eurofins MWG. Melting temperatures were calculated by using the equation $[4 \times (G+C) + 2 \times (A+T)]$ and the PCR annealing temperature calculated $[Tm - 5]$.

The different primers used during polymerase chain reaction (PCR) are listed in Table 2.3. The position of annealing relative to the cloned gene are shown in Appendix I

Table 2.3: The primers used in the experiments for gene cloning and screening of clones

Primer Name	Reference Number	Primer Sequence
Cbei 0751 F*	356	5´-TTATTGAAGATGTTTATGTTGG-3´
Cbei 0751 R*	357	5´-CCAAAACCTATACCTTTTCC-3´
Cbei 4984 F*	434	5´-AATGTACATTATACTATATGTG-3´
Cbei 4983 F*	432	5´-ATCGTATCTGCTAAAAAAGC-3´
Cbei 4982 R*	433	5´-ATTAGACTTGAATTGCACAC-3´
M13F*	371	5´-GTAAAACGACGGCCAGTG-3´
M13R*	372	5´-CAGGAAACAGCTATGACC-3´
T7promoter	373	5´-TAATACGACTCACTATAGGG-3´

* F, forward primer; * R, reverse primer

2.8. Polymerase Chain Reaction (PCR) for gene cloning

Reactions for the amplification were carried out in a mix containing 10 µl of 5x PCR buffer, 1 µl of DNA template, 1 µl of each primer (forward and reverse), 36.5 µl of deionised water and 0.5 µl of DNA polymerase, added after 4.5 minutes to give the final volume 50 µl. Easy-A® High-Fidelity PCR Cloning Enzyme (Stratagene), was used for cloning of *cbei* 0751 (section 2.10.1), cloning of *cbei* 4983/ *cbei* 4982 (section 2.10.2), and also cloning of the *cbei* 4984/ *cbei* 4983/ *cbei* 4982 (section 2.10.3),

Pfu DNA polymerase (Fermentas) was also used in cloning of the *cbei* 4983 / *cbei* 4982 genes with a pJET1.2 cloning kit (section 2.10.2). Samples were amplified on the following cycle; 30 times a cycle of denaturation step at 95 °C for 1 minute, annealing temperature for 1 minute, and elongation step at 72 °C for 3 minutes. Then after 30 cycles of the reaction, a final elongation was performed for 10 minutes at 72 °C, and the reaction kept at 4 °C.

2.9. DNA Gel Electrophoresis

 Samples of DNA were routinely examined using agarose gel electrophoresis. Bioline Agarose gel powder was dissolved to a concentration of 1.0 % (w/v) in 1 x TAE buffer using a microwave, and after cooling 1 µl of 10 mg/ml ethidium bromide was added to 100 ml of gel, and poured into a 10 cm x 7.5 cm or 11 cm x 10 cm gel container sealed with autoclave tape at the edges. A comb was inserted into the gel to form sample wells. After the gel had set, the comb and tape were removed, and the gel was immersed in 1 x TEA buffer (diluted from a 50x stock). 2 µl of 6x loading dye (Fermentas) were mixed with 5 µl of PCR product, and electrophoresis was carried out at 80 volts for 1 to 2 hours, before viewing the bands on a UV-light box. 5 µl of hyperladder I (Bioline) was used as molecular size marker.

2.10. Cloning Reactions

2.10.1. Cloning of *cbei* 0751

A TOPO TA Cloning® Kit was used to perform the cloning of gene *cbei* 0751. Cloning reaction was set up which included 3 µl of fresh PCR product, 1 µl of salt solution, 1 µl of water and 1 µl of pCR® 2.1-TOPO® vector (Appendix IV) giving a final volume of 6 µl. These were mixed gently and incubated for 15 minutes at 25 °C (room temperature). 2 µl of the cloning reaction were added to a vial of *E.coli* TOP10F competent cells. This was mixed gently and then incubated on ice for 15 minutes, the cells were then heat-shocked by placing them for 30 seconds at 42 °C in a water bath and then immediately placed on ice for two minutes. 250 µl of LB broth was added to the tube and then the cells were incubated for 1 hour at 37 °C with shaking horizontally. 50 µl of transformed cells were spread on LB plates with 100 µg/ml ampicillin, the plates were then incubated overnight at 37 °C.

2.10.2. Cloning of *cbei* 4983 and *cbei* 4982

The genes *cbei* 4983 and *cbei* 4982 were cloned using a StrataClone PCR cloning kit. Strata (Agilent) vector (Appendix IV). The cloning reaction was set up which included 2 µl of fresh PCR product, 3 µl of StrataClone Cloning Buffer, 1 µl of pSC-A-amp/kanVector see (Appendix IV), in a final volume of 6 µl. These were mixed gently and incubated for 5 minutes at 25 °C (room temperature) and then kept on ice. 1 µl of the cloning reaction was added to a vial of thawed *E.coli* TOP10 competent cells (Invitrogen). This was mixed gently and then treated as described in section 2.10.1.

Alternatively the *cbei* 4983 and *cbei* 4982 genes were cloned using a pJET1.2 cloning kit (Fermentas). 1 µl of fresh PCR product, 10 µl of 2X Reaction Buffer, 1 µl of pJET1.2/blunt Cloning Vector, (see Appendix IV), 7 µl water and 1 µl of T4 DNA ligase were mixed in a final volume of 20 µl. These were incubated for 5 minutes at 25 °C (room temperature) and kept on ice. 2.5 µl of the cloning reaction were added to a vial of *E.coli* TOP10 cells (Invitrogen). The mixture was mixed gently and then incubated on ice for 15 minutes. The remaining steps were carried out as described above in section 2.10.1.

2.10.3. Cloning of *cbei* 4984/ *cbei* 4983/ *cbei* 4982

The StrataClone PCR cloning kit was also used for cloning of the three genes, *cbei* 4984/ *cbei* 4983/ *cbei* 4982**.** Cloning reaction was set up which included 2 µl of fresh PCR product, 3 µl of StrataClone Cloning Buffer, 1µl of StrataClone Vector Mix amp/kan, see (Appendix IV) in a final volume of 6 µl. The procedure was carried out as described above in section 2.10.2.

2.11. Screening and Analysis of Clones

2.11.1. Screen for presence of cloned genes

Initial screening was for the presence of the gene using cloning primers. Single colonies were picked and streaked on a LB plate containing 100 µg/ml ampicillin and incubated overnight at 37 °C. Samples were then inoculated into 20 µl of sterile deionised water. These samples were heated at 100 °C in a heating block for 10 minutes, and plunged into ice for 3 minutes. The tubes were then centrifuged for 2 minutes at 13000 rpm, and 1 µl of the supernatant was used as the DNA template for PCR reaction.

The PCR reaction contained, 25 µl of BioMix (Bioline), 1 µl of each primer and the volume was made up to 50 µl with distilled water, the PCR cycles were done as described in section 2.8.

2.11.2. Determination of orientation of DNA inserts by PCR

Screening for gene orientation used a combination of primers directed against sequences in the cloned fragment and the vector to determine the orientation of DNA inserts within recombinant clones. Primer combinations depended on the cloned genes and the vector used, as described for individual experiments. The PCR reaction was set up and was run as described in section 2.11.1.

2.12. Preparation of plasmids by miniprep procedure

Plasmids were purified using the Fermentas GeneJET^{TM} Plasmid Miniprep Kit #K0502. Cells were inoculated into 10 ml of LB broth containing 100 µg/ml ampicillin, and the culture was incubated at 37 °C with vigorous shaking overnight. 4.5 ml of bacterial culture was collected in an Eppendorf tube by centrifuging at 13,000 rpm for 5 minutes (1.5 ml each time, three times) and supernatant was removed. The pellet was completely resuspended in 250 µl of the resuspension solution by vortexing. 250 µl of the lysis solution was add to the Eppendorf tube and mixed thoroughly by inverting the tube 4-6 times until the solution was clear. 350 µl of the neutralization solution was added to the tube and mixed immediately and thoroughly by inverting the tube several times. The clear solution was separated by centrifuging at 13,000 rpm for 5 minutes, and the supernatant transferred to the supplied GeneJET^{TM} spin column by pipetting and centrifuged for 1 minute. 500 µl of the wash solution (diluted with ethanol) was added to the column and centrifuged for 60 seconds (twice). The flow- through was discarded and the column was centrifuged for an additional 1 minute to remove residual wash solution. The GeneJET^{TM} spin column was finally transferred into a fresh Eppendorf tube and 50 µl elution buffer was added to the center of it, and incubated for 2 minutes at room temperature, and then centrifuged at 13,000 rpm for 2 minutes. The purified plasmid DNA was stored at -20 °C.

2. 12.1. Large-scale plasmid purification

Plasmids were purified using the QIAGEN plasmid Midi kit. A colony was transferred to 10 ml LB Broth with 50 μg/ml ampicillin and incubated overnight with shaking at 37 °C. 1 ml taken from overnight culture was transferred to a 250 ml flask containing 100 ml LB Broth with ampicillin at the same concentration and incubated with shaking at 37 °C overnight. 50 ml was transferred to a sterile polypropylene tube and centrifuged at 4000 xg for 20 minutes at 4°C, and the supernatant was removed. 4 ml of buffer P1 containing RNase was added to the pellet and mixed by pipetting up and down until no cell clumps remained. 4 ml of buffer P2 was added to the tube and mixed by inverting the tube 4-6 times, then incubated at room temperature for 5 minutes. 4 ml of chilled buffer P3 was then added to the tube and mixed immediately by inverting the tube several times and this was centrifuged at 4000 xg at 4 °C for 20 minutes. 4 ml of buffer QBT was added to the supernatant (cleared lysate) and loaded onto a purification column (QIAGEN-TIP) which was allowed to empty by gravity flow. 20 ml of buffer QC was used to wash the QIAGEN-TIP, then 5 ml of buffer QF was used to elute the DNA. 5 ml of room-temperature isopropanol was added to the eluted sample to precipitate the DNA and centrifuged at 4000 xg at 4 °C for 20 minutes. 3 ml of room-temperature 70 % ethanol was added to wash the DNA pellet, and centrifuged at 4000 xg for 20 minutes at 4 °C. 50 μl of water was added to dissolve the DNA pellet and then mixed by pipetting up and down. The sample was stored at -20 °C. Samples of plasmid DNA were sent for sequencing analysis by Beckman Coulter Genomics. Inserts were sequenced on both strands, starting from primer directed against the vector close to the point of insertion. After completion of the first sequence run, new primers were designed to allow extension of the sequence, until the entire insert has been sequenced.

The different primers used during sequencing analysis are listed in Table 2.4.

Table 2.4: List of primers used in analysis sequencing. The positions for annealing are shown in Appendix I

Primer Name	Reference Number	Primer Sequence
Cbei 0751 F*	510	5´-ACAATGCTTGATACGTATGG-3´
Cbei 0751 R*	511	5´-TATGCAATGCTGCTGTACC-3´
Cbei 4982 F*	450	5´-GAAGGTATTGCAGTAATTCC-3´
Cbei 4983 R* 281	455	5´-AAAGCTGCAATACCTTTCTC-3´
Cbei 4983 F*	508	5´-TTCTTTACAAGTCTTGGTGG-3´
Cbei 4983 R* 181	509	5´-ACAAATATCCAGTGAGAACC-3´

* F, forward primer; * R, reverse primer

2.13. Preparation and transformation of chemically competent cells of *E.coli*

A colony of *E.coli* from a LB plate without ampicillin was inoculated into 10 ml of LB broth. This culture was incubated at 37 °C overnight with shaking. 1 ml of overnight culture was added to 100 ml LB Broth and incubated for about 3 hours with shaking at 37 °C until OD_{600} was between 0.4 and 0.6. 50 ml of culture was then transferred aseptically to a polypropylene tube. This tube was stored on ice for 10 minutes, and the cells were recovered by centrifugation at 4000 xg at 4 °C for 10 minutes. The supernatant was discarded and final traces of medium were removed from the pellet by inverting the tube for 1 minute. The pellet was resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$ and stored on ice for 2-3 minutes and then centrifuged for 10 minutes at 4°C at 4000 xg. The supernatant was removed, and the pellet dried by inverting. The pellet was then resuspended finally in 2 ml of ice-cold 0.1 M $CaCl_2$ and stored on ice. 2 μl of plasmid was added to 200 μl of the cells, and the tubes were mixed several times before being stored on ice for 30 minutes. They were then transferred to a water bath for 90 seconds at 42 °C, and plunged back on ice for 2 minutes. 800 μl of LB medium was added to the tubes and the mixture was incubated for one hour at 37 °C. 50 and 100 μl was spread out onto LB plates containing 100 μg/ml ampicillin and then incubated overnight at 37 °C. Transformed colonies were picked off using toothpicks and scored in a grid like pattern onto petri dishes containing MacConkey agar with the required

carbohydrate in the presence or absence of ampicillin, and incubated for 48 hours at 37 °C.

Fermentation phenotype was compared with the phenotype of untransformed cells on MacConkey agar with or without antibiotic as required.

2.14. Characterization of recombinant *E.coli* cells containing gene *cbei* 0751

2.14.1. Utilization of glucose, mannose and N-acetylglucosamine in growing cultures

A sample of *E.coli* ZSC113 cells was transferred into 10 ml of LB broth with 50 µg/ml ampicillin and incubated overnight with shaking at 37 °C. 1 ml taken from an overnight culture was transferred to a 250 ml conical flask containing 100 ml of LB Broth with 50µg/ml ampicillin and 25mM (glucose, mannose and N-acetylglucosamine) and incubated at 37 ºC. Growth was measured by following $OD_{600.}$ Culture samples (0.5 ml) were taken every hour, transferred to an Eppendorf tube, and then centrifuged at 13,000 rpm for 10 minutes. The supernatants were removed and transferred to fresh tubes and stored at -20 ºC for the measurement of sugar concentration, by high performance liquid chromatography (HPLC). The columns used were a Dionex Carbopac PA-1 Guard column, 4 x 50 mm and Dionex Carbopac PA-100 column, 4 x 250 mm, and the detection was by a pulsed amperometric detector (PAD). Separation was achieved by high performance anion exchange (HPAE). At high pH (14), sugars are partially ionised, and therefore, are separated by anion exchange. Detection was by a pulsed amperometric detector (PAD). Sugars were detected by measuring the electrical current generated by oxidation at the surface of the electrode. Sample was prepared in 1.5 glass vials, 60 µl of cellobiose was added to 300 µl of diluted sample (1:200) and mixed well. A glass vial of fresh calibration standard was putted in position 1 of the instrument and then the samples were load into the tray in sequence. The set detector (PAD) was set up as follows; pulse duration; T1= 2   T2= 2   T3= 5   Range= 2.   Display direction = +   Response time = 0.3 secs.   E1= +0.1V   E2= +0.7V   E3= -0.1v Range= 2.

2.14.2. Glucose uptake by whole cells

A single colony of *E.coli* ZSC113 was transferred to 10 ml of LB broth with 50 μg/ml ampicillin, and incubated overnight at 37 °C with shaking. 2 ml taken from the overnight culture were transferred to a 250 ml flask containing 100 ml of LB Broth with ampicillin and 25 mM glucose. This culture was shaken at 37 °C for 3- 4 hours, until the OD_{600} was 0.6 - 0.8. 100 ml was transferred to 50 ml a polypropylene tube, and centrifuged at 4000 xg for 10 minutes at 4 °C. The cells were washed with 50 mM potassium phosphate buffer pH 7.0, and subsequently resuspended in 10 ml of the same buffer and stored on ice. 20 μl cells in 50 mM potassium phosphate buffer pH 7, were incubated for 3 minutes at 37 ºC, and then radiolabelled sugar [^{14}C glucose (9.5 mM; 1 mCi/mmole)] was added to the tube to give final concentration of 0.2 mM. The total volume was 1000 μl. 150 μl samples were removed, filtered through a glass fibre disc (Whatman GF/F paper) and washed twice with 5 ml of potassium phosphate buffer, and then discs were dried under a heat lamp. Discs were transferred into 5 ml leak-proof scintillation tubes and 4 ml of scintillation ultima gold F (PerkinElmer) was added. The radioactivity was measured using a Liquid Scintillation Analyzer (Packard, 1900CA). All experiments included sample blanks consisting of a 0.2 mM solution of [^{14}C]-glucose in phosphate buffer which was treated as for the experimental samples. Small volumes (20 μl) of the blank solution were spotted directly on glass fiber discs, dried and counted, to allow for conversion of radioactive counts to nmoles of sugar phosphate.

2.15. Preparation of cell extracts

10 ml of LB broth containing 50 μg/ml ampicillin were inoculated with *E.coli* ZSC113 and incubated overnight with shaking at 37 ºC. The entire overnight culture was transferred to a 1000 ml flask containing 500 ml LB Broth with 50 μg/ml ampicillin and 25 mM glucose. The culture was incubated overnight at 37 ºC with shaking, and then the cells were harvested by centrifugation at 12,000 xg at 4 ºC for 10 minutes. The supernatant was removed and the cells were washed twice with 50 mM potassium phosphate buffer pH 7.0. The pellets were resuspended and combined, and centrifuged again. The supernatant was removed and the pellet was weighed and stored at -20 *ºC* until used. The pellet was thawed and suspended (4 ml/g) in 50 mM potassium phosphate buffer pH 7 containing 5 mM $MgCl_2$ and 1 mM DTT. The cells were disrupted by two passages through a French pressure cell press at 20,000 Ibf in^{-2} (138MPa). The cell debris was removed by centrifugation at 12,000 x g at 4 ºC for 10

minutes, and the cell extract (supernatant) was aliquoted and then flash frozen in liquid nitrogen then stored at -70 ℃.

2.15.1. Assay of sugar phosphorylation in cell- free extracts

Glucose phosphorylation with PEP or without PEP was assayed by precipitation of labelled sugar phosphate in barium bromide solution ($BaBr_2$) as described by Mitchell *et al*, (1991). The assay mixture contained 2 mM DTT**,** 12 mM KF**,** 5 mM $MgCl_2$**,** cell extract, 50 mM potassium phosphate buffer, 1 mM PEP and 0.2 mM ^{14}C-glucose. The sample was incubated at 37 ℃ in a water bath for 3 minutes, before the ^{14}C-glucose was added. Samples (0.15 ml) were taken into 2 ml of 1 % $BaBr_2$ in 80 % (vol/vol) ethanol. The precipitates were removed by filtration through a glass fibre disc (Whatman GF/F paper) and then washed once with 5 ml of 80 % (vol/vol) ethanol. The filters were dried under a heat lamp and treated as described in section 2.14.2.

2.16. Dialysis of cell extracts

Samples of extracts were dialyzed by using a Dialysis cassette Slide- A-Lyzer (3 ml, molecular weight cut off 3,500), from Thermo Scientific (Cat# 66330). Up to 1.5 ml of extract was dialysed against 1 liter of 50 mM potassium phosphate buffer pH 7, overnight at 4 ℃.

2.17. Restriction digests

Restriction digests were carried out in Eppendorf tubes. Digests contained 2 μl of 1 μg/μl DNA, 1 μl of each required FastDigest enzyme (Fermentas), 2 μl of 10x FastDigest buffer, and were made up to 20 μl with nuclease-free water. The mixture was vortexed briefly and then incubated for 30 minutes, at 37 °C in a heating block. The 10x FastDigest green buffer was used as loading buffer in gel electrophoresis. The mixture was loaded in the gel and electrophoresed for 1 hour at 80 V, and results were analysed by viewing under UV illumination.

2.17.1. Gel extraction

GeneJET Gel Extraction Kit, # K0691 (Fermentas), was used to purify fragments of DNA from the gel. A gel slice containing the DNA was cut out using a sterile scalpel and then transferred into a pre-weighed 1.5 ml Eppendorf tube. After adding the gel slice the tube was weighed again, and the difference in weight was recorded. 1 volume of binding buffer was added to the gel slice (e.g. 200 μl of binding buffer was added for

every 200 mg of agarose gel). The gel mixture was incubated at 60 °C for 10 minutes in a heating block, with the tube inverted every 2 minutes to facilitate the melting process. When the gel was completely dissolved, the gel solution was transferred to a GeneJET purification column and then centrifuged for 1 minute at 13,000 rpm. The liquid was discarded. 700 µl of washing buffer was added into the GeneJET purification column and centrifuged at 13,000 rpm for 1 minute, the solution was removed and the column was put back into the same tube. To remove the residual washing buffer the empty purification column was centrifuged for 1 minute at 13,000 rpm. The column was then transferred into a sterile 1.5 microcentrifuge tube and 30 µl of elution buffer was added to the centre and centrifuged for 1 minute at 13,000 rpm. The eluted DNA was stored at -20 °C.

2.17.2. Ligation of DNA fragments

Fragments of linear DNA were joined together using a Rapid DNA Ligation Kit (Fermentas, K# 1422). In a 1.5 ml sterile Eppendorf tube, 11 µl of nuclease-free water, 4 µl of 5 x rapid ligation buffer, 1 µl of T4 DNA ligase, and 3 µl of DNA was added with 1 µl vector (3:1 ratio of amount of DNA). The total volume was 20 µl. The ligation mixture was vortexed for a few seconds and incubated for 5 minutes at room temperature and then stored at 4 °C. 5 µl of the mixture was transformed into *E.coli* TOP10 competent cells as described previously in section 2.1. The cells were isolated after transformation on LB plates containing 50 µg/ml ampicillin, and then screened by PCR to confirm the presence of the cloned fragments.

2.18. Ribonucleic acid (RNA) extraction

Spores of *C. beijerinckii* NCIMB 8052 were heated at 80 °C for 10 minutes and then added to 20 ml RCM starter culture and incubated under anaerobic conditions at 37 °C overnight. 1 ml of overnight culture was transferred into 100 ml of CBM with 1% (wt/vol) sugar(s) as required and incubated overnight under the same conditions. 1 ml of overnight culture was then transferred to fresh 100 ml of the same medium and incubated until OD_{600} was between 0.5-0.7.

750 µl of culture sample and 750 µl of RNA stabilization solution (Qiagen) were mixed for 2-3 seconds and stored at room temperature for 7 minutes. The cells were then harvested by centrifuging at 10,000 rpm for 10 minutes, the supernatant was removed and the pellets quickly plunged into liquid nitrogen and stored at – 70 °C.

The RNA purification was carried out using an RNeasy® mini Kit (Qiagen). According to the manufacturer's instructions, 10 µl β- mercaptoethanol (β-ME) was added to 1 ml of RLT buffer, add 44 ml of 96 % ethanol was added to 11 ml of RPE buffer. Lysozyme buffer was prepared as TE buffer containing 3 mg/ml lysozyme. The pellet was thawed on ice and 300 µl of lysozyme buffer was added and the pellet was resuspended by pipetting, then the mixture was incubated at room temperature for 12 minutes with vortexing every two minutes. 700 µl of RLT Buffer containing β-mercaptoethanol was added to the Eppendorf tube and vortexed. 500 µl of 96 % ethanol was then added and mixed by pipetting. The mixture was transferred to an RNeasy Mini spin column and centrifuged for 15s at 10,000 rpm. 700 µl of RW1 Buffer was added to the column and centrifuged for 15s at 10,000 rpm. The column was then removed into a 2 ml collection tube. 500 µl of RPE Buffer was added and centrifuged at 10,000 rpm for 15s, followed by another 500 µl of RPE Buffer with centrifugation at 10,000 rpm for 2 minutes. The spin column was then removed into a new Eppendorf tube, and finally 50 µl of RNase-free water was added into the column and then centrifuged at 10,000 rpm for 1 minute to collect the RNA. The Eppendorf tube containing the RNA was immediately placed in liquid nitrogen and then stored at -70 °C.

To estimate the RNA concentration, a sample of the RNA was diluted 2 : 48 in 1x TE buffer in a sterile PCR tube. Absorbance was measured at 260 nm in an Eppendorf uvette® using a BioPhotometer, with TE buffer as the blank.

2.19. Agarose gel electrophoresis of RNA

Total RNA samples were examined using agarose gel electrophoresis. Agarose was dissolved to concentration of 1 % (w/v) in 1 x MOPS (morpholinopropanesulfonic acid) buffer. 10 ml of 10x MOPS stock buffer (200 mM MOPS, 50 mM sodium acetate, and 10 mM EDTA at pH 7.0,) were added to 80 ml distilled water. 1 g agarose was dissolved by heating in a microwave oven, and after cooling 10 ml of formaldehyde was added to the mixture and poured into the gel container. 5 µl of purified RNA was mixed with 10 µl of the RNA loading buffer, consisting of 10 % (v/v) of 10 x MOPS buffer, 16 % (v/v) formaldehyde, 50 % (v/v) formamide, 0.1 mg/ml ethidium bromide, and 0.01 % (w/v) bromophenol blue, and then denatured for 10 minutes at 70 °C, and placed on ice for 3 minutes. The RNA samples were vortexed before loading into the gel, which was electrophoresed for 30 minutes at 100 volts in 1x MOPS.

2.20. Slot-blot hybridization

2.20.1. DEPC treatment

All solutions used in hybridization procedures were treated with DEPC to remove any RNase. 1000 μl of DEPC was added to 1000 ml of distilled water and then the mixture was stirred for 60 minutes at room temperature, followed by autoclaving the solution to remove any remaining DEPC.

2.20.2. Labeling of DNA probes

DNA probes were labelled using digoxinenin-11-dUTP (Roche Diagnostics GmbH, Germany). Primers were designed for amplification of 200-400 bp DNA, see Appendix I, and are shown in Table 2.5. PCR reaction for DIG-labelled DNA probes used 20 μl of deionized water, 25 μl of 2x BioMix buffer (Bioline), 2 μl of digoxinenin-11-dUTP, 1 μl forward primer, 1 μl reverse primer, and 1 μl of DNA template. The PCR reaction was as described above in section 2.8, and the samples were run on a 1 % agarose gel for 90 minutes at 80 V. Labelling of the probes was confirmed by preparing serial 1:5 dilutions, and 1 μl of each dilution was applied onto a nylon membrane as a dot. The membrane was placed in an ultraviolet cross- linker for 1 minute to fix the DNA, and then was transferred to 10 ml blocking buffer and incubated for 1 hour at room temperature. 1μl of Anti-Dig (Anti-Digoxigenin-AP Fab fragments) (Roche Diagnostics GmbH, Germany) was added to the blocking buffer and the membrane was incubated for 30 minutes under the same conditions. The membrane was then washed 4 times for 10 minutes with DIG-1 buffer and finally the nylon was immersed in DIG-4 buffer for 5 minutes.

For detection, the nylon membrane was placed between two acetate sheets and 800 μl of CDP-star chemiluminescent substrate solution (Sigma) was poured between them, and any bubbles were removed. An X-ray film (Lumi-Film Chemiluminescent Detection Film) was placed on the top and incubated for 60 minutes in the dark, and then the X-ray film was washed with developing solution (GBX developer- replenisher/ Sigma) for 1 minute and fixing solution (GBX fixer- replenisher/ Sigma) for 1 minute, finally rinsing in water and drying at 25 °C.

Table 2.5: Primers used for preparation of hybridization probes

Primer Name	Reference Number	Primer Sequence
Cbei0751dig F*	510	5´-ACAATGCTTGATACGTATGG -3´
Cbei0751dig R*	511	5´-TATGCAATGCTGCTGTACC-3´
Cbei4984dig F*	446	5´-TGAAAGATACCCTGAATTGG-3´
Cbei4984dig R*	447	5´-AAAACTCTGCCATAAGAACG-3´
Cbei4983dig F*	448	5´-TCTATGATATACGGTCTAGC-3´
Cbei4983dig R*	449	5´- ATTCCATATAATGCTGGTGC -3´
Cbei4982dig F*	450	5´-GAAGGTATTGCAGTAATTCC-3´
Cbei4982dig R*	451	5´-TATCTCAGCTCCAGTTTGC-3´

* F, forward primer; * R, reverse primer

2.20.3. Slot Blotting

The purified RNA was diluted to 50 ng/µl with nuclease-free water. The samples were incubated for 10 minutes at 70 °C and immediately placed on ice for three minutes, the blotting stack consisted of three pieces of Whatman 3 MM paper, and a nylon membrane which was immersed in 20 x SSC for five minutes with the orientation marked. After the stack was assembled on the vacuum manifold (Schleicher & Schuell) traces of 20x SSC were removed from the wells by vacuum and 10 µl of RNA samples were loaded in the wells. After 20 minutes under vacuum, the nylon membrane was removed and UV cross-linked two times as described in section 2.20.2.

2.20.4. Hybridisation of RNA

A hybridisation bottle and mesh were treated with RNA-zap, then rinsed with DEPC treated water. 10 ml of hybridisation buffer was poured into the bottle and incubated for 30 minutes at 58 °C. 1.5 µl of DIG-labelled probe PCR product was diluted in 18.5 µl nuclease-free water and heated at 95 °C for 10 minutes and then placed on ice for 5 minutes and poured into the hybridisation bottle. The bottle was incubated overnight at 58 °C.

2.20.5. Washing, blocking and detection

After hybridisation, the nylon membrane was washed three times with 2X SSC high salt concentration buffer and once with 0.2X SSC low salt concentration buffer for 15 minutes. After washing the nylon was rinsed by immersing in DIG-1 buffer, transferred to 10 ml blocking buffer, then treated as described in section 2.20.2.

2.21. Reverse transcriptase (RT) PCR

Trace genomic DNA was removed from RNA samples using the Bioline RNA isolation kit. 5 µg of RNA was mixed thoroughly with 5 µl of 10x DNaseI reaction buffer, 5µl of DNaseI, and DEPC-treated water to make up the volume to 100 µl, and the mixture was then incubated for 10 minutes at 37 °C. The reaction was stopped by adding 1 µl of 0.5 M EDTA and heating for 10 minutes at 75°C.

cDNA (complementary DNA) strands were synthesized from RNA and amplified in a PCR tube using the Bioline cDNA kit. This reaction consisted of two stages. The first stage was synthesis of cDNA using RNA as template, nucleotide primers and reverse transcriptase (RT) enzyme to synthesize a complementary DNA strand. The second stage was copying and amplification of the cDNA product. 1 µg (10 µl) of DNaseI-treated RNA was added to 1 µl of random hexamer primer and 1 µl of 10 mM dNTPs, mixed together then incubated for 10 minutes at 65 °C, and then placed on ice for 2 minutes. 4 µl of 5x reverse transcriptase buffer, 1 µl of RNase inhibitor, 0.25 µl of reverse transcriptase and 2.75 µl of DEPC water were added to the mixture and then incubated at 42 °C for 30 minutes, heated for 15 minutes at 70 °C, and immediately placed at 4 °C. The PCR reaction for cDNA, used 21 µl of deionized water, 25 µl of BioMix (Bioline), 1µl forward primer (Cbei0751dig), 1µl reverse primer (Cbei0751dig), and 2 µl of cDNA product. The PCR reaction was as described above in section 2.8.

2.22. Gene knockout using the ClosTron

DNA sequences required for targeting genes *cbei* 0751 and *cbei* 4983 were designed using the algorithm available on the ClosTron website at the University of Nottingham http://www.clostron.com. Integration vectors were then ordered from DNA 2.0. The plasmids obtained from DNA 2.0, were extracted from a Whatman GFC filters by placing then on a sterile surface, adding 100 µl of 100 mM Tris–HCl, pH 7.5 and incubating for 2 minutes, at room temperature. The bottom of a small tube was punctured, the filter was placed in the tube and it was placed in a 1.5 ml tube, and centrifuged for 1 minute. Approximately 90 µl buffer containing 2 µg plasmid DNA, was expected to be recovered.

2.22.1. Bacterial strain used in gene knockout

E. coli strain C600RK2 was recovered from storage at -70 °C, and was inoculated onto an LB plate without antibiotic and incubated overnight at 37 °C to grow the cells.

2.22.2. Transformation of *E. coli*

A single colony of *E. coli* strain C600RK2 from the LB plate without antibiotic was inoculated into 5 ml of LB broth and incubated overnight at 37 °C. 1 ml of overnight culture was transferred into a flask containing 100 ml LB broth and incubated for approximately 3 hours until the OD_{600} reached 0.6. Competent cells were prepared and transformed as described previously, in section 2.13.

2.22.3. Conjugation

A colony of *E .coli* C600RK2 carrying plasmid was inoculated into 5 ml of LB broth with 250 µg/ml spectinomycin and incubated overnight at 37 °C on a shaking incubator. 1 ml of overnight culture was transferred into each of three 1.5 ml Eppendorf tubes and centrifuged for two minutes at 13.000 rpm at room temperature, and the supernatants were completely discarded. 1 ml of phosphate buffer saline (PBS) was added to each tube to wash the pellet, the supernatant was poured off and the pellet was transferred to the anaerobic cabinet for conjugation. 0.8 ml of *C. beijerinckii* spores had been heated at 80 °C for 10 minutes and then transferred into 10 ml 2X YTG broth medium without any antibiotic and incubated overnight at 37 °C in the anaerobic cabinet. 1 ml of overnight culture was added to fresh 10 ml 2x YTG medium and ten fold (v/v) serial dilutions were prepared and incubated overnight in the same conditions.

The culture which was closest to the required state ($OD_{600} = 0.6$) was used for conjugation. The conjugation was initiated by adding 200 μl of *C. beijerinckii* culture to an *E .coli* cell pellet and gently resuspending by pipetting up and down several times. Ten 20 μl samples of mixture were transferred onto 2X YTG plates (three plates) without antibiotic and incubated at 37 °C under anaerobic conditions for 8-24 hours. All the cells from the plates were recovered using an inoculation loop and resuspended in 1 ml of PBS solution. The mixture of cells was spread onto CBM plates containing 750 μg/ml spectinomycin (selective for clostridia) and 250 μg/ml cycloserine (to kill *E. coli* cells) and incubated anaerobically at 37 °C until colonies appeared.

2.22.4. Purification of transconjugants

Colonies from the conjugation selection plates were picked and re-streaked on fresh plates of the same type and incubated at 37 °C until growth appeared on the plates. Several colonies were then restreaked onto CBM plates containing 10 μg/ml erythromycin four times to purify a transconjugant clone.

2.22.5. DNA isolation from putative mutants

DNA isolation was carried out using a PUREGENE® Bacteria kit (Qiagen).

A single colony from a selective plate was transferred to a 15 ml tube with 10 ml 2X YTG medium containing 10 μg/ml erythromycin, and incubated at 37 °C under anaerobic conditions for 2 - 4 days. 1 ml of the cell culture was transferred into a sterile Eppendorf tube and centrifuged at 13,000 rpm for 5 second and the supernatant was removed carefully by pipetting. 300 μl of cell suspension solution was added into the tube and mixed gently by pipetting up and down. 5 μl of lysozyme buffer (10 mg/ml lysozyme in 10 mM Tris-Cl, pH 8.0) was added immediately, mixed by inverting the tube 25 times and incubated at 37 °C for 30 minutes. The mixture was centrifuged for one minute at 13,000 rpm, and then the supernatant was discarded by pipetting. 300 μl of cell lysis solution was added into the tube and mixed by pipetting. 1.5 μl of RNase A solution was added into the mixture and mixed by inverting 25 times and incubated at 37 °C for 60 minutes then immediately placed on ice for one minute to cool the sample. 100 μl of protein precipitation solution was added to the tube and vortexed for 20 seconds at high speed, and then the tube was centrifuged at 13,000 rpm for three minutes and the supernatant was transferred into a new 1.5 ml tube. 300 μl of isopropanol was added and mixed by inverting gently 50 times, and then after centrifugation for one minute at 13,000 rpm, the supernatant was removed and the tube

was inverted on a clean piece of absorbent paper. 300 µl of 70 % ethanol was added and the tube was inverted ten times to wash the DNA pellet, and centrifuged at 13,000 rpm for one minute at room temperature. The supernatant was carefully discarded, and the tube was drained on absorbent paper for 20 minutes. 100 µl of DNA hydration solution was added and vortexed for 5 seconds, then incubated for 1 h at 65 °C to dissolve the DNA. The DNA was incubated overnight at room temperature with gentle shaking, and the sample was centrifuged briefly and then transferred into a new sterile Eppendorf tube and stored at -20 °C.

2.22.6. Screening of DNA by PCR

PCR was used to screen for the presence of the intron within the target gene. The PCR reaction was carried out as follows: 21 µl of sterile nuclease-free water was added to 25 µl of BioMix (Bioline), 1 µl each of flanking primers or EBS primer plus one flanking primer, and 2 µl of DNA isolated from putative mutants as a template. Primers and their position of annealing are shown in Table 2.6 and Appendix I. The PCR was carried out as described previously in section 2.11.1, and the products were analysed by electrophoresis at 80 V for 60 minutes in a 1% agarose gel in TAE buffer as described in section 2.9.

Table 2.6: List of primers used for analysis of putative mutants

Primer Name	Reference Number	Primer Sequence
Cbei-glcG- F*	435	5´-GTTAATGCATATGTTAGGTGTTGGCGTA GGTAT-3´
Cbei-glcG- R*	436	5´-ATAGCCCGATGCTCCAGTTTGTTTTAACA AAC-3´
Cbei-glcX- F*	437	5´-GTGGTACAAAATTTGTCCCAATGGTTTCT ATTATCG-3´
Cbei-glcX- R*	438	5´-CATAGCCAAAGCTGCCCCTGGCAA-3´
EBS	452	5´-CGAAATTAGAAACTTGCGTTCAGTAAA C-3´

* F, forward; * R, reverse

2.23. Bioinformatics analysis

Protein sequences were obtained using the BLAST programs at National Center for Biotechnology Information (Altschul *et al*., 1997). Multiple alignment of Protein sequences was performed using ClustalW2 of the European Bioinformatics Institute (Chenna *et al*., 2003), and phylogenetic trees were drawn using TreeView (Page, 1996).

CHAPTER 3

RESULTS

FUNCTIONAL OF ANALYSIS OF THE GENE *CBEI* 0751

3. Results

3.1. Characterisation of potential glucose PTS genes in *C. beijerinckii*

As described in the introduction, the whole genome of *C. beijerinckii* NCIMB 8052 has been sequenced and it has been found that *C. beijerinckii* has forty three complete phosphotransferase systems (Shi *et al*., 2010). Seven are members of glucose subfamily, and three are within the phylogenetic branch which is associated with glucose uptake systems. These three permeases are encoded by; *cbei* 0751 which encodes a PTS with three domains IIA, IIB and IIC together; *cbei* 4983 which encodes two domains IIB,IIC and *cbei* 4982 which encodes a separate IIA domain; and *cbei* 4532 which encodes two domains IIBC, and *cbei* 4533 which encodes a IIA domain.

As a preliminary experiment to precede analysis of the potential functions of the glucose subfamily phosphotransferase systems, the ability of *C. beijerinckii* NCIMB 8052 to take up and grow on different sugars was examined. *C. beijerinckii* was grown on CBM plates supplemented with a number of different carbohydrates. As shown in Table 3.1, the bacterium was capable of growth on glucose, mannose, maltose, sucrose and trehalose, all of which are known to be carbon sources which are transported by phosphotransferase systems of glucose-glucoside family.

Table 3.1. Growth of *C. beijerinckii* on CBM plates containing; glucose, mannose, maltose, sucrose, trehalose or no carbon source. Plates were incubated for 24 hours at 37 ℃.

Carbon source	Growth/ No Growth
Glucose	+
Mannose	+
Maltose	+
Sucrose	+
Trehalose	+
No carbon source	-

The sequences of the proteins were obtained from the *C. beijerinckii* genome data base (http://genome.jgi-psf.org/clobe/clobe.home.html). A BLAST analysis was carried out to identify the position of the IIC domain in each protein. This information was used to extract the IIC domain region from the protein and these regions were aligned using

ClustalW2 and analysed in the form of a phylogenetic tree along and with IIC domains from characterised phosphotransferases in other bacteria.

As shown in the phylogenetic tree (Figure 3.1), the IIC domains encoded by *cbei* 0751 and *cbei* 4983 are in a similar place. The *cbei* 4532 IIC domain is clearly different and related to N-acetylglucosamine systems from other bacteria. This *C. beijerinckii* PTS has been shown to transport both N-acetylglucosamine and glucose (Al Makishah and Mitchell, 2013). The *cbei* 0751 domain occupies a position in between maltose and glucose systems, while the *cbei* 4983 domain is closely related to the *Eco*MalX (Frömmel *et al*., 2013). The identity between the IIC domains of the Cbei 0751 protein and Cbei 4983 protein is 45.3 %.

Table 3.2 also shows the relationship between IIC domains included in the phylogenetic tree (Figure 3.1). As was expected, the Cbei 4532 protein domain is the most closely related to N-acetylglucosamine systems of other bacteria. The Cbei 0751 domain is the most closely related to glucose systems of other bacteria. The Cbei 4983 domain is the most closely related to three systems which are (A), BgIP, the β-glucoside specific PTS of *Staphylococcus mutans* (Cote *et al*., 2000), (B), *Eco*MalX the maltose specific PTS of *E. coli* (Reidl and Boos, 1991), and (C), PttB a trehalose specific PTS of *S. mutans* (Webb et al., 2007).

The *cbei* 0751 gene which encodes a complete PTS (IICBA domains) is upstream of a gene encoding a transcriptional antiterminator. Genes encoding the glucose PTS of *C. acetobutylicum* (Tangney and Mitchell, 2007) and *B.subtilis* (Stülke *et al*., 1997), are also associated with an antiterminator. An antiterminator is a protein that binds to mRNA and is involved in regulation of gene expression, and it is therefore likely that expression of the *cbei* 0751 gene may be antiterminator dependent. The *cbei* 4983 gene encoding the IICB domains of a PTS is upstream of a gene encoding a IIA domain, and downstream of a gene encoding a glycoside hydrolase as shown in Figure 3.2. This arrangement strongly suggests the substrate of the PTS should be a disaccharide, since the phosphorylated product of the PTS is probably hydrolysed by the hydrolase enzyme.
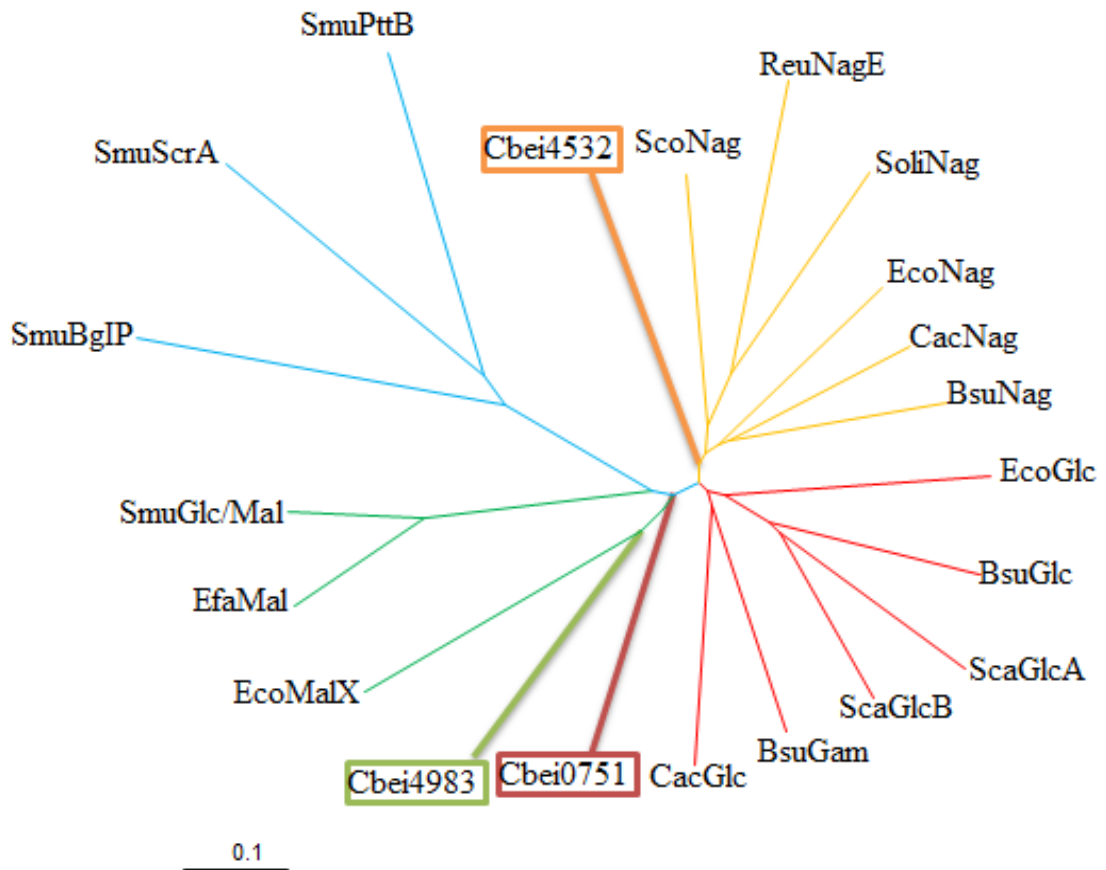
Figure 3.1: Phylogenetic tree of IIC domain including the relationship of glucose-glucoside family PTS systems. A Phylogenetic tree was constructed from the amino acid of different strains included *E. coli* glucose (*Eco*Glc) (WP_000317748), *B.subtilis* glucose (*Bsu*Glc) (NP_389272), *Staphylococcus carnosus* glucose *(Sca*GlcA) (YP_002634092), *S.carnosus* glucose (*Sca*GlcB) (YP_002634093), *B.subtilis* glucosamine *(Bsu*Gam) (NP_388117), *C.acetobutylicum* glucose(*Cac*Glc) (NP_347209), *E.coli* maltose*(Eco*MalX) (P19642), *Enterococcus faecalis* maltose*(Efa*Mal) (NP_814695), *S.mutant* glucose/maltose*(Smu*Glc/Mal) (NP_722340), *S. mutants* β-glucoside (*Smu*BglP) (NP_721375), *S.mutants* sucroseA(*Smu*ScrA) (NP_722158), *S.mutants* putative trehaloseB (*Smu*PttB) (NP_722334), *Streptomyces coelicolor* N-acetylglucosamine (*Sco*Nag) (NP_627133), *Ralstonia eutrophus* N -acetylglucosamine (*Reu*NagE) (YP_724831), *Streptomyces olivaceoviridis* N-acetylglucosamine (*Soli*Nag)(CAD29623), *E. coli* N-acetylglucosamine (*Eco*Nag)(NP_415205), *C. acetobutylicum* N-acetylglucosamine (*Cac*Nag) (NP_347981), *B.subtilis* N-acetylglucosamine (*Bsu*Nag) (NP_388651). All member are of the glucose subfamily, and they have been characterized as glucose (Glc), glucoseamine (Gam), maltose (Mal), N-acetylglucosamine (Nag), and β-glucoside phosphotransferase, *Cbei* 0751, is a glucose PTS. *Cbei* 4532 is N-acetylglucosamine PTS, *Cbei* 4983 might be a maltose PTS. 0.1, amino acid substitutions per sequence site.
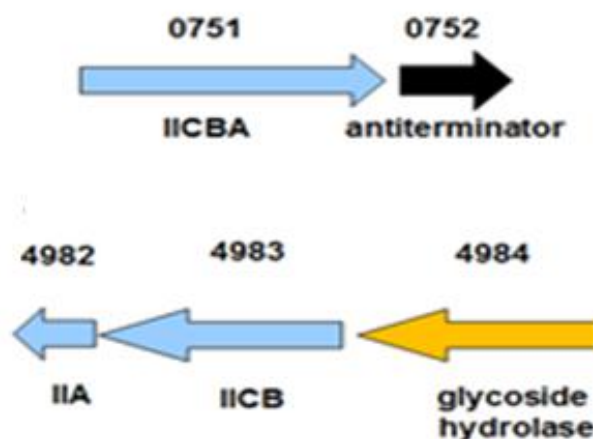
Figure 3.2: The organization of genes, *cbei* 0751 and *cbei* 4983, *cbei* 0752 encodes a putative anti-terminator protein, and *cbei* 4984 encodes a putative glycoside hydrolase.

Table 3.2: BLAST homology results for the deduced amino acid sequences of the IIC domain of glucose, β-glucoside, maltose and N-acetylglucosamine PTSs and glucose subfamily PTSs of *C. beijerinckii.*

Species/system	Cbei 0751		Cbei 4983		Cbei 4532	
	% Identity	% Similarity	% Identity	% Similarity	% Identity	% Similarity
E.coli Glc	40	57	38	55	39	58
B.subtilis Glc	47	64	43	62	35	53
Staphylococcus carnosus GlcA	45	64	42	62	41	57
S. carnosus GlcB	44	62	41	60	39	54
B.subtilis Gam	45	60	40	56	38	58
C. acetobutylicum Glc	40	55	34	50	35	52
Streptomyces coelicolor Nag	40	54	31	50	41	58
Ralstonia eutrophus NagE	45	59	37	56	46	62
Streptomyce solivaceoviridis Nag	38	55	35	54	43	61
E.coli Nag	40	54	33	48	42	59
C. acetobutylicum Nag	40	53	38	51	42	59
*B.subtilis*Nag	39	53	34	49	45	62
E.coli MalX	42	61	43	62	32	49
Enterococcus faecalis Mal	35	50	33	51	33	44
S. mutants Glc/Mal	34	50	33	51	30	45
S. mutants BgIP	26	42	45	51	25	42
S. mutants ScrA	41	51	33	48	25	40
S. mutants PttB	28	45	37	63	27	41

3.2. Cloning and characterization of gene *cbei* 0751

3.2.1. Amplification of gene *cbei* 0751 by PCR

As demonstrated in the previous section, the protein encoded by gene *cbei* 0751 was identified as the most likely glucose phosphotransferase system in the organism. This protein encodes a PTS protein with three domains IIA, IIB and IIC together therefore encodes the entire phosphotransferase system. Therefore, the aim was to investigate whether the encoded protein could function as a glucose PTS. The *cbei* 0751 gene was amplified by PCR as described in Materials and Methods section 2.8. The size of obtained product was between 2000 and 2500 bp, in agreement with the expected size of 2364 bp, as shown in Figure 3.3. The amplified DNA was then cloned in pCR$^®$ 2.1-TOPO$^®$ vector and transformed into *E.coli* and plated onto LB agar containing ampicillin.
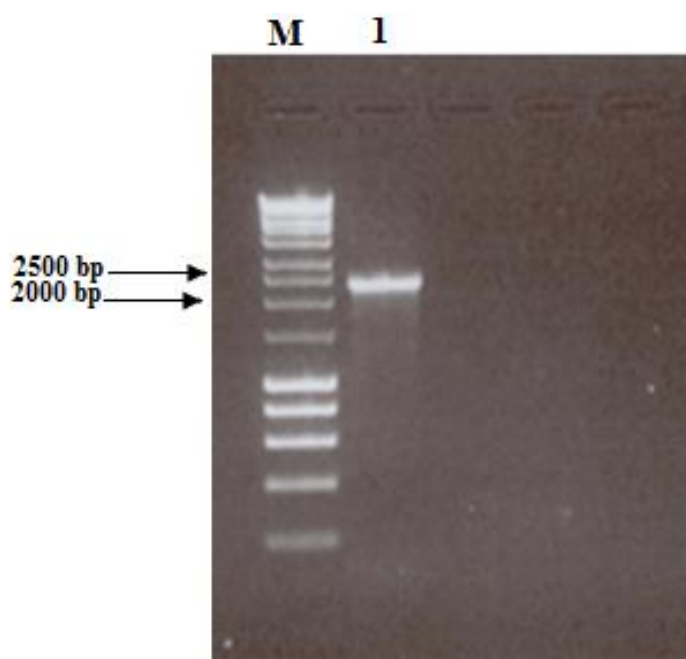
Figure 3.3: PCR amplification of gene *cbei* 0751; lane M, Hyperladder I used as a marker; lane 1, amplified product from PCR reaction using the forward primer 356 and the reverse primer 357.

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3.2.2. Screening and analysis of clones

40 colonies were picked off and transferred onto fresh LB agar plates and incubated under the same conditions. These were given reference numbers, and incubated overnight at 37 ℃. Colonies were then screened to identify the gene insert within the vector (Figure 3.4). Only two colonies (numbered ME15 and ME20) were found to contain the gene insert with the same size as the cloned PCR fragment. The gene had been successfully cloned into the pCR® 2.1-TOPO® vector, although the number of recombinants recovered was small.
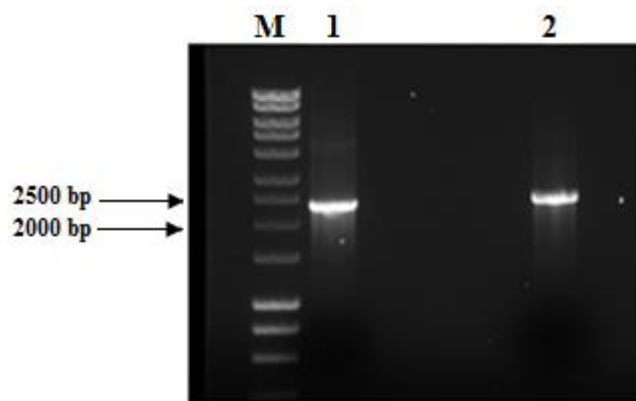
Figure 3.4: Screening of transformants by PCR. The presence of *cbei* 0751 was determined using the forward primer 356 and the reverse primer 357; lane M, Hyperladder I; lane 1, sample ME15; lane 2, sample ME20.

3.2.3. Determination of orientation of DNA inserts by PCR

In the next stage to determine the insert orientation, a new PCR was carried out, using different combinations of primers directed against the vector and the insert. The cloned DNA could be inserted in 5′- 3′ or 3′- 5′ orientation with respect to the *lac* promoter on the vector. The 5′- 3′ orientation would be in the suitable direction for expression by the *lac* promoter within the pCR® 2.1-TOPO® vector. If the combination of the M13 reverse primer and the reverse cloning primer 357 gives a product, that indicates the insert is under the control of the *lac* promoter. However, the primer combination of the M13 reverse and forward cloning primer 356 should not give a PCR product if the insert is under control of the *lac* promoter (Figure 3.5(i)). Likewise, the combination of the T7 promoter primer 373 and the primer 357 should not give a product if the insert is under the control of *lac* promoter, but the combination of the T7 promoter primer and the primer 356 will give a product if the insert is under the control of the *lac* promoter

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(Figure 3.5(i)). On the other hand, different products will be obtained if the insert is in the opposite orientation, under control of the T7 promoter (Figure 3.5(ii)).

The results of the analysis are shown in Figures 3.6 and 3.7. According to the results, the inserts were cloned in opposite orientation in the two vectors. Thus, ME15 gave product with the M13 reverse primer and forward primer 356, and no amplification with M13 reverse primer and reverse primer 357; the cloned gene was therefore in the 3´- 5´ orientation (Figure 3.6). ME20 did not give product with the M13 reverse primer and forward primer 356, but gave product with the M13 reverse primer and reverse primer 357, so the orientation was 5´- 3´ and under the control of *lac* promoter as shown (Figure 3.6). As expected, ME15 did not give product with forward primer 356 and T7 promoter primer, but gave product with reverse primer 357 and T7 promoter primer (Figure 3.7). ME20 gave product with forward primer 356 and T7 promoter primer, but did not give product with reverse primer 357 and T7 promoter primer. The results clearly indicated that the orientation of the insert in the two plasmids was different (Figure 3.7).

One gene was potentially expressed under control of *lac* promoter and the other one was under the control of the T7 promoter. Therefore, it was expected that ME20 will be expressed in *E. coli* because it is under the *lac* promoter whereas the other gene may not be expressesd because it is in the opposite orientation.
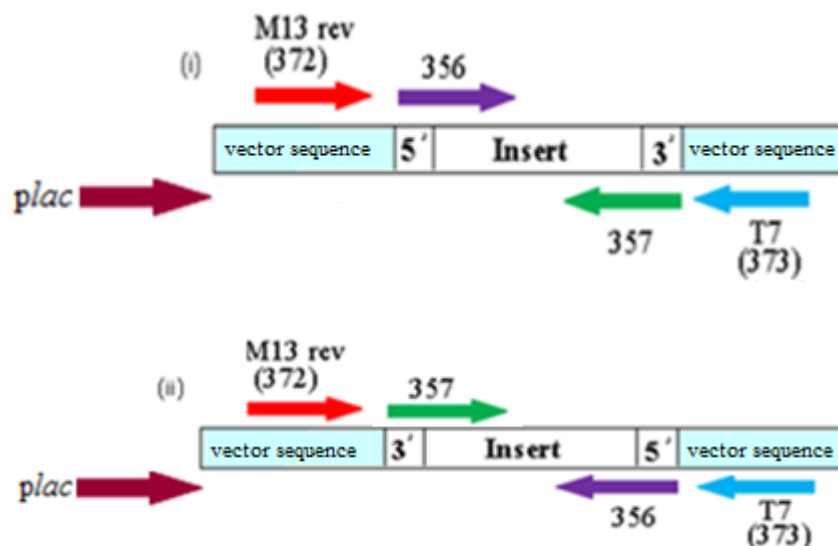
Figure 3.5: The annealing of primers 356,357 and M13 reverse (372), and the T7 promoter primer (373) with recombinant pCR® 2.1-TOPO® vector, p*lac*, promoter of the *lac* operon, ▪ blue colour, vector sequence.
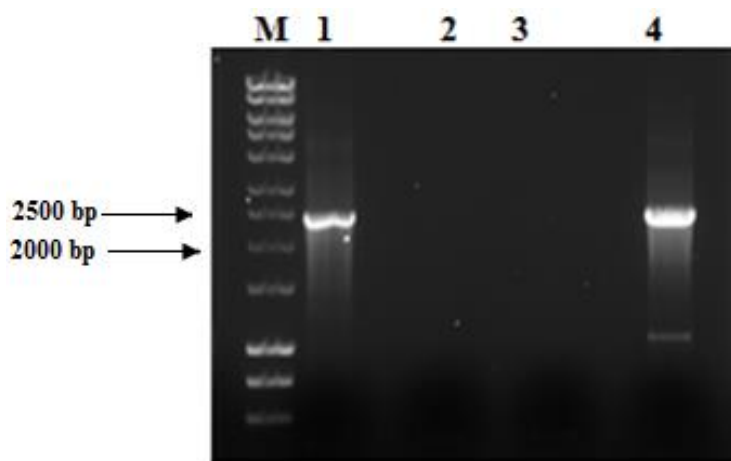
Figure 3.6: Determination of orientation of clones by PCR; lane M, Hyperladder I; lane 1, ME15 with M13 reverse primer and primer 356; lane 2, ME20 with M13 reverse primer and primer 356; lane 3, ME15 with M13 reverse primer and primer 357; lane 4, ME20 with M13 reverse primer and primer 357.
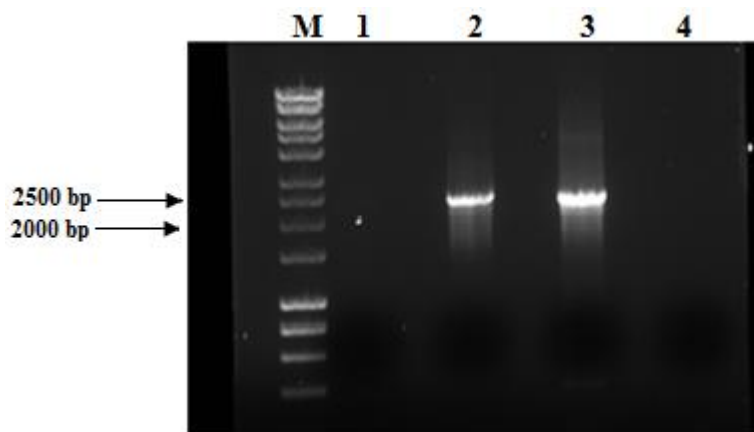
Figure 3.7: Determination of orientation of clones by PCR; lane M, Hyperladder I; lane 1, ME15 with primer 356 and T7 promoter primer 373; lane 2, ME20 with primer 356 and T7 promoter primer 373; lane 3, ME15 with primer 357 and T7 promoter primer 373; lane 4, ME20 with primer 357 and T7 promoter primer 373.

3.2.4. Preparation of plasmids

The next step was to introduce the plasmids (ME15 and ME20) into *E. coli* mutant ZSC113 which lacks transport and phosphorylation of glucose or mannose, in order to determine whether the fermentation phenotype of the mutant would be complemented. Plasmids were purified using the Fermentas GeneJET™ Plasmid Miniprep Kit. Recombinant plasmid was prepared from ME15 and ME20 grown in LB Broth containing 100 µg/ml ampicillin, and then incubated overnight at 37 °C with vigorous

shaking at 180 rpm. Successful plasmid isolation was confirmed by agarose gel electrophoresis as shown in Figure 3.8.
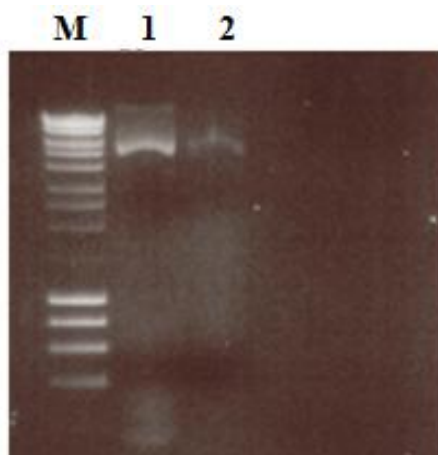
Figure 3.8: Isolation of plasmids ME15 and ME20 from *E.coli* TOP-10F; lane M, Hyperladder I; lane 1, plasmid ME15; lane 2, plasmid ME20.

3.2.5. Fermentation phenotype tests

The purified plasmids were next transformed into mutant *E. coli* ZSC113 as described in Materials and Methods. The transformants were isolated on LB plates containing 100 µg/ml ampicillin. Colonies were then transferred onto MacConkey agar plates with 1% glucose or 1% mannose as the fermentable carbon source. The transformed cells showed a positive fermentation phenotype for both sugars, thus showing the ability to ferment glucose and mannose (Figures 3.9 and 3.10). Also the positive phenotype was found for both plasmids independent of the orientation of the cloned gene, which indicates that the gene is expressed whether or not it is under the control of the *lac* promoter. This suggests that, there is a functional promoter which is driving the expression of pME15 in which the insert is in the opposite orientation. On the other hand, *E.coli* ZSC113 was unable to ferment glucose and mannose. The presence of the plasmids in the transformed cells was demonstrated by PCR using primers 356 and 357 (data not shown). The results therefore showed that gene *cbei* 0751 encoded a phosphotransferase system which was capable of uptake of both glucose and mannose.

The ability of the PTS encoded by *cbei* 0751 to transport N- acetylglucosamine was also tested by transforming into an *E.coli nagE* mutant lacking NAG PTS activity and screening on MacConkey agar plate supplemented with 0.5% N- acetylglucosamine. The results shown in Figure 3.11, demonstrated that the transformed cells were unable to ferment the N- acetylglucosamine substrate.
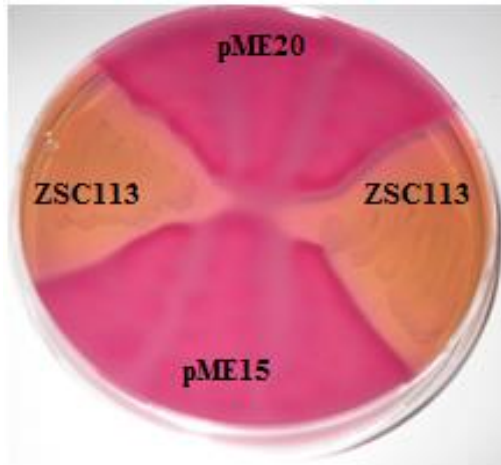
Figure 3.9: Fermentation of glucose by *E.coli* ZSC113 containing plasmids pME15 and pME20 on MacConkey agar.
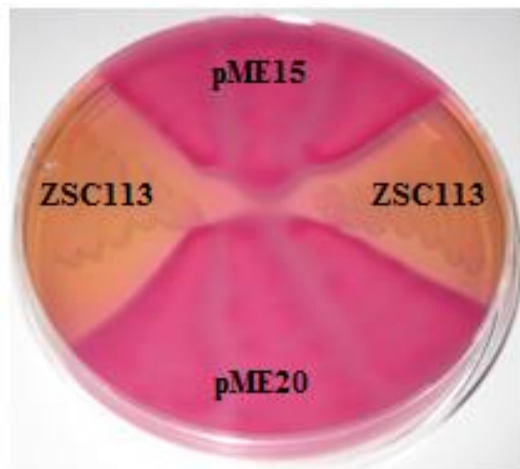
Figure 3.10: Fermentation of mannose by *E.coli* ZSC113 containing plasmids pME15 and pME20 on MacConkey agar.
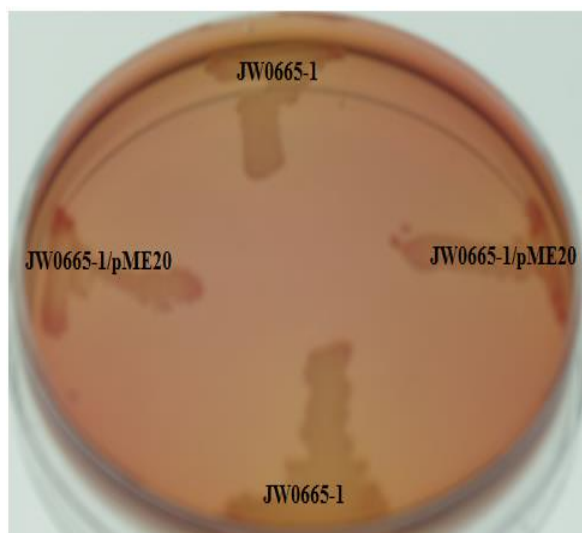
Figure 3.11: Fermentation of NAG by *E.coli* JW0665-1 mutant containing plasmid pME20 on MacConkey agar contained 0.5 % N- acetylglucosamine.

3.2.6. Large scale preparation of plasmid

Plasmids were purified using the QIAGEN plasmid Midi kit as described in Materials and Methods, section 2.12.1. An agarose gel electrophoresis was run to check the presence of the plasmid from ME15 and ME20 (Figure 3.12). Samples of these plasmids were sent for sequencing to Beckman Coulter Genomics. The sequence was obtained as a result of two reactions working along the genes. The results indicated that the complete sequence was 100% correct, when compared with the sequence from the genomic database (http://genome.jgi-psf.org/clobe/clobe.home.html), which confirmed the structure and orientation of the recombinants. Further characterization with respect to glucose uptake and phosphorylation was carried out.
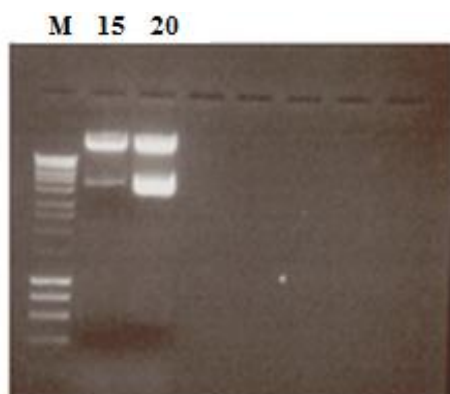
Figure 3.12: Plasmid purification of ME15, ME20 using the Qiagen midi kit; lane M, Hyperladder I; lane 15, plasmid ME15; lane 20, plasmid ME20.

3.3. Characterization of recombinant *E.coli* cells containing gene *cbei* 0751

The successful complementation of *E.coli* ZSC113 for fermentation of glucose and mannose, suggested that recombinant cells should now had the ability to take up and phosphorylate these sugars. Therefore, this ability was measured in various ways. Firstly, utilization of glucose and mannose was examined in LB broth. Secondly, glucose uptake and phosphorylation by whole cells and cell extracts was investigated.

3.3.1. Utilization of glucose and mannose in growing cultures

The strains were examined for the ability to take up and utilise glucose and mannose when growing in cultures. Strains ZSC113/pME15 and ZSC113/pME20, and the untransformed mutant ZSC113, were grown in LB broth containing 25 mM glucose and mannose, and 100 µg /ml ampicillin in the case of the recombinant strains only. The growth was measured by reading optical density at 600 nm, and supernatant samples were collected for sugar analysis. Both recombinant strains were able to utilise glucose and mannose, as shown in Figures 3.13 and 3.14 (glucose) and in Figures 3.16 and 3.17 (mannose), whereas the mutant *E.coli* ZSC113 showed very poor ability to remove the sugar from the growth medium (Figures 3.15 and 3.18 for glucose and mannose respectively). Therefore the utilization of glucose and mannose was evident only in ZSC113/pME15, ZSC113/pME20, and was absent in the untransformed mutant.

The strain JW0665-1/pME20 was also investigated for the ability to uptake and utilise NAG in LB broth under the same conditions. The results showed that the recombinant strain could not utilise NAG (Figure 3.19), had been demonstrated previously for untransformed JW0665-1 strain (Al Makishah and Mitchell, 2013).
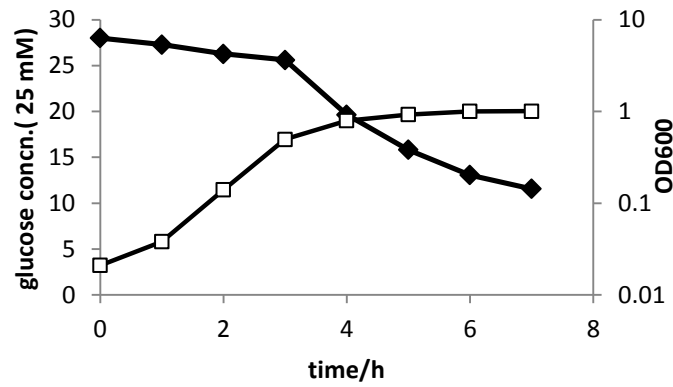
Figure 3.13: Growth and glucose utilization by strain ZSC113/pME15 in LB broth. Results from average value of independent duplicate cultures are shown. The initial glucose concentration was 25 mM and indicated by (♦), optical density of cells by (□). Error bars (standard deviation (SD)) are hidden by the symbols.
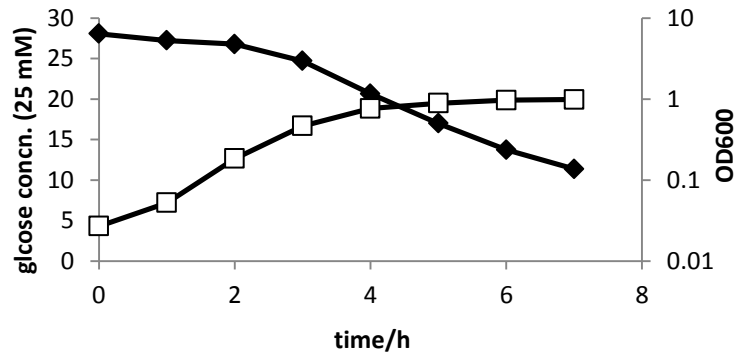
Figure 3.14: Growth and glucose utilization by strain ZSC113/pME20 in LB broth. Results from average value of independent duplicate cultures are shown. The initial glucose concentration was 25 mM and indicated by (♦), optical density of cells by (□). Error bars (SD) are hidden by the symbols.
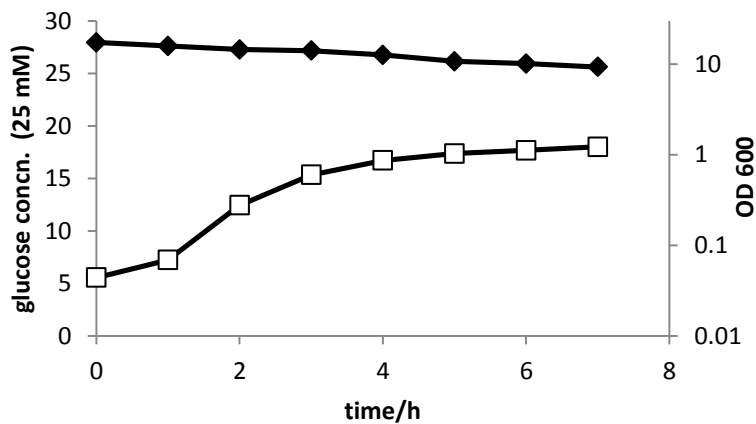
Figure 3.15: Growth and glucose utilization by strain *E.coli* ZSC113 in LB broth. Results from average value of independent duplicate cultures are shown. The initial glucose concentration was 25 mM and indicated by (♦), optical density of cells by (□). Error bars (SD) are hidden by the symbols.
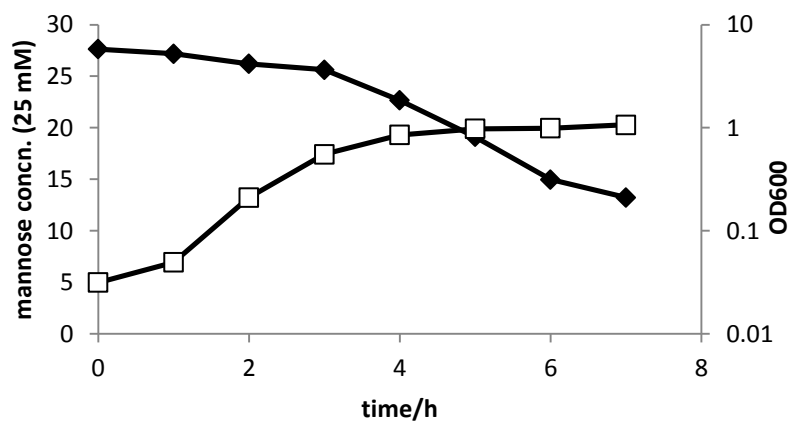
Figure 3.16: Growth and mannose utilization by strain ZSC113/pME15 in culture. Results from average value of independent duplicate culture are shown. The initial mannose concentration was 25 mM and indicated by (♦), optical density of cells by (□). Error bars (SD) are hidden by the symbols.
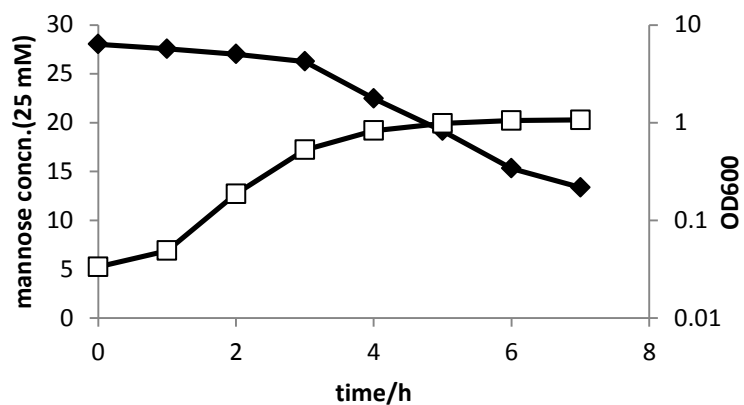
Figure 3.17: Growth and mannose utilization by strain ZSC113/pME20 in culture. Results from average value of independent duplicate culture are shown. The initial mannose concentration was 25 mM and indicated by (♦), optical density of cells by (□). Error bars (SD) are hidden by the symbols.
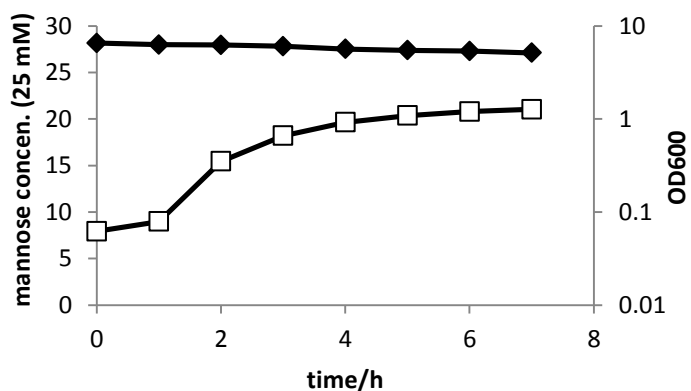
Figure 3.18: Growth and mannose utilization by strain *E.coli* ZSC113. Results from average value of independent duplicate culture are shown. The initial mannose concentration was 25 mM and indicated by (♦), optical density of cells by (□). Error bars (SD) are hidden by the symbols.

66
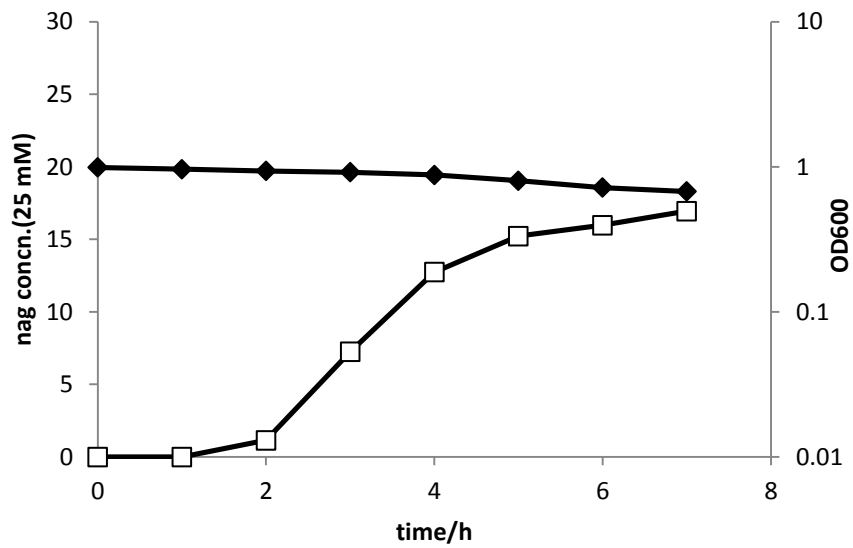
Figure 3.19: Growth and N- acetylglucosamine utilization by strain JW0665-1/pME20. Results from average value of independent duplicate cultures are shown. The initial N-acetylglucosamine concentration was 25 mM and indicated by (♦), optical density of cells by (□). Error bars (SD) are hidden by the symbols.

3.3.2. Glucose uptake by whole cells

Accumulation of glucose by whole cells was measured by following uptake of radiolabelled sugar ^{14}C- glucose. That strains ZSC113/pME15 and ZSC113/pME20 were able to accumulate glucose at a significant rate indicating that a transport system for glucose was active, as shown in Figure 3.20. On the other hand, mutant *E.coli* ZSC113 did not appear to be able to transport glucose during the experiment (Figure 3.21). Therefore, the result clearly shows that the recombinant strains are able to take up glucose but this activity is not seen in mutant *E.coli* ZSC113. Therefore, the results are in agreement with the results of the culture studies and the fermentation studies, which indicate that the *cbei* 0751 gene has given the *E.coli* strain the ability to take up glucose.

(a)   **(b)**
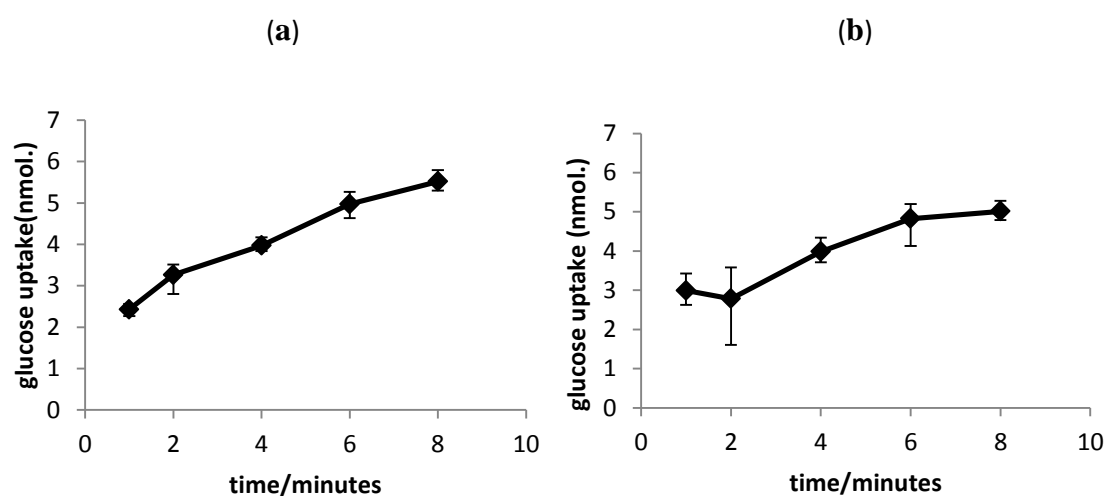
Figure 3.20: Uptake of glucose by whole cells of strains ZSC113/pME15 (a) and ZSC113/ pME20 (b). Results from average value of independent triplicate assays are shown, and error bars indicate standard deviation of the means.
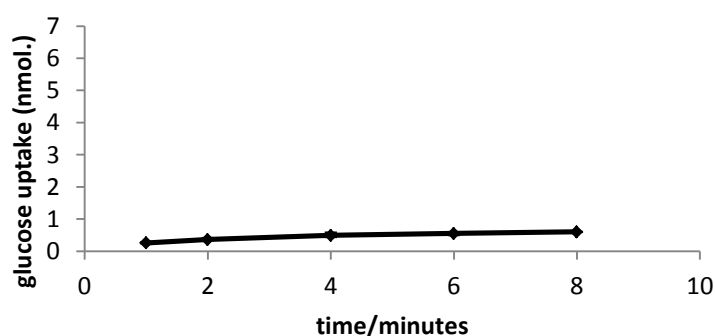
Figure 3.21: Uptake of glucose by whole cells of strain *E.coli* ZSC113. Results from average value of independent triplicate assays are shown, and error bars indicate standard deviation of the means.

3.3.3. Assay of glucose phosphorylation by cell extracts

As a final step in characterization of recombinant cells carrying the *cbei* 0751 gene, glucose PTS activity was assayed directly by following the rate of phosphorylation by cell free extracts. Cell extracts were prepared from strains ZSC113/pME15, ZSC113/pME20 and ZSC113 growing in LB broth. PTS activity is expected to be dependent on PEP. However, in initial assays the rate of sugar phosphorylation by the ZSC113/pME15 and ZSC113/pME20 extracts was similar in the presence or absence of PEP. The extracts were therefore dialysed to remove any PEP or other metabolites which might be present and interfering with the assay.

Following dialysis, the sugar phosphorylation assays were repeated. For both ZSC113/pME15 and ZSC113/pME20 extracts glucose was phosphorylated at a rapid rate in the presence of PEP. However, in the absence of PEP, the phosphorylation rate was considerably lower (Figure 3.22). Therefore, the results provide evidence for glucose PTS activity in both extracts.

(a) (b)
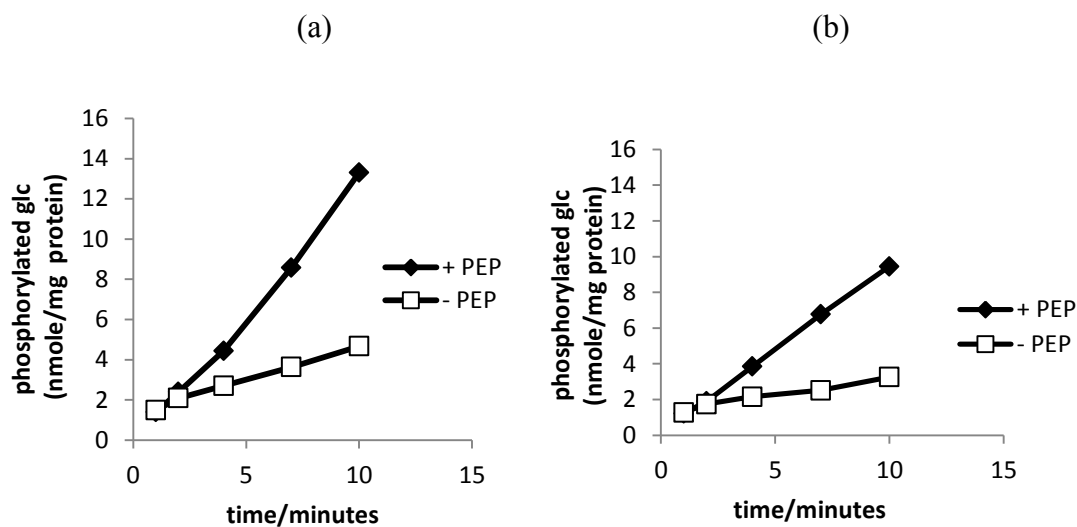
Figure 3.22: Phosphorylation of glucose by extracts of strains ZSC113/pME15(a) and ZSC113/ pME20 (b) with PEP, and without PEP as a control. Error bars (SD) are hidden by the symbols.

On the other hand, cell extract prepared from *E.coli* ZSC113 showed no activity with or without PEP even without being dialysed (Figure 3.23).
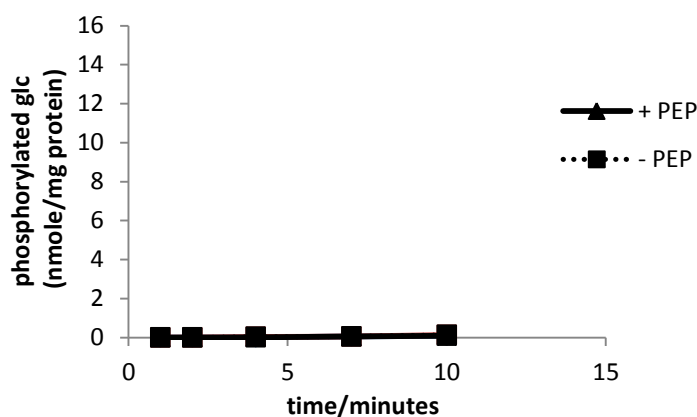
Figure 3.23: Glucose phosphorylation by extract of strains *E.coli* ZSC113 in the presence and absence of PEP. Error bars (SD) are hidden by the symbols.

Experiments investigating fermentation of glucose and mannose on MacConkey agar, and utilization of glucose and mannose in LB broth, provided evidence that the gene *cbei* 0751 encodes a glucose phosphotransferase system which also recognises mannose as a substrate. Given the apparent dual substrate specificity of the system, it was clearly of interest to examine whether mannose could inhibit phosphorylation of glucose. Therefore, experiments were set up in order to look at the inhibition of glucose phosphorylation by mannose and other glucose analogues.

3.3.4. Inhibition of glucose phosphorylation by other sugars

Glucose phosphorylation by cell extracts was examined in the presence of other related sugars in order to identify which sugar(s) might be recognised as substrates of the glucose PTS. The analysis showed that in extracts of ZSC113/pME20 glucose phosphorylation was inhibited by mannose (Figure 3.24). The glucose analogues methyl α –glucoside, (α MG) and 2-deoxyglucose also caused a strong inhibition of glucose phosphorylation, but inhibition by 3-O-methyl glucose was much less severe as shown in Figure 3.25. Glucose phosphorylation was most strongly inhibited by mannose, which is consistent with previous observations showing that the cloned *cbei* 0751 complemented strain *E.coli* ZSC113 for fermentation of both glucose and mannose, and confirming that mannose is a substrate of the PTS encoded by the *cbei* 0751 gene.
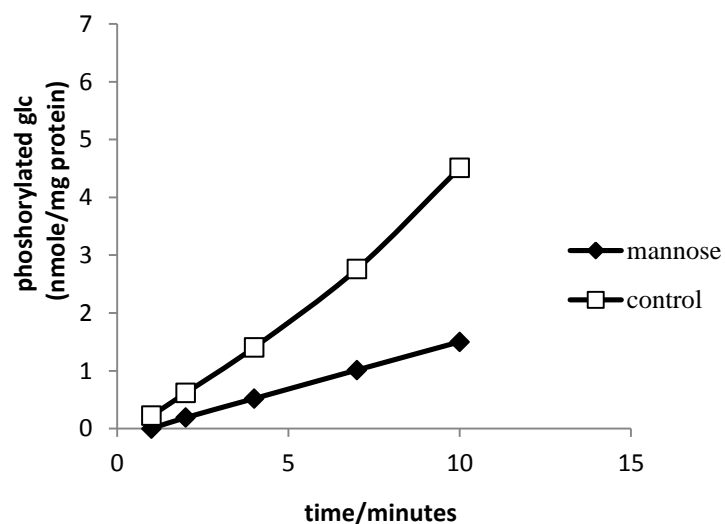
Figure 3.24: Effect of mannose on glucose phosphorylation by cell extract of strain ZSC113/ pME20. The results average of two experiments. The glucose concentration was 0.2 mM and the inhibitor at the concentration of 10 mM. Error bars (SD) are hidden by the symbols.
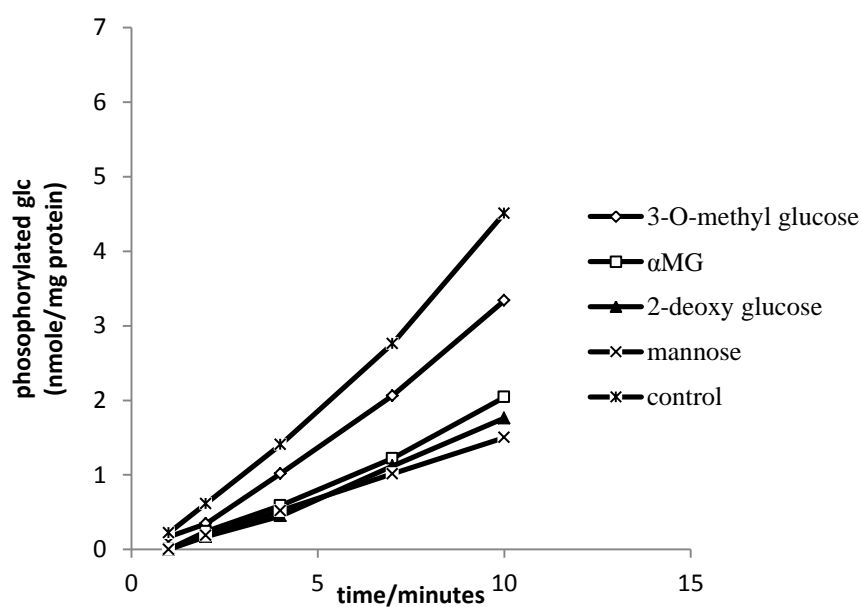
Figure 3.25: Effect of glucose analogues on glucose phosphorylation by cell extract of strain ZSC113/pME20.The results average of two experiments. The glucose concentration was 0.2 mM and the inhibitor at the concentration of 10 mM. Error bars (SD) are hidden by the symbols.

Since *cbei* 0751 is clearly a glucose PTS and gene arrangement next to an antiterminator is similar to the glucose PTS (*glcG*) in *C. acetobutylicum*, it is proposed that the *cbei* 0751 gene should be named *glcG*.

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3.4. Analysis of expression of *cbei* 0751 in *C. beijerinckii*

3.4.1. RNA isolation

According to the previous experiments the *cbei* 0751 PTS is a glucose uptake system. It was therefore of interest to determine whether the system is induced by glucose. RNA was isolated from *C. beijerinckii* NCIMB 8052 cells that were grown in CBM containing different carbon sources; glucose, sucrose, galactose, N- acetyglucosamine, mannose and maltose. RNA samples were examined by agarose gel electrophoresis, and results indicate the RNA was of good quality as shown in Figure 3.26.
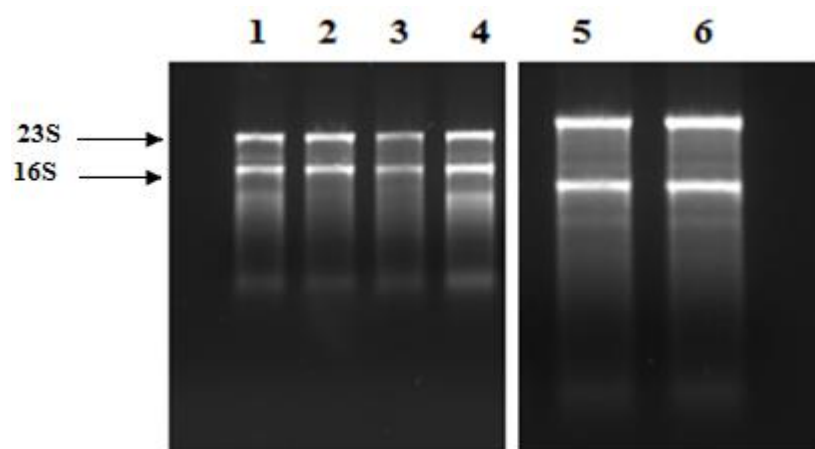
Figure 3.26: Total RNA isolation from *C. beijerinckii.* Cultures were grown on CBM containing: lane 1, glucose; lane 2, sucrose; lane 3, galactose; lane 4, N- acetylglucosamine; lane 5, mannose; lane 6, maltose. RNA samples were run on denaturing agarose gel containing ethidium bromide.

3.4.2. Preparation of hybridization probe

A hybridization probe was prepared by digoxigenin-labelling of PCR amplified product using *C. beijerinckii* NCIMB 8052 DNA as the template, and primers directed agents internal regions of the gene, as mentioned previously in Materials and Methods in section 2.20.2. In agarose gel electrophoresis, the unlabelled control reaction gave a product of a size in agreement with the expected size of 350 bp.

The DIG-labelled product had a higher molecular mass, which suggesting successful incorporation of DIG (Figure 3.27).
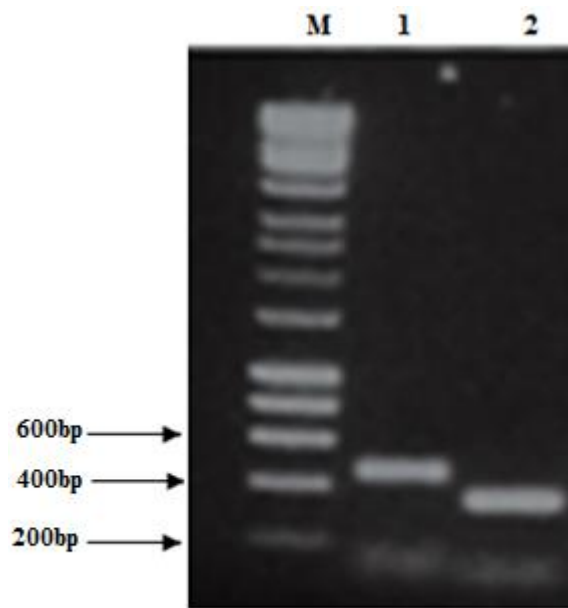
Figure 3.27: PCR amplified hybridization probe for *cbei* 0751. Lane M, Hyperladder I;   lane 1, gene *cbei* 0751 DIG- labelled PCR product; lane 2, control, unlabelled PCR product.

The probe was examined directly on a nylon membrane by dot blot as shown in Figure 3.28. Detection of the probe from undiluted sample to the fifth dilution, confirmed that it was suitable for hybridization analysis.

Figure 3.28: Dot blot of DIG- labelled section of the gene *cbei* 0751. The sample was serially diluted x5(from left to right) and samples were processed and detected with CDP-star as described in Materials and Methods.

3.4.3. Expression of the *cbei* 0751 gene during growth on several carbon sources

The DIG-labeled PCR amplified DNA product (DNA probe) was used in slot blot hybridization with RNA samples extracted from *C. beijerinckii* cultures grown on glucose, sucrose, galactose, N- acetylglucosamine, mannose and maltose. The results are shown in Figure 3.29. As can be seen, hybridization was observed following growth on all substrates tested. The strongest hybridization signal was obtained from RNA prepared from cells grown on galactose, and surprisingly the weakest signal was from cells grown on glucose. Therefore, while the slot blot hybridization does not provide a quantitative analysis of gene expression, there is clearly no evidence for induction of *cbei* 0751expression by glucose.
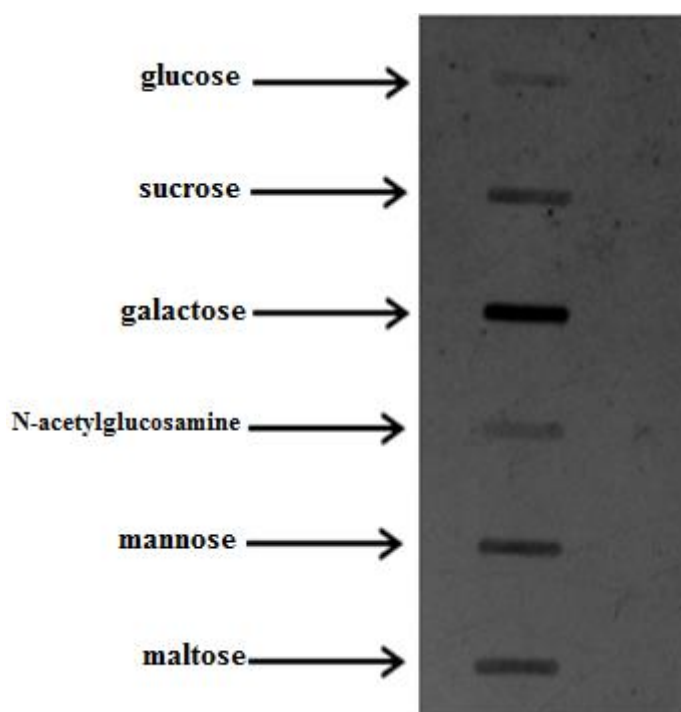
Figure 3.29: Slot blotting and hybridization of RNA samples for monitoring gene *cbei* 0751 expression. RNA samples were isolated from cells grown on different carbon sources; glucose, sucrose, galactose, N-acetylglucosamine, mannose and maltose.

3.4.4. DNaseI treatment

To remove any trace amount of DNA present in RNA samples, New England Biolabs protocol was used as described in the Materials and Methods section 2.21. Following the treatment RNA samples were reanalysed by agarose gel electrophoresis. The result shown in Figure 3.30 indicated that the RNA was still intact. A PCR reaction was carried out using the *cbei* 0751dig forward primer and *cbei* 0751dig reverse primer as described in the Materials and Methods. As can be seen in Figure 3.31, the RNA before treatment gave product indicating that DNA was present, but after treatment there was no PCR product indicating DNA had successfully been removed.
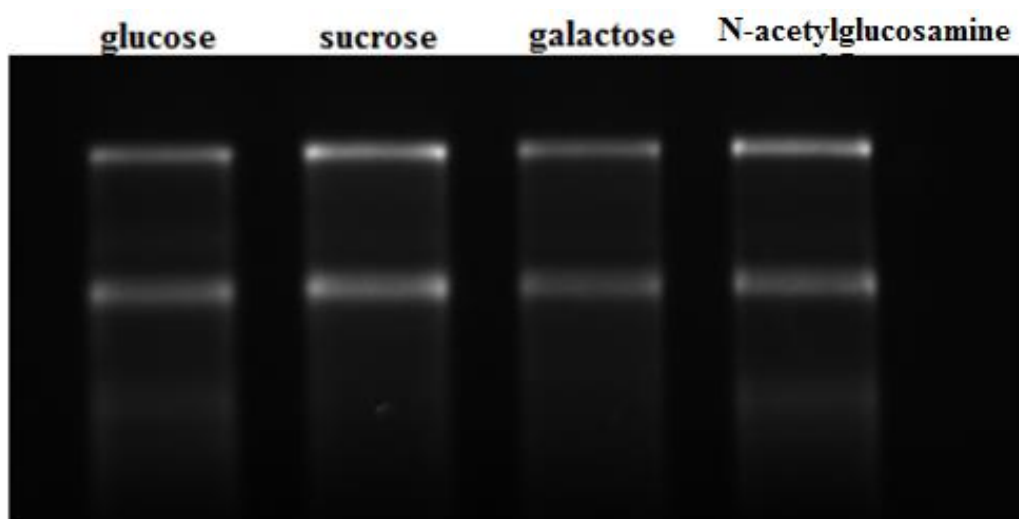
Figure 3.30: Some RNA samples after treatment to remove any trace of DNA present. Samples were run on denaturing agarose gel containing ethidium bromide.

.
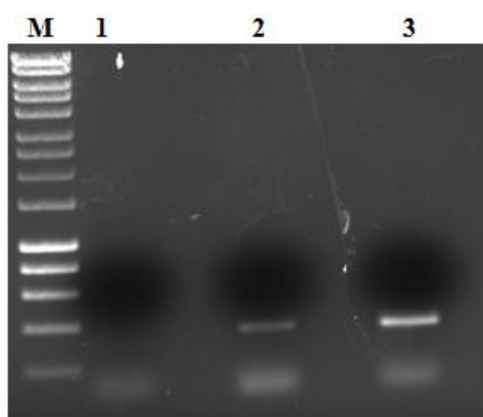
Figure 3.31: PCR amplification of RNA isolated from cells grown on glucose; lane M. Hyperladder I; lane 1, RNA after treatment with DNase; lane 2, RNA before treatment with DNase; lane 3, PCR amplification with genomic DNA as control.

3.4.5. Analysis of gene expression by reverse transcriptase (RT)-PCR

To re-examine the expression of the gene *cbei* 0751 in different growth conditions, a reverse transcriptase (RT)-PCR analysis was carried out. In this procedure, reverse transcriptase is used to create a complementary DNA (cDNA). The RT-PCR reaction consists of two steps. The first one is reverse transcription of RNA which can be performed using a primer that anneals to the RNA template to create a complementary DNA (cDNA) by using the reverse transcriptase (RT) enzyme, hence, this reaction is termed the RT reaction. The second step is then amplification of the cDNA by PCR. The results were analysed on an agarose gel shown in Figure 3.32. In each case a band of the expected size was obtained when reverse transcriptase had been included to generate the cDNA. On the other hand in the control samples when the reverse transcriptase step was eliminated, there is no product. This shows clearly that there was no DNA present in the samples and it confirms that the expression of the gene had taken place in samples extracted from cells grown on different carbon sources.
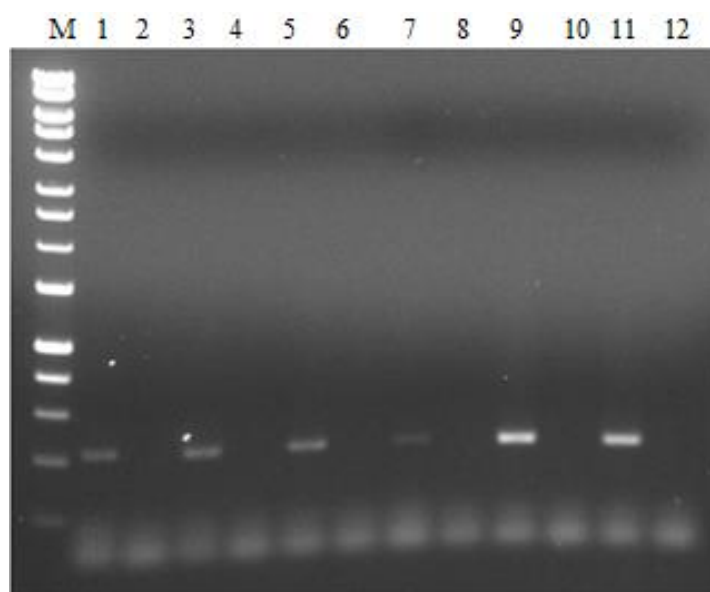
Figure 3.32: Reverse transcriptase (RT)-PCR of *C. beijerinckii* RNA isolated following growth on different carbon sources. lane M, Hyperladder I; lane 1, cDNA of glucose; lane 3, cDNA of sucrose; lane 5, cDNA of galactose; lane7, cDNA of N- acetylglucosamine; lane 9, cDNA of mannose; lane11, cDNA of maltose. Lanes, 2, 4, 6, 8, 10 and 12, were a control for each sample respectively in which the RT-step was eliminated.

3.5. Gene Knockout using the ClosTron

To assess the importance of the *glcG* gene in *C. beijerinckii,* one experimental approach would be to inactivate it and examine the effect on cellular behaviour. The plasmid used in *C. beijerinckii* is pMTL007S-E2 as shown in Figure 3.33(a) (Heap *et al*., 2010).

Three critical features are present in the plasmid. First, the intron which contains the *ermB* gene (encoding resistance to erythromycin) which is inactive due to the insertion of an intron from the phage td as shown in Figure 1.6. Second, the *specR* gene encoding resistance to spectinomycin. Third a gene encoding for LtrA, the protein which catalyses the incorporation of the retargeted intron into the target gene. By incorporating a specific short sequence in the intron, it can be targeted to a particular gene and transfer to the target is accompanied by activation of erythromycin resistance which allows for selection of stable mutants. The activation is due to the splicing out of the phage (td) intron during transfer of the *ermB-* containing intron to the target site.

Following insertion of the target intron and selection for erythromycin resistance, different combinations of primers directed against either chromosomal DNA or the intron can be used in PCR to establish whether mutants have been correctly constructed. In the wild type the combination of forward primer 435 and reverse primer 436 should give product of 368 bp. However, for a mutant the primer combination of EBS primer (which is specific to the intron sequence and present within the insert) and 435 forward primer should give a PCR product of 490 bp, but the combination of EBS primer and the 436 reverse primer should not give a product. After putative clones were isolated and purified several times on CBM plates supplemented with 10 μg/ml erythromycin, DNA was extracted and the various PCR amplification were carried out. The results of the analysis are shown in Figure 3.34. The only products which were obtained from the combination of the forward primer and reverse primer as describe above these not expected for mutant so, this indicated that the DNA was still from a wile type bacterium, on the other hand the product which was expected from mutant which the reverse primer and EBS primer did not give product for any of the mutant.

(a)
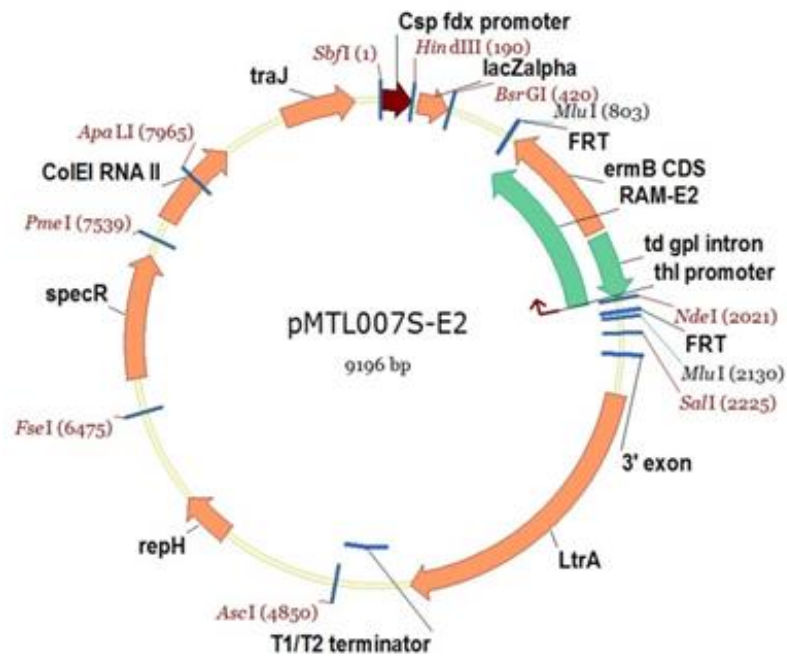
(b)
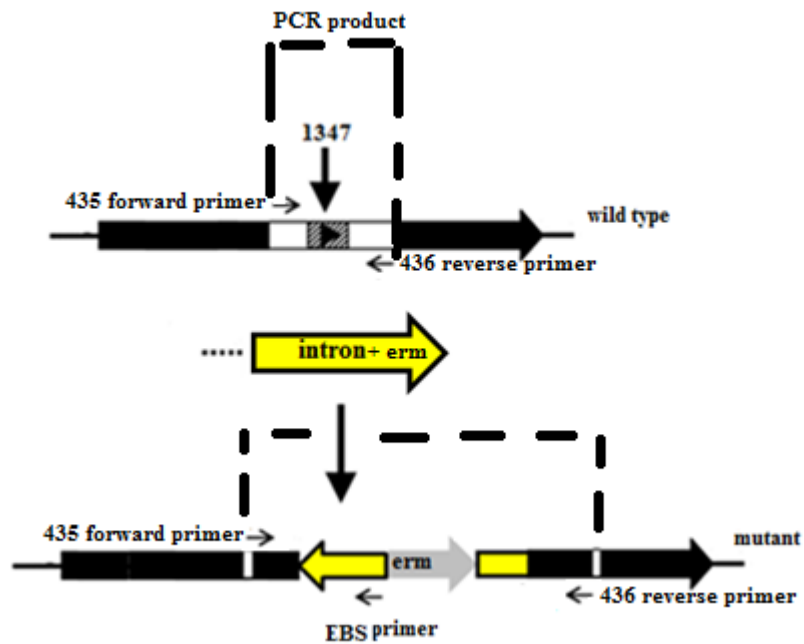
Figure 3.33: (a) Plasmid pMTL007S-E2 (from Muhammad Ehsaan, Nottingham University); (b) PCR used to screen for intron insertion in gene *glcG*. The specified insertion into *glcG* was at nucleotide 1347 on the sense strand. ▶ = ClosTron target, - - PCR product using the forward primer 435 and the reverse primer 436 with wild type, - - PCR reaction using the forward primer 435 and the EBS primer and also used reverse primer 436 and EPS primer with mutant. Only one of these reactions will give product depending on the orientation of the intron insertion.
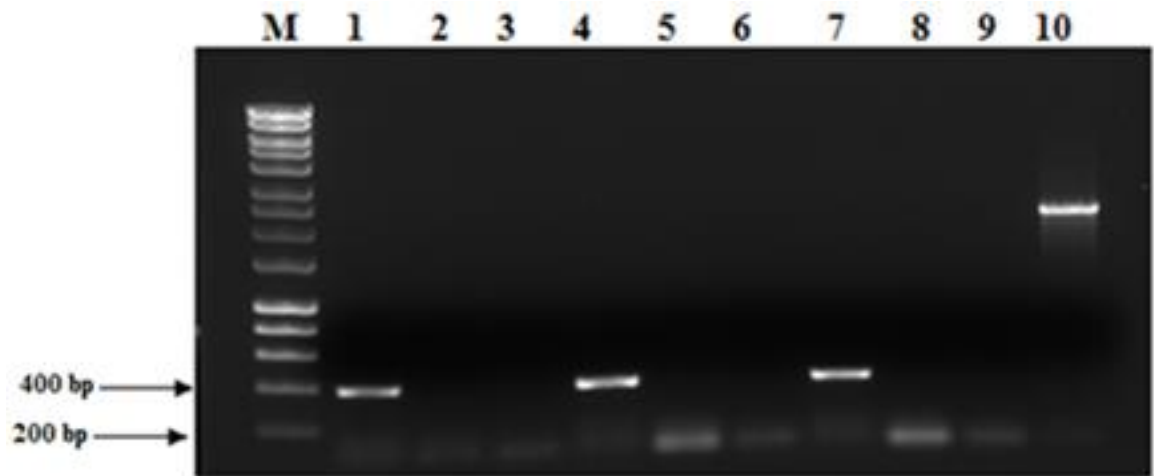
Figure 3.34: Mutant screening by PCR used to screen for intron insertion in gene *glcG*. lane M, Hyperladder; lane 1, sample 2 with forward primer 435 and reverse primer 436; lane 2, sample 2 with forward primer 435 and EBS primer; lane 3, sample 2 with reverse primer 436 and EBS primer; lane 4, sample 4 with forward primer 435 and reverse primer 346; lane 5, sample 4 with forward primer 435 and EBS primer; lane 6, sample 4 with reverse primer 436 and EBS primer; lane 7, sample 6 with forward primer 435 and reverse primer 436; lane 8, sample 6 with forward primer 435 and EBS primer; lane 9, sample 6 with reverse primer 436 and EBS primer; lane10, wild type with forward primer 356 and reverse primer 357; these are the cloning primers as a control, and the expected size of 2364 bp, the numbers on the gel are reference numbers for the samples (individual colonies).

3.6. Cloning of the genes *cbei* 4983 and *cbei* 4982

3.6.1. Amplification of genes *cbei* 4983 and *cbei* 4982 by PCR

In the previous section the gene *cbei* 0751 was successfully cloned and it was shown it at codes for a glucose PTS which also recognises mannose as a substrate. This leaves the question of what is the function of the other system coded for by the genes *cbei* 4983 and *cbei* 4982. This system is in a phylogenetic position that suggests that it may be a system for a disaccharide uptake, and the fact that the genes are located next to a gene encoding for a hydrolase also suggests that a disaccharide may be the substrate. The genes *cbei* 4983 and *cbei* 4982 were amplified by PCR using 432 forward primer and 433 reverse primer, and Easy-A® High-Fidelity PCR Cloning Enzyme (Stratagene). As described in Materials and Methods in section 2.8, the product obtained was just lower than 2500 bp, in agreement with a product expected to be 2263 bp, as shown in Figure 3.35. Therefore, the PCR- generated DNA fragment encodes the entire PTS; the gene *cbei* 4983 encodes the two PTS domains IIB and IIC, and the *cbei* 4982 gene encodes a separate PTS IIA domain. These genes could be cloned in the StrataClone PCR Cloning Vector and transformed into *E.coli* TOP10.
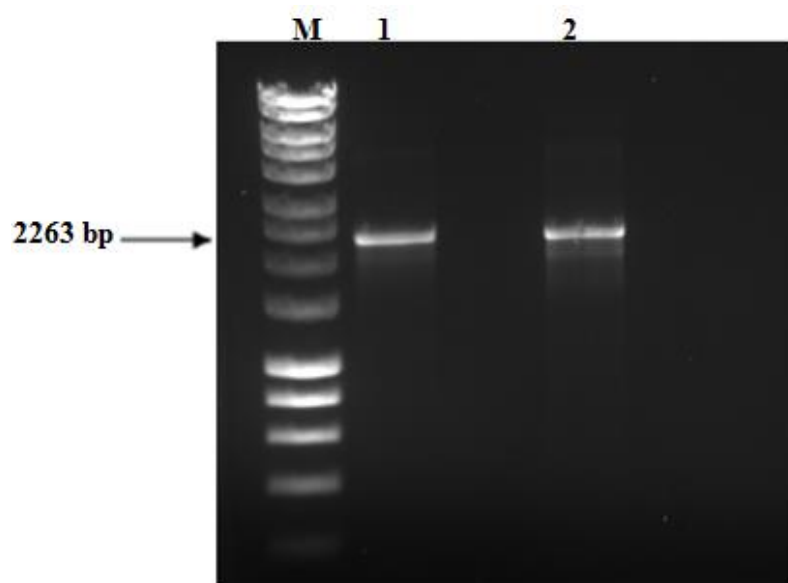
Figure 3.35: PCR amplification of genes *cbei* 4983 and *cbei* 4982; lane M, Hyperladder I; lane 1 and lane 2, amplified products from PCR reaction using the forward primer 432 and the reverse primer 433.

3.6.2. Screening and analysis of clones

The PCR amplified product was inserted into the StrataClone PCR Cloning Vector (see Appendix IV) from the StrataClone PCR Cloning Kit, and then transformed into *E.coli* TOP10 competent cells. Transformed cells were plated onto LB plates containing 100 µg/ml ampicillin and incubated at 37 ℃ overnight. 56 colonies were picked off onto fresh LB plates containing the same amount of ampicillin and incubated again at 37 ℃ overnight. These isolates were then screened for the presence of the cloned genes. 38 of the 56 clones showed the presence of the genes (as shown in Figure 3.36), since they had an amplified PCR product of the expected size of around 2263 bp. This indicated that the *cbei* 4983 and *cbei* 4982 genes had been successfully inserted into the StrataClone PCR Cloning Vector.
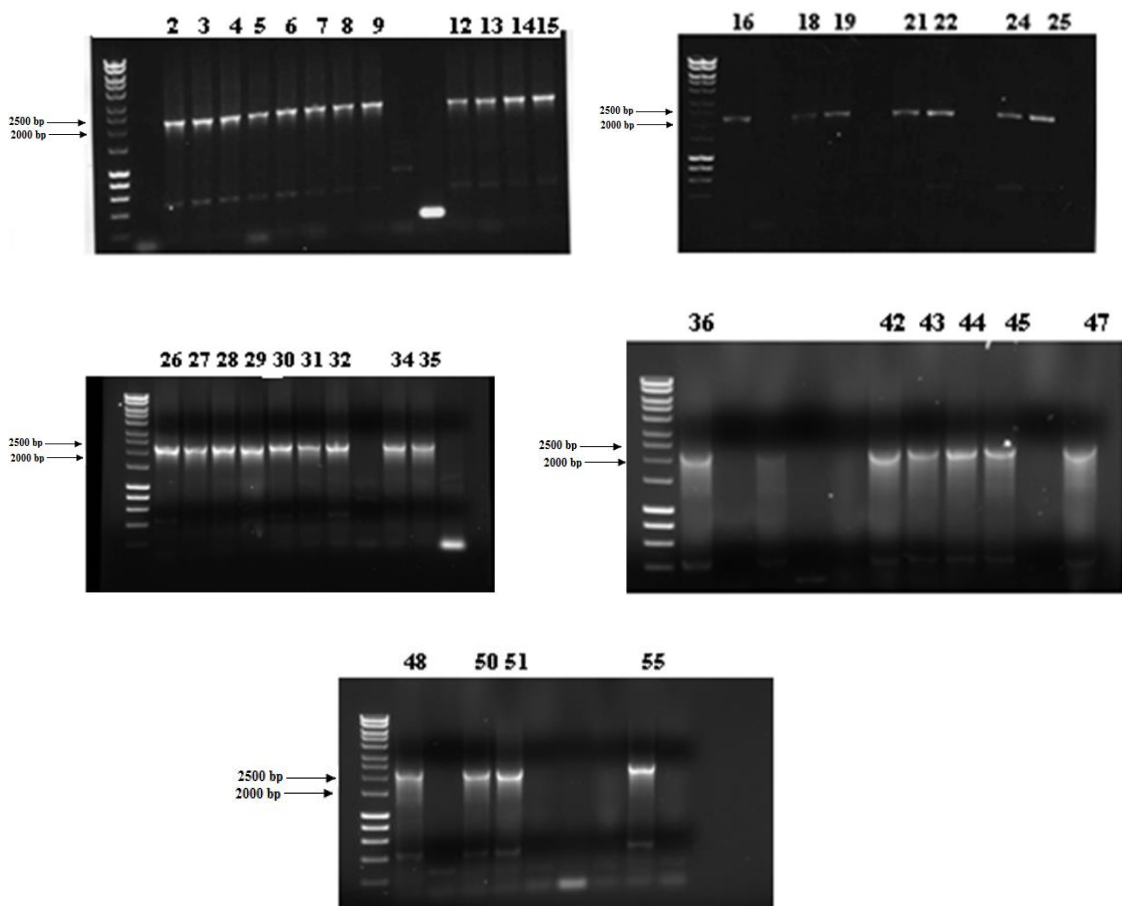
Figure 3.36: Screening of transformants for the presence of genes *cbei* 4983 and *cbei* 4982 by PCR using the cloning primers (forward primer 432 with the reverse primer 433). The numbers on the gel are reference numbers for the samples (individual colonies) and the expected size of the insert to be 2263 bp.

3.6.3. Determination of orientation of DNA inserts by PCR

The orientation of the inserted genes *cbei* 4983 and *cbei* 4982 in the StrataClone PCR Cloning Vector was determined by PCR was described in section 2.8, using a combination of a primer directed against the vector (see Appendix IV), and a primer directed against the insert. If the primer combination of M13 reverse primer and the reverse primer 433 gives a product, the insert is under the control of *lac* promoter. However, the primer combination of M13 reverse and the forward primer 432 should not give product if the insert is under control of the *lac* promoter (Figure 3.37(i)). On the other hand, the combination of the T7 promoter primer and the forward primer 432 will give a product if the insert is under the control of *lac* promoter, but the combination of the T7 promoter primer and the reverse primer 433 should not give a product if the insert is under control of the *lac* promoter (Figure 3.37(ii)). The results of the analysis are shown in Figures 3.38-3.41. Of the 38 colonies which had the gene insert, only one colony (number 25) gave a product with M13 reverse primer and 433 reverse primer (Figure 3.38), and also gave product with T7 promoter primer and 432 forward prime (Figure 3.40). However, this isolate did not give product with M13 reverse primer and 432 forward primer (Figure 3.39), nor with T7 promoter primer and 433 reverse primer (Figure 3.41). The other 37 colonies gave product with M13 reverse primer and 432 forward primer (Figure 3.39), and most, but not all, of these also gave a product with T7 promoter primer and 433 reverse primer (Figure 3.41). It was therefore concluded that one clone (number 25) contained the insert under the control of the *lac* promoter, and the others contained the insert under the control of the T7 promoter.
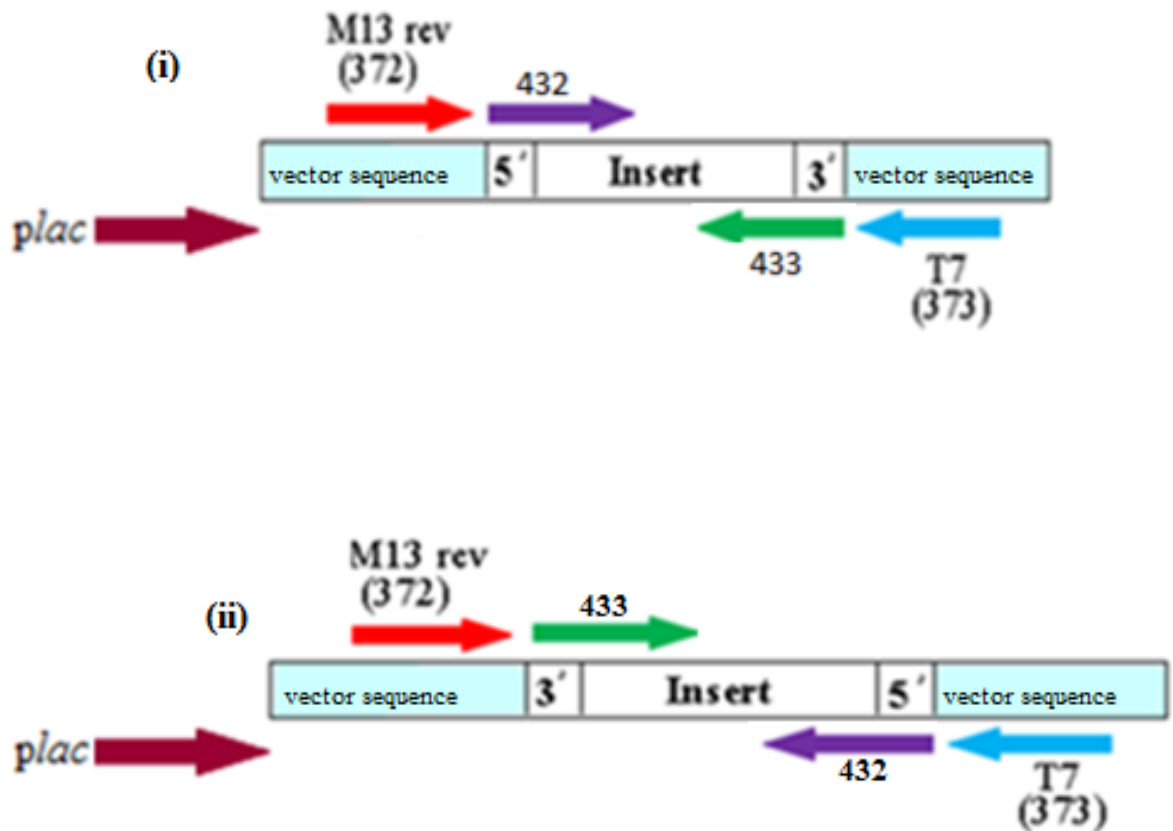
Figure 3.37: The annealing of primers 432,433 and M13 reverse (372), and the T7 promoter primer (373) with recombinant StrataClone PCR cloning vector. p*lac*, promoter of the *lac* operon, ▢ blue colour, vector sequence.
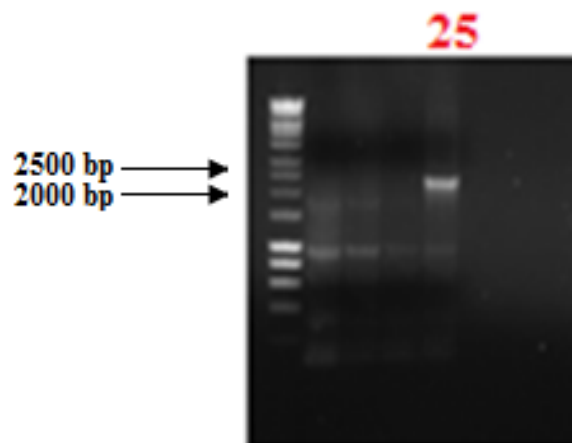
Figure 3.38: Screening of transformants for the orientation of genes *cbei* 4983 and *cbei* 4982 by PCR using the M13 reverse primer with 433 reverse primer. Only sample 25 gave product with the M13 reverse primer and 433 reverse primer.
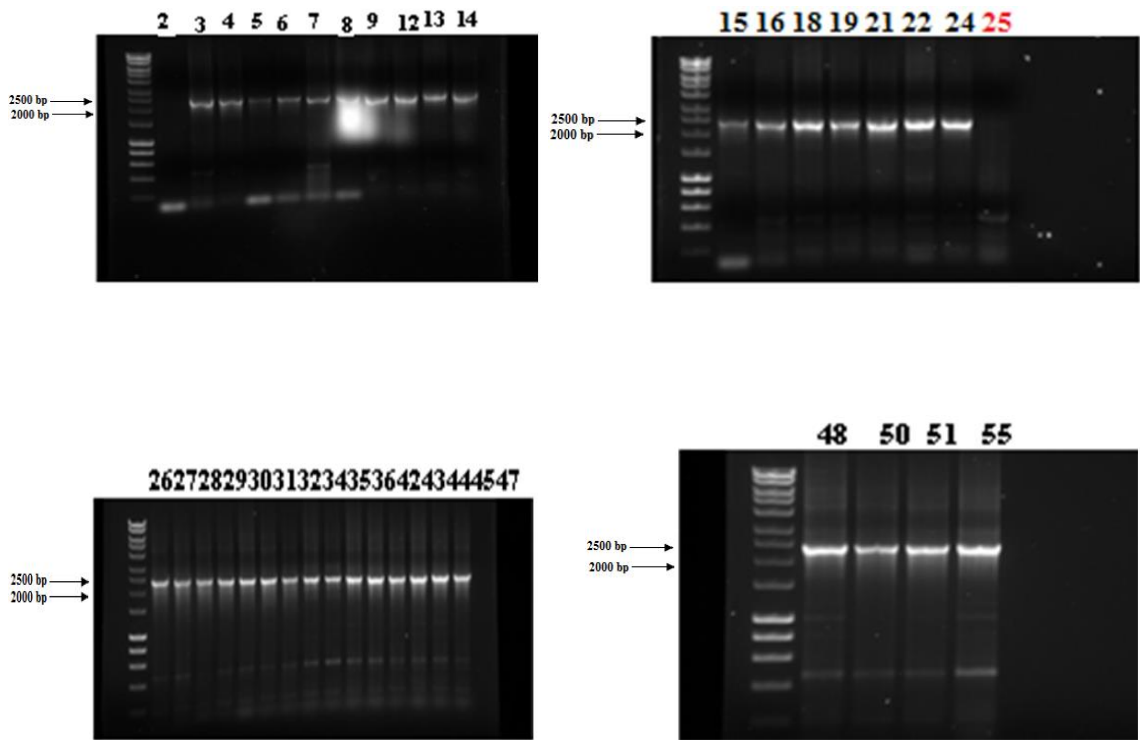
Figure 3.39: Screening of transformants for the orientation of genes *cbei* 4983 and *cbei* 4982 by PCR using the M13 reverse primer with 432 forward primer. Sample 25 did not give product with M13 reverse primer and 432 forward primer. The numbers on the gel are reference numbers for the samples (individual colonies) and the expected size of the insert to be 2263bp.
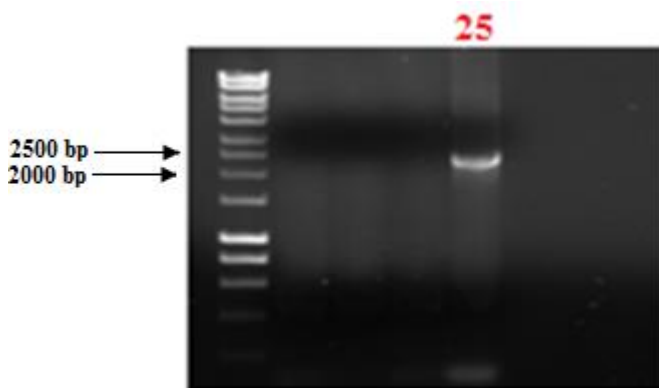
Figure 3.40: Screening of transformants for the orientation of genes *cbei* 4983 and *cbei* 4982 by PCR using T7 promoter primer with 432 forward primer. All samples did not give product except sample 25 give product with T7 promoter primer and 432 forward primer. And the expected size of the insert to be 2263bp.
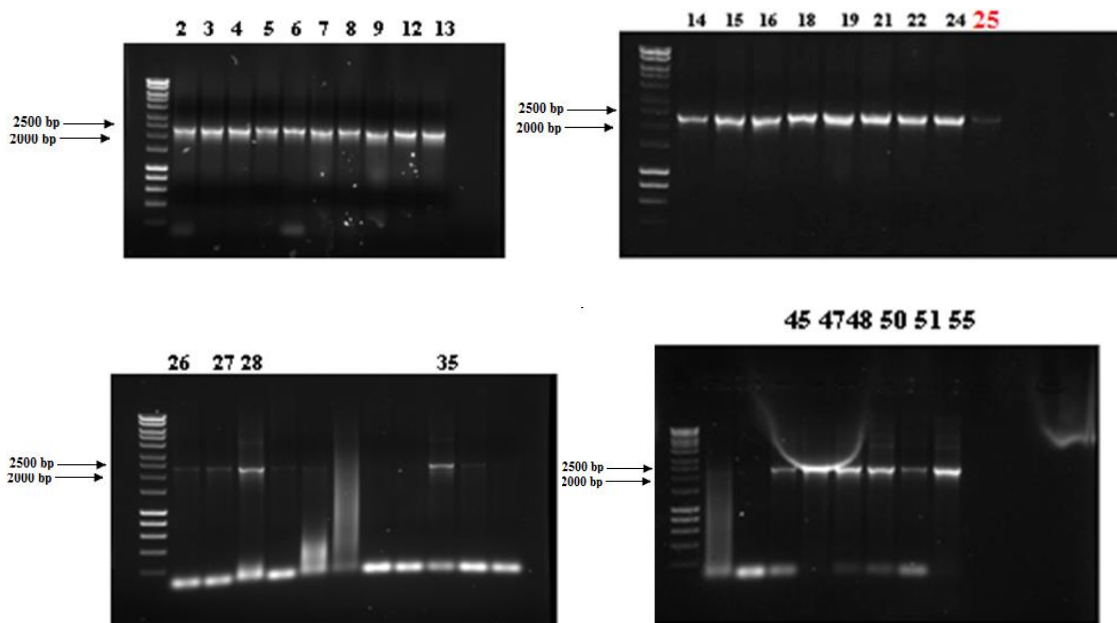
Figure 3.41: Screening of transformants for the orientation of genes *cbei* 4983 and *cbei* 4982 by PCR using T7 promoter primer with 433 reverse primer. Sample 25 did not give product with T7 promoter primer with 433 reverse primer. The numbers on the gel are reference numbers for the samples (individual colonies) and the expected size of the insert to be 2263bp.

3.6.4. Preparation of Plasmids

Plasmids were prepared from recombinants carrying the *cbei* 4983 and *cbei* 4982 genes using the Fermentas GeneJET™ Plasmid Miniprep Kit. The samples chosen were pME25, because it was in preferred orientation with the insert under the control of the *lac* promoter, and pME26 selected at random as a sample that had the insert in the opposite orientation. Successful plasmid isolation was confirmed by gel electrophoresis as shown in Figure 3.42.
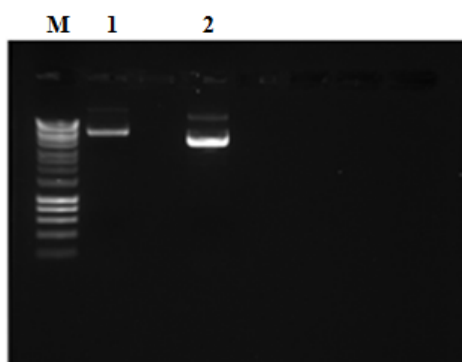
Figure 3.42: Isolation of plasmids pME25 and pME26 from *E.coli* TOP-10; lane M, Hyperladder I; lane 1, pME25; lane 2, pME26.

3.6.5. Transformation of chemically competent cells of *E. coli*

The purified pME 25 and pME26 were transformed into mutant *E. coli* ZSC113 which is unable to phosphorylate glucose or mannose (see section 3.2.5), and the colonies were transferred onto MacConkey agar plates containing 100 µg/ml ampicillin and 1 % glucose or 1 % mannose. The results were that pME25 and pME26 did not have any effect on glucose fermentation as shown in Figure 3.43, and they also had no effect on mannose metabolism (not shown). The cells had a negative fermentation phenotype as shown by pale yellow colonies, which indicated that the plasmids did not provide the ability to ferment glucose and mannose.

pME 25   pME 26

Figure 3.43: Fermentation of glucose on MacConkey agar by *E. coli ZSC113* containing plasmid pME25 or pME26 showing the inability to ferment glucose, all samples on the plates from clones pME25 and pME26.

pME25 was transformed into other *E. coli* mutants including JW3381- 4 which is unable to metabolise maltose, JW3701-2 unable to ferment cellobiose and JW4199- 1 unable to phosphorylate trehalose. The transformants were screened on MacConkey agar containing 30 µg/ml kanamycin and 100 µg/ml ampicillin and also 1 % maltose, 1 % cellobiose or 0.1 % trehalose. The trehalose concentration was lower because it was found that strain JW4199-1 gave a positive fermentation phenotype when the concentration of trehalose was 1 %. After incubation at 37 °C for up to 48 h, the strains containing pME25 demonstrated a negative fermentation phenotype for maltose, and trehalose as shown in Figures 3.44 and 3.45, and also cellobiose (not shown). The *nagE* mutant JW0665-1 was able to ferment maltose, but not trehalose under the condition tested. The *nagE* mutant was used as a control because it is resistant to kanamycin.
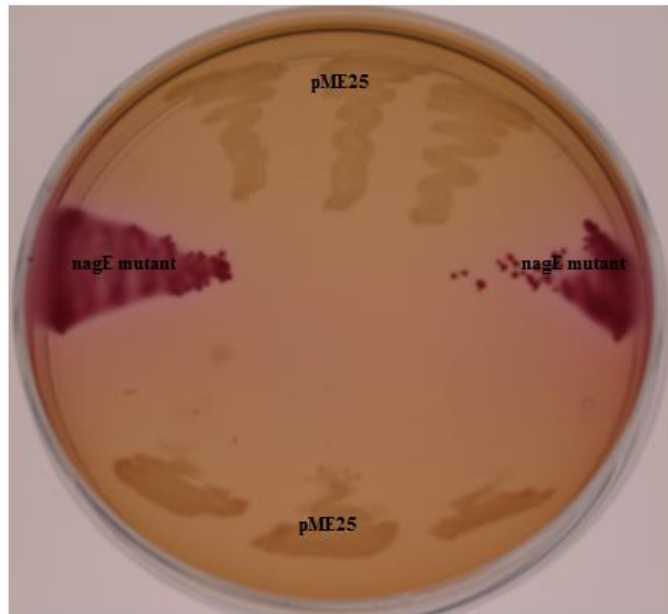
Figure 3.44: Fermentation of maltose on MacConkey agar by mutant JW3381- 4 containing pME25 showing the inability to ferment maltose. The *nagE* mutant JW0665-1 was able to ferment maltose under these conditions.
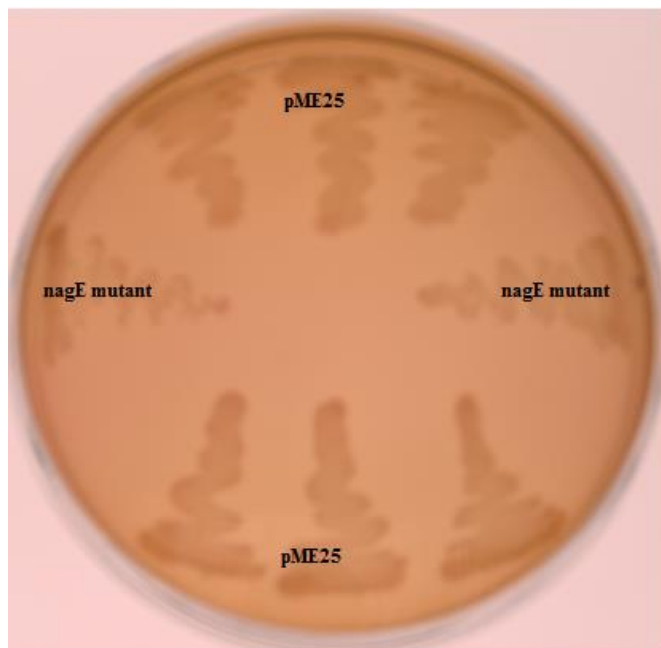
Figure 3.45: Fermentation of trehalose on MacConkey agar by *E. coli* JW4199- 1 containing pME25 showing the inability to ferment trehalose.

3.6.6. Large scale preparation of plasmid

The pME25 and pME26 plasmids were purified in a higher yield, using the QIAGEN plasmid Midi kit, as described previously in section 2.12.1, and agarose gel electrophoresis was used to check the presence of the plasmids. The plasmids were then sent for sequencing to Beckman Coulter Genomics.

The sequence of the insert in pME26 was found to be correct, and it confirmed that it was in the orientation with the *cbei* 4983/*cbei* 4982 genes under control of the T7 promoter. For pME25 the insert was in the opposite orientation. However, although the sequence of the genes was correct in the reverse direction, the upstream region in the vector was correct only until the *Kpn* I site as shown in Figure 3.46. The sequence then had no relationship to the vector sequence. In the forward direction from the M13 reverse primer, the sequence had no relationship at all to the vector or the cloned genes. Both of the unknown sequences showed a relationship to a transposable element. Therefore it appeared possible that some sort of DNA transfer may have taken place perhaps from the genome of the host cell into the plasmid, and this would obviously affect expression of the cloned genes.
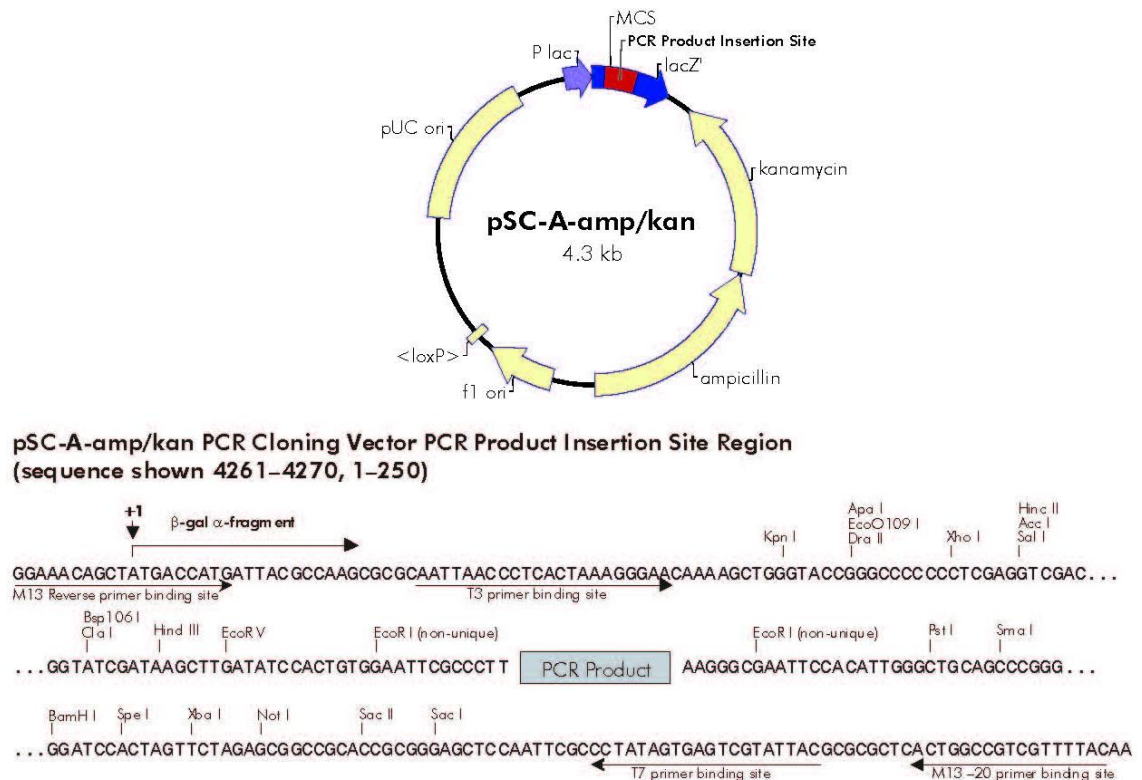
Figure 3.46: StrataClone PCR cloning vector pSC-A-amp/kan.

3.6.7. Screening and analysis of clones in the vector pJET 1.2/blunt

The clones of genes *cbei* 4983/ *cbei* 4982 that were obtained by the previous methods, were predominantly in the wrong orientation so would not be expected to be expressed. The one clone that was apparently under the control of the *lac* promoter was found not to contain the correct DNA sequence. Therefore, an alternative strategy was to try to clone the genes in a different vector. The genes *cbei* 4983 and *cbei* 4982 were amplified by PCR using 432 forward primer and 433 reverse primer, and *Pfu* DNA polymerase (Fermentas). The blunt-ended PCR product was inserted into the pJET 1.2/blunt Cloning Vector (see Appendix IV). The transformation of *E.coli* TOP10 resulted in a number of colonies on LB agar plates in the presence of ampicillin. 50 colonies were picked off and transferred onto new LB agar plates containing ampicillin and incubated at 37 ºC overnight. To show the gene insert within the clones a screening was carried out. Figure 3.47, shows some of the results of the screening. 12 of the 50 colonies contained a gene insert of between 2000 bp- 2500 bp, but other colonies did not have the gene insert. The gene had been successfully cloned in some but not all of the colonies.
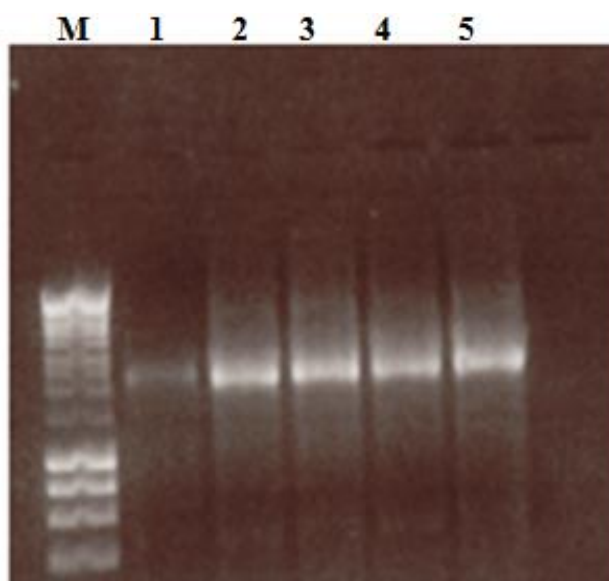
Figure 3.47: Screening of transformants for the presence of genes *cbei* 4983 and *cbei* 4982 by PCR using the forward primer 432 and the reverse primer 433; lane M, Hyperladder I as a marker; lane 1, DNA fragment as a control; lane 2, sample 13; lane 3, sample 14; lane 4, sample 15; lane 5, sample 16.

3.6.8. Determination of orientation of DNA inserts by PCR

The next stage was to determine the insert orientation. A new PCR was carried out, using different combinations of primers against the insert and the vector. The primer combination of the T7 promoter primer and the reverse primer 433 should give a product that indicates the insert is under the control of the T7 promoter while the combination of the T7 promoter primer and the forward primer 432 should not gave product if the insert is under the control of T7 promoter primer (Figure 3.48(i)).

The combination of the T7 promoter primer and the reverse primer 433 should not give product if the insert is under the control of the *lac* promoter, however the primer combination of the T7 promoter primer and the forward primer 432 should give product if the insert is under the control of the *lac* promoter (Figure 3.48(ii)).
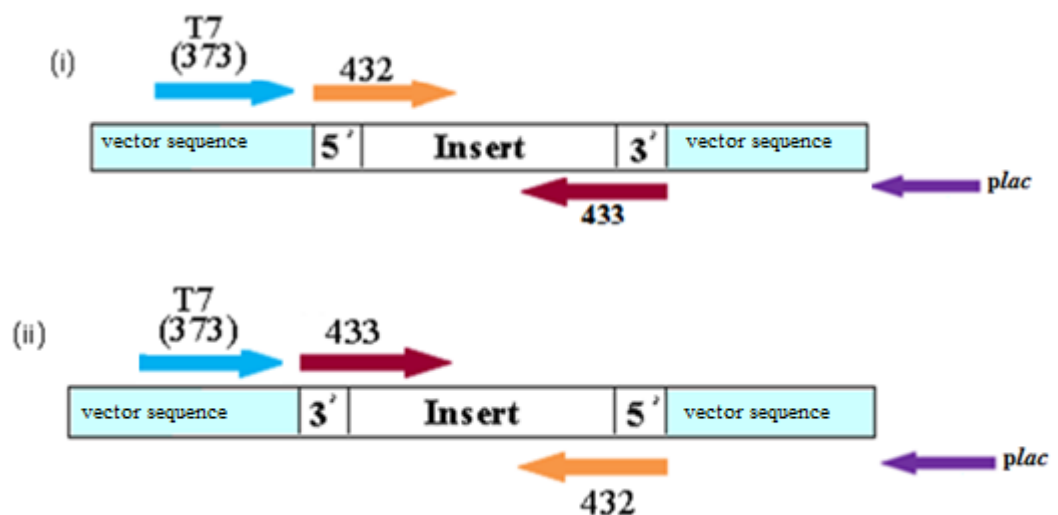
Figure 3.48: The annealing of primers 432, 433 and the T7 promoter primer (373) with recombinant pJET 1.2/blunt Cloning Vector, p*lac*, promoter of the *lac* operon, blue colour, vector sequence.

The results of the analysis are shown in Figure 3.49. 4 of 12 colonies gave product with reverse primer 433 and T7 promoter primer, but they did not give product with forward primer 432 and T7 promoter primer. Therefore, the orientation of all cloned genes was under the control of the T7 promoter.
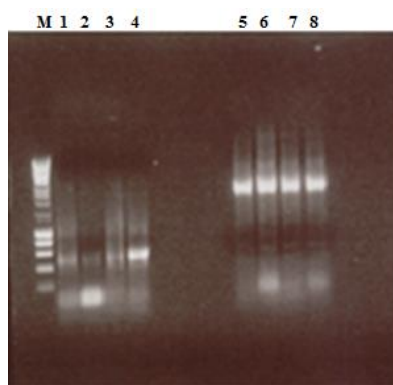
Figure 3.49: Determination of orientation of clones by PCR; lane M, Hyperladder I; lane1, sample 13 with forward primer 432 and T7 promoter primer; lane 2, sample 14 with forward primer 432 and T7 promote primer; lane 3, sample 15 with forward primer 432 and T7 promoter primer; lane 4, sample 16 with forward primer 432 and T7 promoter primer; lane 5, sample 13 with reverse primer 433 and T7 promoter primer; lane 6, sample 14 with reverse primer 433 and T7 promoter primer; lane 7, sample 15 with reverse primer 433 and T7 promoter primer; lane 8, sample 16 with reverse primer 433 and T7 promoter primer. The numbers on the gel are reference numbers for the samples (individual colonies).

At this stage, plasmids were tested for complementation of strain ZSC113 for fermentation of glucose and mannose, but they were not tested for the complementation of mutants unable to ferment maltose or trehalose. No complementation was observed as expected because there did not appear to be a promoter to drive expression of the genes. That meant it was decided to consider a different option and this was to transfer the genes into a different vector in which they would be expressed. But first, the sequence of the cloned genes was determined.

3.6.9. Large scale preparation of plasmid

Plasmid pME14 containing genes *cbei* 4983 and *cbei* 4982 was purified using the QIAGEN plasmid Midi kit as described in Materials and Methods in section 2.12.1. A sample of the plasmid was sent for sequencing to Beckman Coulter Genomics (Appendix I). The sequence was found to be correct, therefore, it was decided to try to move the cloned genes from the pJET 1.2/blunt Cloning Vector to the pUC18 vector, in which they would be under the control of the *lac* promoter, before carrying out any further fermentation tests.

3.6.10. Recombination of the *cbei* 4983 and *cbei* 4982 genes

As shown in the previous section the genes *cbei* 4983 and *cbei* 4982 were successfully cloned in the pJET 1.2 blunt vector. Following verification of the sequence of the cloned genes, the vector containing the *cbei* 4983 and *cbei* 4982 genes was restriction digested as described in Materials and Methods in section 2.17. The vector pME14 carrying the *cbei* 4983 and *cbei* 4982 genes was double–digested with *Xba*I and *Bgl*II. It was also digested with *Xba*I and *Bgl*II individually to show that both enzymes were functioning. As shown in Figure 3.50, digestion with *Xba*I produced a linear fragment, whereas digestion with *Bgl*II gave two fragments, one of them representing the plasmid (2900 bp) and the other one representing the insert (2300 bp). Finally double–digestion with *Xba*I and *Bgl*II gave fragments of the same size as shown in Figure 3.50. These results are consistent with the restriction map of pJET 1.2 blunt vector (Appendix IV). The DNA fragment carrying *cbei* 4983 and *cbei* 4982 was then purified (Figure 3.51), as described in 2.17.2.
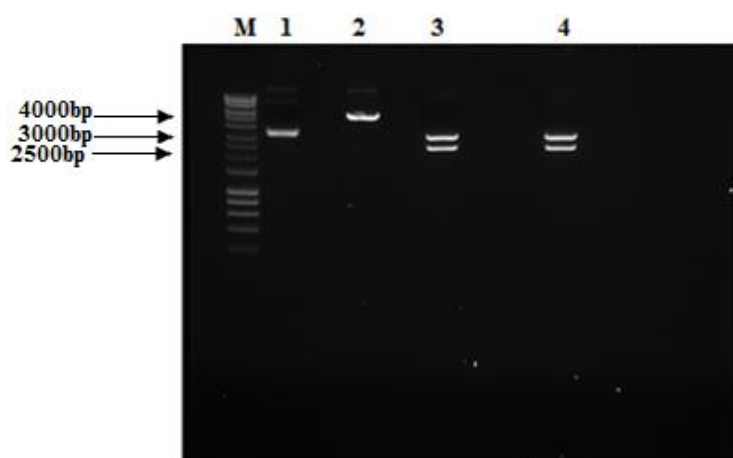
Figure 3.50: Restriction digestion of pME14. pME14 was digested using *Xba*I and *Bgl*II; lane M, Hyperladder I; lane 1, undigested pME14 containing *cbei* 4983 and *cbei* 4982; lane 2, pME14 digested with *Xba*I; lane 3, pME14 digested with *Bgl*II; lane 4, pME14 digested with both enzymes *Xba*I and *Bgl*II.
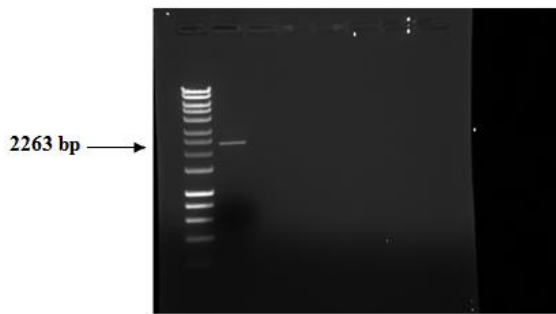
Figure 3.51: Purification of *Xba*I and *Bgl*II fragment containing gene *cbei* 4983 and *cbei* 4982.

The vector pUC18 was digested, by enzymes *Xba*I and *Bam*HI as shown in Figure 3.52. The double-digested plasmid (approximately 2700 bp) was purified as shown in Figure 3.53, and the purified plasmid was ligated to the purified fragment carrying *cbei* 4983 and *cbei* 4982. The *Xba*I ends of pUC18 and the DNA fragment will ligate together and the *Bam*HI terminus of pUC18 will ligate with the *Bgl*II terminus of the DNA fragment because they have the same sticky end. Therefore, the fragment can only be inserted into pUC18 in one specific orientation (see Figure 3.54).
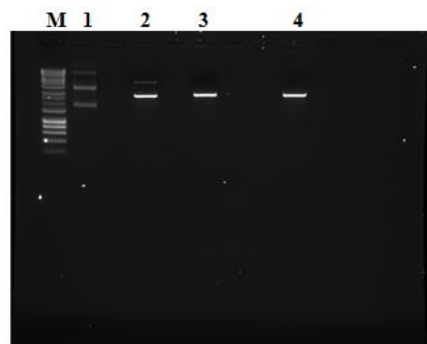
Figure 3.52: Restriction digestion of pUC18; lane M, Hyperladder I, lane 1, undigested plasmid; lane 2, after restriction by *Xba*I; lane 3, after restriction by *Bam*HI; lane 4, after restriction by *Xba*I and *Bam*HI.
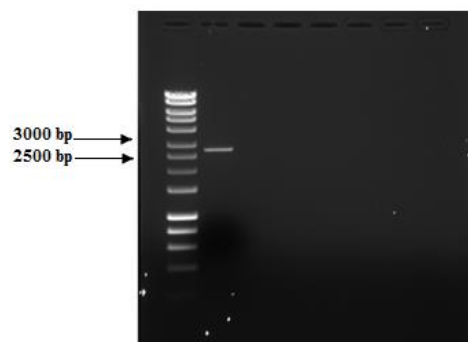
Figure 3.53: Purification of pUC18 digested with *Xba*I and *Bam*HI.
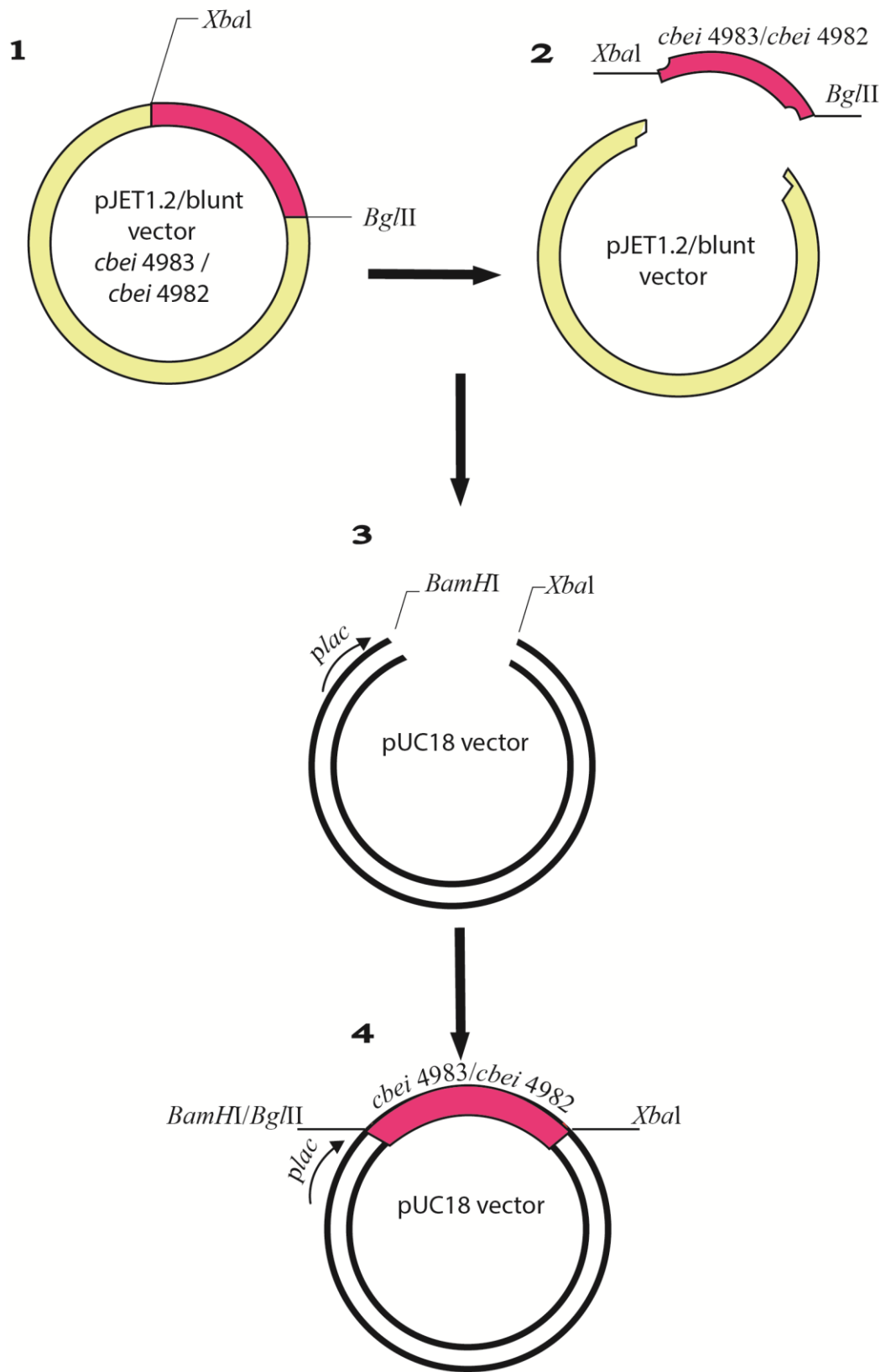
Figure 3.54: Restriction digestion strategy for cloning of *cbei* 4983 and *cbei* 4982 in pUC18.

Following ligation as described in Materials and Methods section 2.17.2, the mixture was transformed into *E.coli* TOP10 as described in Materials and Methods, and plated on LB supplemented with 100 µg /ml ampicillin and 40 µg /ml X-gal for blue-white screening (without ITPG). The plate had white and blue colonies, the white were transferred and grown on new plates with the same amount of antibiotic. The isolates were tested by PCR to check whether they contained the DNA insert. No positive results were obtained. The ligation and transformation were repeated several times but the results were still negative. It is not clear why this procedure was unsuccessful.

3.7. Cloning of the genes *cbei 4984, cbei 4983* and *cbei 4982*

3.7.1. PCR reaction for genes *cbei 4984 /cbei 4983/cbei 4982*

The previous experiments were designed to provide any evidence for the function of the PTS encoded by the genes *cbei* 4983 and *cbei* 4982. One obvious limitation of the approach is that the genes only code for the PTS, so that since the PTS will produce a phosphorylated substrate the transformed *E.coli* cells will have to have the enzyme necessary for metabolism of that sugar phosphate. Because the gene immediately adjacent to these genes seems to code for a hydrolase enzyme, it was decided effectively to begin again and to try to clone all three genes instead of only the PTS genes. Genes *cbei* 4984, *cbei* 4983 and *cbei* 4982 were amplified together in a PCR reaction. The primer combination used was 434 forward primer and 433 reverse primer, and the size of product obtained was around 4000 bp (expected size to be 3738 bp) as shown in Figure 3.55 (a), For comparison, the amplified product containing only genes *cbei* 4983 and *cbei* 4982 was between 2000 bp and 2500 bp in agreement with a product expected to be 2263 bp as shown in Figure 3.55 (a). The arrangement of genes is shown in Figure 3.55 (b).
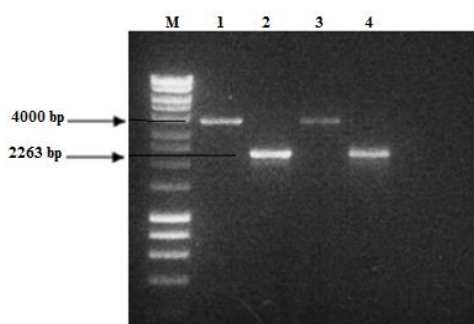
Figure 3.55 (a): PCR amplification of genes *cbei* 4984, *cbei* 4983 and *cbei* 4982; lane M, Hyperladder I; lane 1, amplified product from PCR reaction using the forward primer 434 and the reverse primer 433; lane 2, amplified product from PCR reaction using the forward primer 432 and the reverse primer 433; lane 3, amplified product from PCR reaction using the forward primer 434 and the reverse primer 433; lane 4, amplified product from PCR reaction using the forward primer 432 and the reverse primer 433.
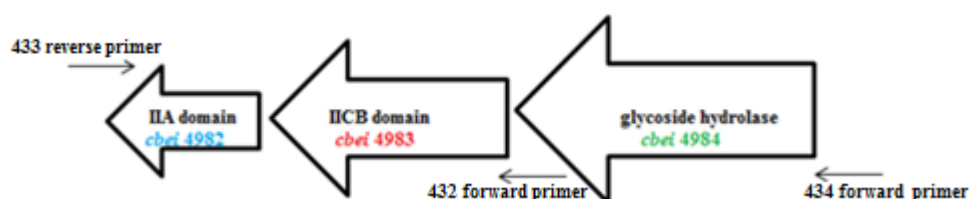
Figure 3.55(b): Arrangement of genes encoding phosphotransferase system in *C. beijerinckii.*

3.7.2. Transformation, screening and analysis of clones

Unlike when cloning the two genes *cbei* 4983 and *cbei* 4982, when three genes *cbei* 4984, *cbei* 4983 and *cbei* 4982 are cloned they will have an upstream promoter concerned with expression of the genes. Therefore, in principle it is not necessary to isolate clones in which the insert is under control of the *lac* promoter. The next step was to insert the PCR- amplified product into the StrataClone Vector, transform into StrataClone SoloPack competent cells, and spread onto LB plates containing 100 µg/ml ampicillin, as described in section 2.10.2. 44 colonies were picked off and after plating on LB containing ampicillin were incubated overnight at 37 °C. To show the gene insert within the vector, a screening was done (Figure 3.56). 35 of the 44 colonies contained the gene insert, as shown by a product of around 4000 bp. The genes had apparently been successfully cloned in these samples.
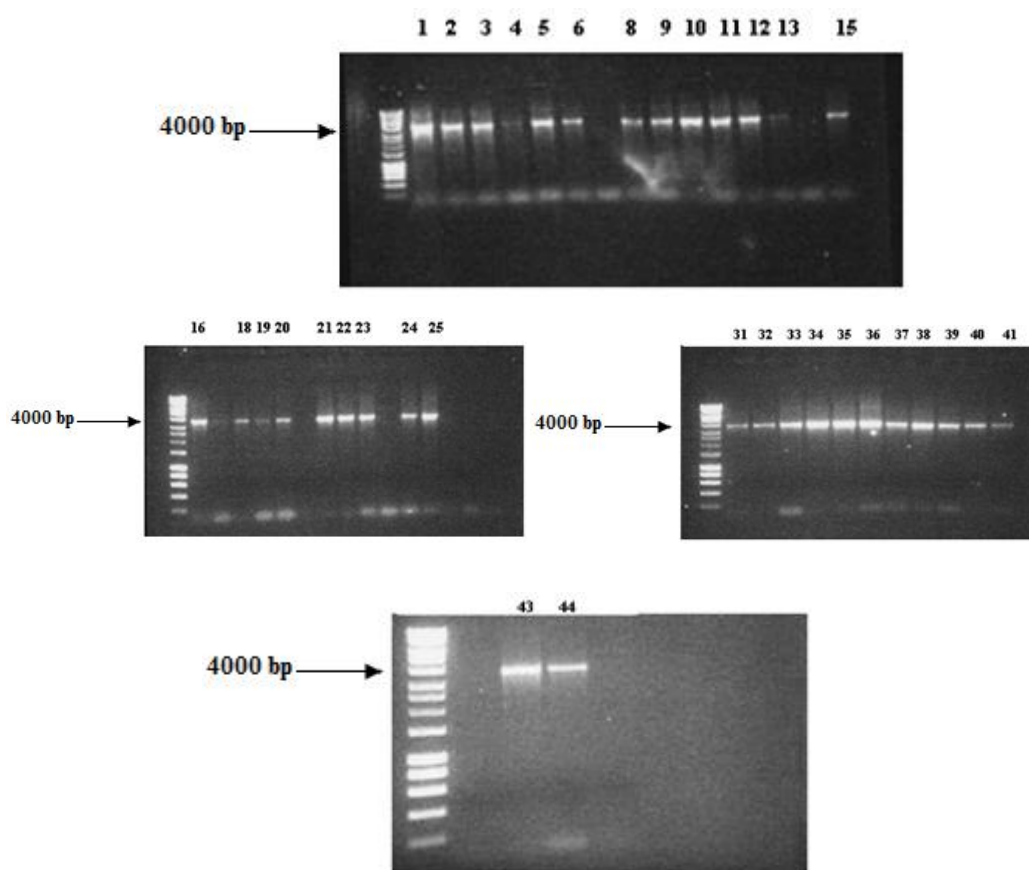
Figure 3.56: Screening of transformants for the presence of genes *cbei* 4984, *cbei* 4983 and *cbei* 4982 by PCR using the forward primer 434 with the reverse primer 433. The numbers on the gel are reference numbers for the samples (individual colonies) and the expected size of the insert to be 3738 bp.

3.7.3. Determination of orientation of DNA inserts by PCR

The strategy for determination of the orientation of the cloned DNA inserts was similar to that used earlier for genes *cbei* 4983 and *cbei* 4982 in the StrataClone Vector. In the StrataClone Vector, the genes are placed under the control of the *lac* or T7 promoter. Therefore, PCR was used to determine the insert orientation by using different combinations of primers directed against the vector and the insert. The primer combination of the reverse primer 433 with M13 forward primer 371, should give product if the insert is under the control of the T7 promoter. The primer combination of the forward primer 434 with M13 forward primer 371 should not a give product if the insert is under control of the T7 promoter primer. On the other hand, the primer combination of the reverse primer 433 with M13 reverse primer 372 should give a product if the insert is under the control of the *lac* promoter. However, the primer combination of forward primer 434 with M13 reverse primer 372 should not give a product if the insert is under control of the *lac* promoter.

35 colonies containing the gene insert were screened. Seven of the thirty five colonies gave a product with M13 forward primer 371 and reverse primer 433, but did not give product with M13 forward primer 371 and forward primer 434 as shown in Figure 3.57. 22 colonies gave a product with M13 forward primer 371 and forward primer 434, but did not give product with M13 forward primer 371 and reverse primer 433 as shown in Figure 3.58. Four of these 22 colonies were used to confirm that the genes were inserted under control of the *lac* promoter, and gave a product with M13 reverse primer 372 and reverse primer 433 (Figure 3.59), but did not give product with M13 reverse primer 372 and forward primer 434 (not shown). Six of the thirty five colonies did not give product at all in any of the PCR amplifications.
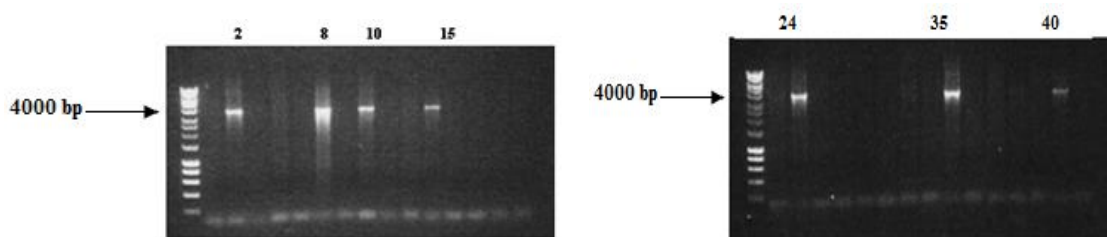
Figure 3.57: Screening of transformants for the orientation of genes *cbei* 4984/ *cbei* 4983/ *cbei* 4982 by PCR using M13 forward primer 371 with 433 reverse primer. The numbers on the gel are reference numbers for the samples (individual colonies) and the expected size of the insert to be 3738 bp.
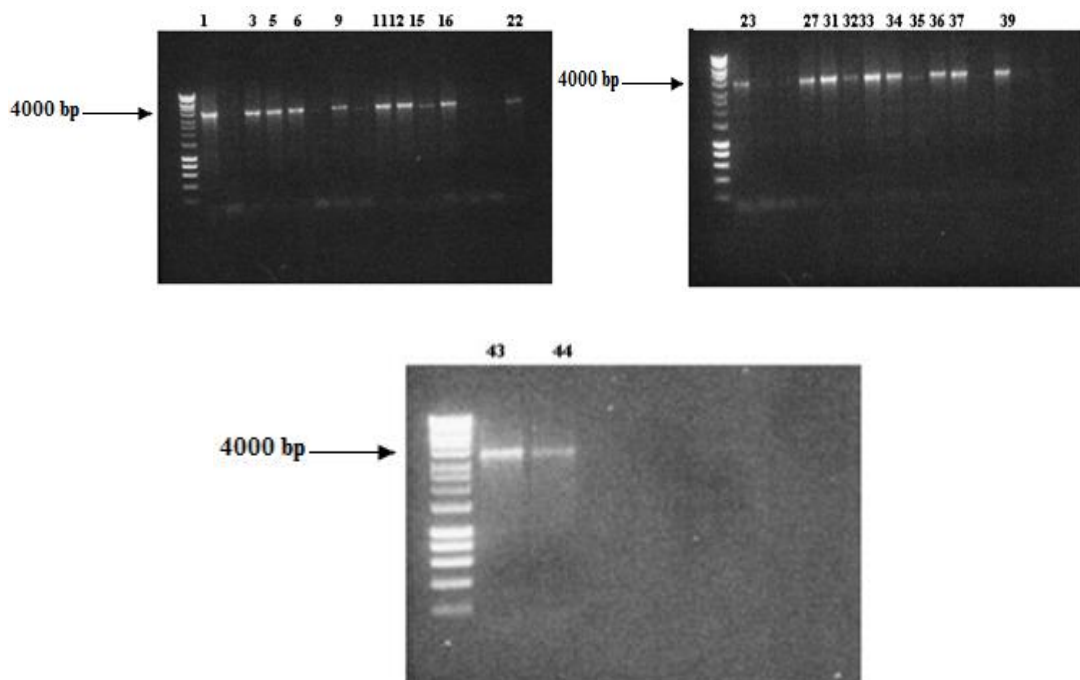
Figure 3.58: Screening of transformants for the orientation of genes *cbei* 4984/ *cbei* 4983/ *cbei* 4982 by PCR using M13 forward primer 371 with 434 forward primer. The numbers on the gel are reference numbers for the samples (individual colonies) and the expected size of of the insert to be 3738 bp.
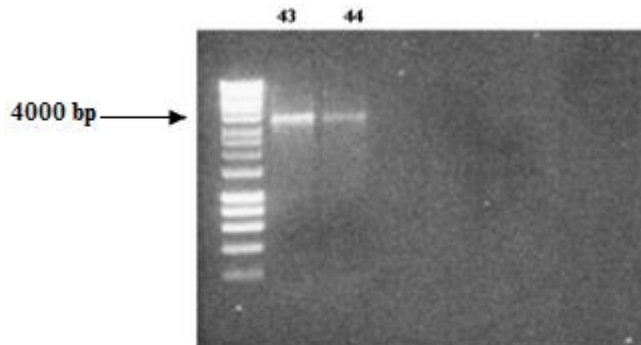
Figure 3.59: Screening of transformants for the orientation of genes *cbei* 4984/ *cbei* 4983 / *cbei* 4982 by PCR using M13 reverse primer 372 with 433 reverse primer. The numbers on the gel are reference numbers for the samples (individual colonies).

3.7.4. Preparation of plasmid and DNA sequencing

As a result of the analysis (Figure 3.59), three transformants (pME1, pME3, pME11) were selected for plasmid preparation and sequencing. The pME1 sample did not provide any sequence data, and pME3 showed two errors within in the sequence. Therefore, only pME11 which had the correct sequence, was used in the following fermentation tests.

3.7.5. Transformation and phenotype screening

The plasmid pME11 was transferred into different *E.coli* mutants including JW3381- 4, JW3701-2, and JW4199-1, which are unable to metabolise maltose, cellobiose, and trehalose as demonstrated in section 3.6.5. The transformants were screened on MacConkey agar containing 30 µg/ml kanamycin and 100 µg/ml ampicillin and supplemented with 0.1 % trehalose, 1 % maltose, or 1 % cellobiose, and incubated at 37°C for up to 48h.The strains containing pME11 demonstrated a negative fermentation phenotype for cellobiose and maltose as shown in Figures 3.60 and 3.61 and also trehalose (not shown), indicating that pME11 apparently does not encode for uptake and metabolism of trehalose, maltose or cellobiose. As in the previous set of experiments, the *nagE* mutant JW0665-1 was shown to be able to ferment maltose as a control (Figure 3.61), but not cellobiose.

pME11 was also transformed into a mutant *E.coli* JC10279 deficient in maltose transport (see section 2.1), and then the colonies were transferred onto MacConkey agar supplemented with 1 % maltose to examine the phenotype. Transformants grew as small colonies that gave a positive fermentation phenotype (not shown), but on further restreaking the cells showed a negative result, indicating that these samples were again unable to ferment maltose.
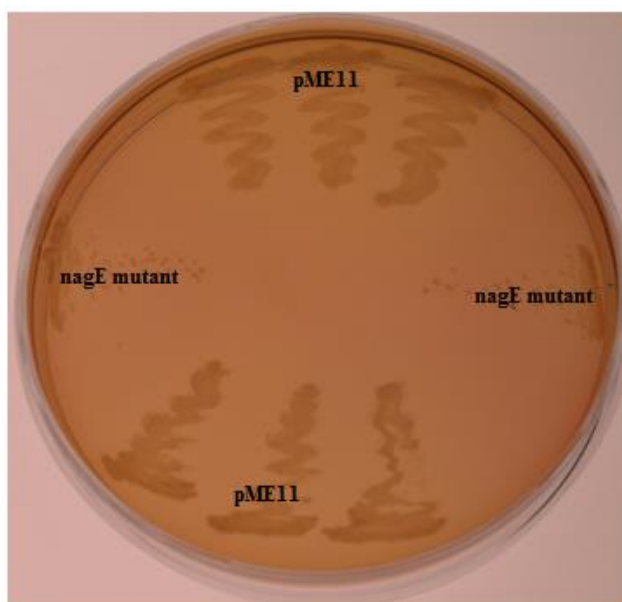
Figure 3.60: Fermentation of cellobiose on MacConkey agar by *E. coli* JW3701-2 containing pME11. The recombinant strain is unable to ferment cellobiose.
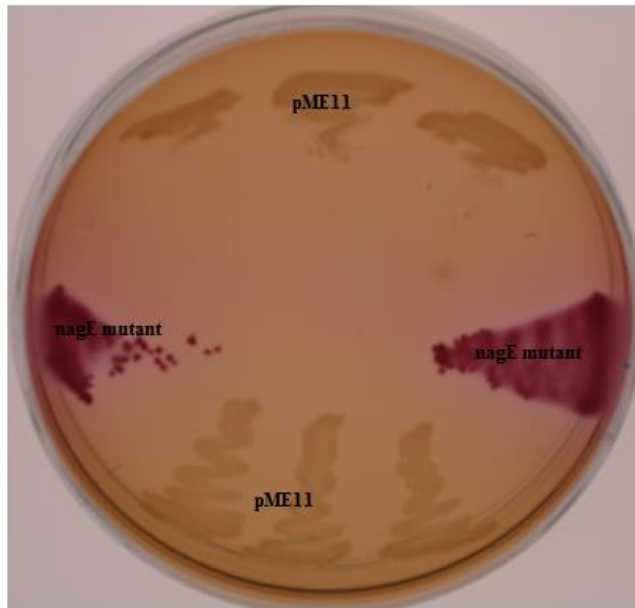
Figure 3.61: Fermentation of maltose on Macconkey agar by mutant JW3381- 4 containing pME11. The recombinant strain is unable to ferment maltose. *E.coli* strain JW0665-1(*nagE*), however was able to ferment maltose.

3.8. Gene Knockout using the ClosTron

As was done for gene *cbei* 0751 an attempt was made to create a knockout mutant of the *cbei* 4983 gene to examined the effect on growth of *C. beijerinckii* could be determined, as described in section 3.5, site for insertion was selected based on the analysis of ClosTron website http://www.clostron.com. The required plasmid was ordered from DNA 2.0, the plasmid was treated as described previously and transformed into *E.coli* strain and then conjugated into *C. beijerinckii.* In the wild type the combination of forward primer 437 and reverse primer 438 should give product of 368 bp. However, for a mutant the primer combination of EBS primer and 437 forward primer should give a PCR product of 490 bp, but the combination of EBS primer and the 438 reverse primer should not give a product. After putative clones were isolated and purified several times on CBM plates supplemented with 10 µg/ml erythromycin, DNA was extracted and the various PCR amplifications were carried out. The results were as was found in the previous case with the *cbei* 0751 gene. The only products which were obtained were from the combination of the forward primer 437 and reverse primer 438. It was therefore clear that nothing was inserted in between as shown in Figure 3.62.
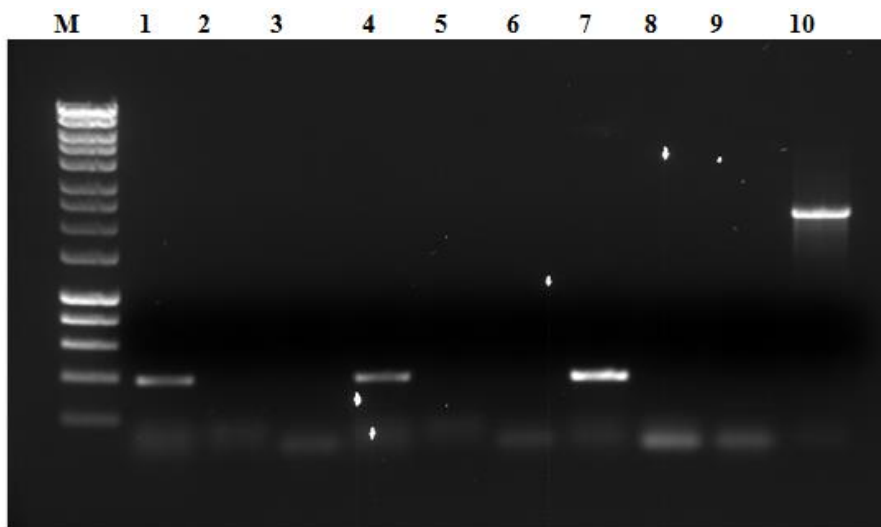
Figure 3.62: Mutant screening by PCR used to screen for intron insertion in gene *cbei* 4983; lane M, Hyperladder as a marker; lane 1, sample 1 with forward primer 437 and reverse primer 438; lane 2, sample 1 with forward primer 437 and EBS primer; lane 3, sample 1 with reverse primer 438 and EBS primer; lane 4, sample 5 with forward primer 437 and reverse primer 438; lane 5, sample 5 with forward primer 437 and EBS primer, lane 6, sample 5 with reverse primer 438 and EBS primer; lane 7, sample 7 with forward primer 437 and reverse primer 438; lane 8, sample 7 with forward primer 437 and EBS primer; lane 9, sample 7 with reverse primer 438 and EBS primer; lane 10, wild type with forward primer 356 and reverse primer 357 those are the cloning primers as a control, and the expected size to be 2364 bp, the numbers on the gel are reference numbers for the samples (individual colonies).

CHAPTER 4

DISCUSSION

4. Discussion

Biofuels are attracting interest as a kind of fuel at present due to the economic advantages and environmental concerns. Also they are renewable energy sources (Paul, 2009). Butanol is a potentially important biofuel of the future. It has been produced by fermentation in the past, but by the early 1960's the solvent fermentation began to decline in USA and UK, because the ABE fermentation processes could not compete economically compared to the petrochemical industry (Jones and Woods, 1986, Gibbs, 1983). Price increases of substrates such as molasses, wheat and maize after the second World War, low production of solvent in the fermentation, and solvent toxicity for clostridia especially from butanol, were factors that played a major role in the decline of the ABE fermentation (Hastings, 1971). In South Africa and the former USSR, ABE fermentation plants operated until 1982, and in China the production of butanol by fermentation was 350,000 tons/year until 2004 (Durre, 2008). Reintroduction of the ABE fermentation will depend on a number of factors including use of cheap, renewable material, and improved butanol tolerance, production and recovery from the solvent fermentations.

The market price of fermentable organic material is one of the major considerations in the economics of fermentative biofuel (Gapes, 2000). Lignocellulose is available from many sources such as forestry and agriculture including fruit, grains, oil seeds, and vegetables (Howard *et al*., 2004). Other waste material products consist of many carbohydrates that can be used as a carbon source to produce huge quantities of chemicals acetone and butanol by fermentation (Durre 2008, Durre 2007). Resources are dependent on the area of the world (Kim and Dale, 2005), which will dictate the availability of substrates for use in biofuels production. After hydrolysis, sugars including mono- and disaccharides are released form lignocellulose biomass or other waste material, and it is these molecules which are fermented by bacteria.

In fact, it has been demonstrated that *C. beijerinckii* has the ability to use a range of carbon sources such as glucose, mannose, maltose, sucrose, which could be present in the majority of waste materials (Tangney and Mitchell, 2005), to produce the solvents acetone, butanol and ethanol (Ezeji and Blaschek, 2007).

The first step of sugar metabolism is the transport of the sugar across the cytoplasmic membrane (Mitchell, 1996). Sugar transport systems in bacteria operate by a variety of mechanisms. However, the dominant system in obligate anaerobes is the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS), which is characterized by transport and metabolism of its substrate as it enters the cell (Mitchell, 1998). The advantage of substrate transport by the phosphotransferase system is that phosphorylation of the sugar during entry into the cells alters the substrate configuration, so that the sugar is unable to leave the cells and also both transport and metabolism are accomplished by a single metabolic step (Tangney *et al*., 2001).

Most of the clostridial species including *C. beijerinckii* and *C. acetobutylicum* depend greatly on the PTS for uptake and metabolism of different carbon sources such as glucose, which is the preferred carbon source. Bacteria prefer to utilise one carbon source over another, a phenomenon called carbon catabolite repression (CCR), the mechanism of which is discussed in section 1.7. The PTS phosphocarrier protein HPr is considered to be a key element of catabolite repression in low G-C Gram- positive bacteria (Tangney *et al.,* 2003). In the solventogenic clostridia preferential metabolism of glucose over several alternative carbon sources has been observed (Mitchell, 1996, Mitchell *et al*., 1995). Full redevelopment of the ABE fermentation will require a more complete characterization of the PTS and its metabolic function in clostridia.

In the genome sequence, 43 complete PTS have been identified by sequence homology in *Clostridium beijerinckii* NCIMB 8052. Of these, three PTSs have been characterised. One has been shown to be a sucrose system (Reid *et al*., 1999), a second one has been shown to be a glucitol system (Tangney *et al*., 1998) and the third one has been shown to be a N- acetylglucosamine system which also transports glucose (Al Makishah and Mitchell, 2013).

 Bacteria which are developed for making biofuel by fermentation will ideally be able to take up sugars as fast as possible and in an unregulated way. Because glucose is generally a preferred sugar which represses metabolism of other substrates, it is necessary to understand how the cells respond to glucose (Alpert *et al*., 1985). Therefore, identifying which systems are involved in glucose uptake is important because they are the systems which are likely to be involved in sensing glucose in the environment and causing catabolite repression.

4.1. The PTS system encoded by gene *cbei* 0751

In the first part of this project, the gene *cbei* 0751 was amplified from genomic DNA of *C. beijerinckii* NCIMB 8052. The gene *cbei* 0751 encodes a PTS with three domains IIA, IIB and IIC together, therefore it is encodes a complete PTS which should be functional in *E. coli*. The PCR product was ligated into the pCR® 2.1-TOPO® vector and transformed into *E. coli*. Two (ME15 and ME20) of forty colonies were found to have the insert. The colonies were screened by PCR to determined the orientation of the insert and plasmids pME15 and pME20 were found to contain *cbei* 0751 under the control of T7 promoter and *lac* promoter respectively. These plasmids were isolated and transformed into *E. coli* ZSC113 to investigate the fermentation phenotype on MacConkey agar containing either glucose or mannose. The transformants demonstrated a positive fermentation phenotype for both sugars. Although the gene *cbei* 0751 in pME15 was in an orientation under control of the T7 promoter, it appeared that it was also expressed. The T7 promoter in *E. coli* is supposed to be silent, because it is recognised by T7 RNA polymerase but not by *E. coli* RNA polymerase. *E. coli* ZSC113 does not contain the T7 RNA polymerase. It could be that there is a low level of transcription activity from the T7 promoter but a more likely explanation for expression of the gene is that it was cloned with about 210 bp of upstream DNA, which may contain a promoter or a DNA sequence that is recognised as a promoter in *E. coli*. This is not unique, because in many cases in the laboratory cloned genes have been predominantly isolated in the orientation that appears to be unfavourable for expression. DNA base composition of clostridia is around 70 % A-T, and *E. coli* promoters are highly A-T rich, so any segment of clostridial DNA just might act like a promoter as far as the *E. coli* RNA polymerase is concerned. A low level of expression from a non-natural promoter might be compatible with growth and survival, allowing clones with unfavourable orientation to be readily isolated.

After the initial demonstration of complementation of glucose and mannose fermentation, additional experiments were carried out which demonstrated that the fermentation phenotype was in fact due to activity donated to *E. coli* ZSC113 by the *cbei* 0751 gene. Firstly, glucose and mannose were shown to be utilised in broth culture by pME15 and pME20 but not by *E. coli* ZSC113. Therefore, the recombinants but not the mutant were able to take up the sugars from the growth medium. Secondly, uptake of radiolabelled glucose was demonstrated by the recombinants but not the *E. coli* ZSC113 mutant. Thirdly, extracts made from the recombinants, but not *E. coli* ZSC113,

showed PEP- dependent phosphorylation of glucose that indicated the presence of a PTS. Glucose phosphorylation was strongly inhibited by mannose, suggesting that both sugars are phosphorylated by the same PTS. Therefore, it can be concluded that the PTS encoded by *cbei* 0751 can transport and phosphorylate both sugars glucose and mannose. An attempt was made to demonstrate competitive inhibition of glucose phosphorylation by mannose, by altering the concentrations of glucose and mannose in a series of experiments. Although different reaction rates were observed as expected the data did not allow for a definitive conclusion.

Inhibition of glucose phosphorylation by a number of other analogues was studied as shown in Figure 3.25. The activity was inhibited by 2-deoxyglucose and methyl α–glucoside (α MG), but not well by 3-O-methyl glucose. When these results were compared with experiments carried out previously using crude extract of *C. beijerinckii* (Mitchell *et al*., 1991), the pattern was similar with the exception of the effect of methyl α–glucoside. The difference between the experiments in this study and the earlier experiments is that in extracts of *C. beijerinckii* there could have been two, or more PTSs contributing to glucose phosphorylation activity (Mitchell *et al*., 1991), with the result that the total activity was not inhibited strongly by α MG. The N-acetylglucosamine system also transports glucose (Al Makishah and Mitchell, 2013), and some members of the mannose family might also transport glucose because it is well known that systems in the mannose family often have the ability to transport glucose as a substrate. The mannose system of *E. coli* has been shown to transport glucose (Tchieu *et al*., 2001), and the glucose system of *E. coli* is able to transport mannose (Saffen *et al*., 1987, Erni and Zanolari, 1986). *Staphylococcus carnosus* contains two related glucose phosphotransferase systems which are inhibited by different analogues (Knezevic *et al*., 2000). Therefore, phosphotransferases show different specificity with different combinations of sugars being recognised as substrates.

In the recombinant strains, the components of the PTS from different organisms must interact with one and other to make up the complete system. That is *E. coli* proteins must interact with clostridial proteins. The point of the connection is not entirely clear because the mutation in *E.coli* ZSC113 is not well defined by modern standards.

This interaction is likely to be at the level of HPr transferring phosphate to IIA. However, it could also be at the level of interaction between proteins within the enzyme II complex. Interaction between the components of the *C. beijerinckii* PTS and the PTS in *C. pasteurianum*, *B.subtilis* and *E.coli* has been demonstrated previously (Mitchell *et al*., 1991). Also more recently the complementation of an *E.coli* N- acetylglucosamine PTS mutant by genes from *C. beijerinckii* also showed that interaction could occur between proteins from the two species (Al Makishah and Mitchell, 2013).

As part of this project experiments were carried out to examine the expression of the *cbei* 0751 gene in cultures grown on different carbon sources. The carbon sources tested were glucose, sucrose, galactose, N-acetyglucosamine, mannose and maltose. As shown in slot blot hybridization (Figure 3.26), expression of the gene appears to occur under all growth conditions tested. The limitation of the hybridization approach is that it is not fully quantitative but the results did indicate the gene was expressed constitutively to some extent. The initial experiments were followed up by using RT-PCR after removing DNA contamination from the RNA samples, and again the expression was seen under all conditions as shown in Figure 3.32, but this experiment did not include a control gene for RT-PCR. Wang *et al*., (2011), showed that the *cbei* 0751 gene is not the most highly expressed gene encoding a PTS of the glucose family in a typical *C. beijerinckii* fermentation growing on glucose; the most highly expressed gene was in fact *cbei* 4532 which encodes the N- acetyglucosamine PTS (Al Makishah and Mitchell, 2013). They found that the *cbei* 0751 gene was expressed at a lower level, which did not change much over the course of the fermentation. Therefore, a more detailed study of the expression of *cbei* 0751 under different condition would add to understanding of the importance of the gene.

Regulation of expression of *cbei* 0751 is assumed to be a function of the antiterminator coded for by *cbei* 0752. Inspection of the sequence of the putative antiterminator protein shows that it contains the conserved histidines which may be phosphorylated to control its activity. Therefore, it would be expected that gene *cbei* 0751 may be induced by the substrate of the PTS it codes for as a result of effects on the phosphorylation of the antiterminator protein. The association of the glucose PTS gene with an antiterminator is similar to the gentic organization in *C. acetobutylicum* (Tangney and Mitchell, 2007), and *B.subtilis* (Stülke *et al*., 1997). In *C. acetobutylicum* (Tangney and Mitchell, 2007), a difference is that the gene encoding the antiterminator is situated upstream of the PTS gene whereas in *C.beijerinckii* it is downstream of the PTS gene.

Servinsky *et al*., (2010) carried out a transcription analysis using *C. acetobutylicum* grown on eleven different carbohydrates. Expression of the glucose PTS was regulated at transcriptional level, with a strong signal during growth on maltose, starch and glucose but lower expression for other substrate. In *B.subtilis,* antiterminator is association with *pts*G, *pts*H and *pts*I which encode for HPr and EI (Stülke *et al*., 1997). The expression of these genes has been shown to be under the control of the antiterminator. In contrast, to the clostridia and *B.subtilis,* in *E.coli* there is no antiterminator involved in regulation of expression of the glucose PTS. The glucose PTS is constitutively expressed although there is a difference in expression of the *ptsG* gene in *E. coli* dependent on the growth conditions (Postma *et al*., 1993). The precise role of the antiterminator coded by *cbei* 0752 remains to be established by further experiments.

In most clostridia, glucose is the preferred carbon source and its presence inhibits transport and phosphorylation of many other carbohydrates. This is an important aspect of cell physiology because it has implications for metabolism of sugars in a fermentation. One way to identify the importance of a gene is to inactivate it and then look at the effect on cell behaviour. As already discussed, there are several potential systems for glucose uptake in *C. beijerinckii,* so that knock out of one particular system might not have much effect on glucose utilization and sensing, because other systems can take over. However, it has been shown by Xiao *et al*., (2011), that inactivation of *glcG* (the *C. acetobutylicum* gene that is equivalent to *cbei* 0751) did reduce catabolite repression but at the same time it had no effect on the rate of glucose metabolism. Therefore that study demonstrated that is was possible to see an effect on catabolite repression by inactivation of one particular gene.

In this study, attempts were made to knock out *cbei* 0751 using the ClosTron technology, developed at the University of Nottingham (Heap *et al*., 2010, Heap *et al*., 2007). Although putative mutants were isolated on plates containing erythromycin further characterisation showed that the *glcG* gene had not been disrupted. Several attempts were made to obtain a *glcG* mutant, but without any success. One explanation is that the intron has inserted into a different site in the chromosome, which could have been proved by Southern blotting. In this laboratory attempts to use the ClosTron to mutate the *nag*E gene were also unsuccessful. Also at the University of Cape Town several attempts have been made to isolate mutants of genes involved in nitrogen metabolism, but no mutants were obtained (S. Reid, personal communication). The

ClosTron may not be as efficient in *C. beijerinckii* compared with *C. acetobutylicum* and *C. difficile.*

4.2. The PTS system encoded by genes c*bei* 4983 and *cbei* 4982

The second part of the project concerned the phosphotransferase system encoded by the c*bei* 4983 and *cbei* 4982 genes. Unlike the PTS encoded by *cbei* 0751, which is a single protein containing the IIA, IIB and IIC domains together, two genes are required because *cbei* 4983 encodes a protein with the IIB and IIC domains and *cbei* 4982 encodes a separate protein with the IIA domain. Therefore, both the proteins are needed to constitute an intact PTS. The first attempt at cloning used StrataClone Vector with the PCR product generated using 432 forward primer and 433 reverse primer. When the cloning was done it was expected that 50 % of clones would be in the favourable orientation that is under control of the *lac* promoter in the vector. But all except one of the clones were found to have the genes inserted under the control of the T7 promoter. The one exception was pME25 containing *cbei* 4983/ *cbei* 4982, under the control of the *lac* promoter. This plasmid was isolated and used to transform *E. coli* ZSC113 which is unable to metabolise glucose and mannose, but the negative phenotype was not complemented. The plasmid was also transformed into the mutants JW3381-4 which is unable to metabolise maltose, JW3701-2 which is unable to ferment cellobiose and JW4199-1 which is unable to phosphorylate trehalose. The transformants were streaked onto LB plates and transferred onto MacConkey agar. The transformants demonstrated a negative phenotype on the MacConkey agar containing maltose, cellobiose and trehalose respectively, which indicated that the cloned genes did not complement the mutation. Leter testing of the plasmid found that the gene sequence was in fact correct, the plasmid contained an unexpected DNA sequence upstream of the cloned genes. Therefore, this provides a possible explanation for why no activity was observed in the transformants.

Unlike *cbei* 0751, these genes c*bei* 4983 and *cbei* 4982 will require a vector promoter for expression, because the genes are downstream of *cbei* 4984 which codes for a putative hydrolase enzyme and that gene appears to be part of an operon with c*bei* 4983 and *cbei* 4982. Therefore, since no clones were obtained in the required orientation a different approach was adopted.

The c*bei* 4983 and *cbei* 4982 genes were then cloned in a different vector pJET1.2 blunt. As for the previous cloning, all clones isolated were found again to contain the insert in the unfavourable orientation, under the control of the T7 promoter. One plasmid pME14 was selected and sequenced and shown to carry the correct genes.

This plasmid was transformed into *E. coli* ZSC113 but had no effect on glucose or mannose fermentation. However, the genes may simply not be expressed due to lack of an active promoter in the plasmid. It was therefore, decided to transfer the genes to the vector pUC18, in a way that would place them under control of the *lac* promoter by unidirectional cloning. Although white colonies were isolated on LB plates supplemented with X-gal, suggesting that they contained recombinant plasmid, PCR screening did not show the presence of the expected insert. The reasons why this approach did not succeed are not known.

The phylogenetic tree including the IIC domain of *cbei* 4983 strongly suggested that the system may be a maltose PTS. A problem in proving this function may be that *E. coli* does not generally metabolise maltose by a PTS. Instead maltose metabolism in *E. coli* consists of uptake by an ATP dependent transport system, and then metabolism by two principal enzymes amylomaltase (MalQ) and maltodextrin phosphorylase (MalP) (Schwartz, 1987, Freundieb and Boos, 1986, Reyes *et al.*, 1986), As a result, *E. coli* does not have an enzyme able to hydrolyse maltose 6- phosphate. This is important because this is the product of the phosphotransferase system and cells would have to metabolise it to give a positive fermentation phenotype on MacConkey agar. Therefore, if the genes *cbei* 4983/ *cbei* 4982 are expressed in *E. coli* so that cells can take up and phosphorylate maltose it may be that the cells will not be able to metabolise the maltose 6-phosphate which is generated. *E. coli* does in fact have a maltose PTS called MalX, but this is a minor system that is only synthesised under certain conditions and under normal conditions does not play a role in maltose metabolism. MalX has been suggested to transport maltose without modifying the substrate, so that a maltose 6-phosphate hydrolase is not required to metabolise the sugar (Reidl and Boos, 1991).

One mutant used in this study is mutated in *malT*, which is the gene that encodes the activator of the maltose regulon and so the cells should not have any activity associated with uptake and metabolism of maltose. The other mutant *E. coli* JC10279 has a mutation called *malA* which affect the region containing the *malQ* and *malP* genes.

As for maltose, complementation of metabolism of cellobiose and trehalose may be a problem in *E. coli*. The mutant used to screen for cellobiose fermentation was mutated in *bglG* which codes for an antiterminator. The mutation therefore affected expression of the β-glucoside specific PTS. The cellobiose PTS in *E.coli* is cryptic, and is induced by chitobiose (Soto-Gil and Zyskind, 1989), so *E.coli* cannot grow on cellobiose under normal circumstances. Similar to maltose, *E.coli* would require an enzyme able to hydrolyse the product of a cellobiose (or β-glucoside) PTS. Earlier study of a β-glucoside PTS from *C. longisporum* (Brown and Thomson, 1998), used a strain LP100 which had a constitutive *bglA* gene encoding phospho-β-glucosidase. This strain was not available for the present study.

The metabolism of trehalose in *E.coli* involves accumulation by a PTS followed by hydrolysis of trehalose 6-phosphate. The mutant JW4199-1 lacks trehalose PTS activity, and could be potentially complemented by a cloned PTS. However, *E.coli* also has a protein call trehalase which is present in the periplasm and which can hydrolyse trehalose to glucose. This enzyme is probably the reason why the mutant fermented trehalose when it was added to the medium at a concentration of 1 %. This fermentation did not occur when the trehalose concentration was decreased to 0.1 % presumably because this is below the concentration required for trehalase activity. At this concentration, the affinity of a cloned trehalose PTS must be fairly high to take up the substrate even when it is present at low concentration. Most phosphotransferases do have a high affinity for their substrates, but the properties of the PTS encoded by *cbei* 4983 /*cbei* 4982 are unknown.

The final strategy of the project attempted to clone the PTS-encoding genes together with the gene encoding the putative hydrolase enzyme, that is the three genes *cbei* 4984/*cbei* 4983/*cbei* 4982. A clone was successfully isolated and verified by sequencing, with the genes under the control of the *lac* promoter. However, when tested for complementation of *E. coli* mutants no positive fermentation was observed for maltose, cellobiose or trehalose. However, it was not actually demonstrated in the experiments that the genes were expressed.

In summary, none of the approaches used allowed for identification of the function of the PTS encoded by *cbei* 4983 /*cbei* 4982. Therefore in future a different strategy should be taken. *B.subtilis* has a maltose PTS and an associated glucoside 6-phosphatase providing the mechanism for metabolising maltose 6-phosphate (Schönert *et al*., 2006).

This suggests that a future study may be better to concentrate on cloning in *B. subtilis* because there is better chance that the bacterium will be able to metabolise a product of the phosphotransferase system successfully. Also *B.subtilis* accumulates cellobiose and trehalose by phosphotransferase systems (Tobisch *et al*., 1997, Schock and Dahl, 1996), so that these sugars will also be metabolised following accumulation by a PTS.

A totally different approach would be to try to identify the function of the hydrolase enzyme encoded by *cbei* 4984 since this would potentially identify the product of the PTS encoded by *cbei* 4983/ *cbei* 4982. Therefore, the hydrolase gene could be cloned and expressed in *E.coli.* In the later part of the project an attempt was made to clone the hydrolase gene but this attempt was unsuccessful in the time available. *C. acetobutylicum* has got two genes encoding proteins which show a high relationship (identity of about 50 %) to the product of *cbei* 4984 (Thompson *et al*., 2004). One of them MalH has been shown to hydrolyse maltose 6-phosphate and also a range of other glucoside phosphates. The other one PagL only showed the ability to hydrolyse p-nitrophenyl-α-D-glucopyranoside 6-phosphate (pNPαGlc6- phosphate), and it did not hydrolyse other glucoside phosphates. The PTSs associated with the *malH* gene and with the *pagL* gene show about 35 % indentity to the PTS coded by *cbei* 4983, and so it is possible that *cbei* 4983 has a similar substrate. However, it should be noted that the phylogenetic tree in Figure 1.5 shows a better candidate for a maltose PTS in *C. beijerinckii* which is the one with a IIC domain encoded by *cbei* 4705. This PTS is closely related to maltose PTSs of other bacteria, including *B.subtilis* and *C. acetobutylicum.*

Another totally different approach to looking at function of a gene is to knock it out and examine the effects. As for *cbei* 0751, in this study attempts were made to inactivate the *cbei* 4983 gene using the ClosTron, but these were also unsuccessful. As discussed for *cbei* 0751, the limitation of this approach is that there are 43 different PTSs in *C. beijerinckii* and if one is knocked out another one may still be able to take up the substrate.

4.3. Final conclusion

Understanding of sugar uptake in clostridia is a very important part of optimising fermentation. Very few transport systems in clostridia have been fully characterised. In *C. beijerinckii* three systems have previously been characterised encoded by *cbei* 4532/*cbei* 4533, *cbei* 0336/*cbei* 0337 and *cbei* 5012. In this study it was shown the *cbei* 0751 encodes a glucose/mannose phosphotransferase system, therefore it has been demonstrated that there are at least two glucose phosphotransferase systems in *C. beijerinckii*, encoded by *cbei* 0751 and *cbei* 4532/ *cbei* 4533. Both of these systems could potentially contribute to catabolite repression. Ability to knock out these genes and examination of the behaviour of mutant strains, may be useful in understanding their contribution. Even if these systems are more fully characterised there are many other systems which still have to be characterised, and therefore a great deal of work has to be done in order to fully understand the area of sugar uptake. In part of this study, experiments were done to look it the function of the *cbei* 4983/ *cbei* 4982 system. However at the present time is not possible to identify what the substrate of this system is.

A full characterisation of sugar uptake in *C. beijerinckii* will be extremely useful in designing strains which will be capable of metabolising different substrates. This will improve the economics of the ABE fermentation based on use of different substrates.

5. Appendices

Appendix I. Gene sequences

Clostridium beijerinckii NCIMB 8052 (*cbei* 0751)

TGCCCGCGATAATATTTTATGAAAACATTGACATGCAGTTATAGATATGCTAGAGTGAAAAATATGATAT
AGTATGTGACTGGTAGGTAGGCATTAACTATACATAAATAT**TTATTGAAGATGTTTATGTTGG**TTAATGT 356fwdprimer
TTTTTTGCACAAAAAAGGCATAATAAAGCATTAATAATGTGCAAAAATAATCCTATGAAGGAGAAGAATT
ATGAAAGACAAAGTTTTTGGCGTTTTGCAACGTGTAGGAAGATCTTTTATGCTTCCAATTGCTATTTTAC
CAGTAGCAGGTTTATTTTTAGGTATAGGTGAGTCATTCACTAATAAAACAATGCTTGATACGTATGGAAT 510Digfwd primer
TACAGGTTTAATTGGACCTGGCACATTTGTCAATGCACTTCTTTCAGTAATGAATGATGCAGGAAATATT
GTGTTTGAAAATTTGCCTTTAATATTTGCAATAGGGGTTGCAATAGGCATGTCTAAGAAAGAAAGAGAAG
TTGCAGCGTTAGCAGCAGGAATTGCATTTCTTATAATGCATGCATCAATTGGTGCCATGATTAAAATTCA
TGGTGGAACAGAAGCTCTTTTAAGTGGCGCATCAACTGAAGTACTTGGTATAATTTCATTACAAATGGGT
GTTTTCGGTGGGATTATCGTAGGGCTT**GGTACAGCAGCATTGCATA**ATAGGTATTATAAAATTGAATTAC 511Digrev primer
CACAAGTATTATCGTTTTTTGGTGGAACTAGATTTGTTCCTATTATATGTTCAATTGTCTATTTAATAGT
TGGAATTTTAATGTTTTATATTTGGCCTCCAGTTCAAGGTGCTATTTACAAAGTTGGAAATATAGTGCTT
GCATCAAGTTATGCAGGAACGTGGGTTTATGGTTTGATGGAACGGCTACTAATACCGTTTGGGCTTCATC
ACGTATTTTATTTACCATTCTGGCAAACAGCAGTTGGAGGTACAGCTCAAGTAGGAGACAAAGTTATTGA
AGGTGCTCAAAATATTTTTTTTGCAGAACTAGGAACGCCAGGAATAACACACTTTAGTGTTTCTGCAACA
AGATTTATGTCGGGTAAATTTCCGCTTATGATATTTGGCTTGCCAGGAGCAGCACTAGCAATGTATAAAT
GTGCTAAGCCAGAAAAGAGAAAAGCAGTAGGTGGATTATTACTATCAGCAGCATTAACGTCAATGCTCAC
TGGTATAACAGAACCAATTGAATTCACATTTCTATTTGTAGCACCAGTATTATATGGAATTCACTGTGTG
CTTGCTGGACTTGCTTATAT**GTTAATGCATATGTTAGGTGTTGGCGTAGGTAT**GACCTTCTCAGGTGGAT 435glcGfwd primer
TTATTGATTTATTCTTATTTGGTATTTTACAAGGAAATGCTAAAACAAGCTGGATACTGATTGTTATTGT
AGGAATCGTATATTTTGTAGTATACTATTTATTATTCACCTTCTTAATTAAGAAGCTTGATTTAAAGACT
CCAGGTCGTGAAGATTCTGGTGAAGTTAAGCTTTATACTAGAAGTGATTTAGAAGCAAAGAAAAATGATC
AAAATGAAAACGCAGACGAACTTTCGGCCATGATATGCAGAGGTCTCGGAGGAAAGAACAATATTTCTGA
TGTTGATTGCTGTGTAACTAGATTGCGTTGTACTGTACATAACTCTGAATTGGTGAATGAAG**GTTTGTTA** 436glcGrev primer
AAACAAACTGGAGCATCGGGCATATTTCACAAAGGCGTAGGCGTCCAAATAATGTATGGGCCAAGAGTAA
CTGTTATAAAATCAAATTTAGAAGATTACTTAGTTACAGCACCAGATAAAGAAGATACTGGTTATGCAAT
AATAAAAAAAGAATCAGAGAAAGATACTGAAAAAGGTATTGAAAAAAAGGTTCAAGGAAAAGTTATAAGT
ACAGTGATTTTAAATAGTCCATTGACAGGTGAAGCAAAAGATTTATCCGAAGTACCAGATGAAGTATTTG
CGAGCAGGATAATGGGAGATGGAGCTGTGGTTGTCCCAAGCGATGGAAATGTTATAGCTCCGGCAGATGG
AGTAATAAGTTTTGTATTCCCATCAAAACATGCATTGGGATTAACAACAACTGATGGACTTGAATTACTT
ATTCATATAGGAATAGATACAGTAAAGCTTGATAAAAAAGCCTTTGAAACTTATGTGGAAGAGGGAGATA
AAGTACAAGCTGGAGATAAAATATTAAGCTTTGACTTAGAATTTATAAAAAATAATGCACCATCTATTGC
ATCACCATGCATATGCACTGCATTGAATAGCAATCAAAAGGTGCGTCTATTAAAAACTGGAGATATAAAA
GCCGGAGAAGCTTTAATTGCAGTTGATGCATTTGAATAATATATAAGGGATGAGGAGGCATAAAATGTTT
AGAGTGATAAAAGCACTAAATCATAATGGTGTACTCGCTATAAATATGGATAACTATAAAGAGTATATCT
TGCTC**GGAAAAGGTATAGGTTTTGG**TAAAAAGGTCAATGAACGTATTGAAGCTCCAGAAAATGCACATAT 357rev primer
ATATCTCTTGCAGCAAGAAACTGAAAGAG

Figure 5.1: Sequence of the *cbei* 0751gene and annealing positions of primers used in this project. The sequence of the strand of the positive chromosome is shown.

■ Amplification primers for cloning of gene *cbei* 0751

■ Primers for Dig-labelling of hybridization probe for gene *cbei* 0751

■ Primers, 435 forward primer and 436 reverse primer (*glcG*) for gene knockout for *cbei* 0751

Clostridium beijerinckii NCIMB 8052 (*cbei* 4984/ *cbei* 4983/*cbei* 4982)

AATGTACATTATACTATATGTGAAAAAATTTTCTAAAGCTGTTTAAACCCTTCTCTTATTGATAATATCA 434fwd primer
CATTATACTAAAAGTATCATGAATGGTACCACATGAAAGAGTGTTAAAATTTCTATGACAAGGGATTAAT
AATACCTTGTACAGTAGATATATAAGGGGATGTTTTAGATGAAAAAATTCAATGTATCATTAGTTGGTGG
AGGTAGTACATGGACTCCAGGACTTCTCGCAAGTTTATGTAAATTAAAGGACAGGTTTCCTATAAATAGA
TTGATTTTATTCGATATAGATAAAGAACGTCAAGAAACAATTGCAAATTATGCAAAGTATTGTTTAATG
AAAGATACCCTGAATTGGATTTTAGATACACTACAAAGGTTGAAGAAGCTTATATAGATGTTGACTTTGT446-Digfwdprimer
AATTATGCAAATGAGAACCGGTGGTTATAAGATGAGGGAAAAGGATGAAAAATTCCTTTAAGCTTAGGC
GTAATAGGGCAAGAAACCTGTGGTGCTGGAGGATTTGCGTATGGTCTTAGATCTATAAAAGACATGATTA
AAACAATAAAGGATATAAGAAAGTATTCTAAAGATGCTTGGATATTAAATTATACAAATCCAGCTGCTAT
AGTGGCAGAGGCATTAAGAAGAGAATTTCCAGAAGATAAGAGAGTTTTAAATATATGTGATCAACCAGTT
AATTTATTACGTTCTTATGGCAGAGTTTTGGGAAAAGATTCGAAAAACTTTGAACCAGTTTATTTTGGAT447-Digrevprimer
TAAATCATTTTGGATGGTTTACTAATTTATATGATGAAAATGGAAACGATTTGGTTCCAGAACTTAGAAA
AGTAATAAAAGAAAGGGGGATTTATTCCTGTAGATGCAGAGCAAAGAGATAAATCATGGTTAGATACTTAT
GCAATGGTACAGGATATGGTTAATGATTTCCCAGAATATCTTCCAAATACTTATTTACAATATTATTATT
ATCCTGATTATAAAGTTAGTCATTTAAATCCAGATTATACAAGAGCAAATGAAGTTATGGATGGAAGAGA
AAAGAGAGTTTTTGAAGAATGTAGAAGAGTCGCTAAAGCTGGTACTGTAGAAGGTTCTGTGATGGTTCAT
AATGATGCACACAGTGAATTTATGATAGAAGTGGCTGAATCTATTGCTTATAATCTTAAAAAAATATATA
TCGTAATTGTAAAAAATAATGGAATAATTTCAAATTTAGATGATGATGTAATGGTTGAAGTTGCTTCGAT
AATGACAGCAAATGGACCAAGGCCTCTTTCAGTAGGTAAAATCCCTACATTTTACAAAGGTCTAATTGAA
TCACAATCAGCTTATGAAAAATTAACAGTAGATGCTTATTTTGAAGGTTCATATGAAAAGCGTTACAGG
CATTAACTATGAACAGAACT**ATCGTATCTGCTAAAAAGC**AAGAGCGGTACTTGATAAATTAATAGAAGC 432fwd primer
CAACAAGGAGTATTGGCCTGAATTTAAATAATAAGATTTAAATAGAATATTGGTATATTAAAAAACAGTA
CCAATATTCTAATAAAATATGAAATAATTATTATATTAGGAGGAAGAATAAATGACAAATACAAGCTATT
TCTCTAAATTTCAACAATTAGGAAAAGTATTAATGACCCCAATACTTATATTGCCAATTGCTGGTATATT
AATGGGAATTGGGTCTGCATTTTTAAGCCCATCTGTAATGAAAACGATGCCTTTTTTGCAAATGCCATTT
TTCAAGTTGTTTTTTAGTTTGCTTAAATCATCAGGATCAATTGTATTTAATAATTTGCCGGCAATCTTTG
CAATATCAATAACTATAGGTTATGCAAAAAAAGAGAAAGGTATTGCAGCTTTATCTGCTTTTTTAGGATA455rev primer
TATGGTTATGAATGTAACCATGAGCGCCTTACTTATTAATCTTGGTAAAATAAACCCAGATAAACTGCTT
GTAGGACAATCAAATATACTAGGAGTTCCAACAGTTGATACAGGAGTTTTTGGTGGAATAATAGTAGGAT
TTTTAATCGTTTATCTACATAATAAATATTATAATATATCTTTACCTCCTGTTTTATCAATATTTAGTGG
TACAAAATTTGTCCCAATGGTTTCTATTATCGGATCATTAATTTTAGGATTAGCATTGTCTGTAGTATGC437glcXfwd primer
CCTTTTATTCAAGGTATATTAATTGAATTAAGTATATTAATTAAAAACTCTGGTGCTTATGGATCTATGA448-Digfwd primer
TATACGGTCTAGCTGAGCGAGCTCTTCTTCCTTTTGGACTTCATCATTTTGTATATCTTCCATTTTTCTT
TACAAGTCTTGGTGGTAGTATGGAGATAGGTGGAAAAGTAGTTGAAGGTGCTGTTAATATTTATCAGGCT 508fwd primer
CAGTTAGCTACTCCAGGAGAAATGTTTAATATTGATGTCACTAGATTTGCTATGAATGGTAAAGTTATAG
AATCTATGTTTGGATTGCCAGGGGCAGCTTTGGCTATGTATAAGTGTGCAAAGCCTGAAAGAAAAAAAGT438glcXrev primer
TATAGGGGGATTTTTTCTTGCAGCAGTAATTCCAGCCTTCTTTTCTGGAATTACAGAACCAATTGAATTT
GCATTTTTATTTGTAGCACCAGCATTATATGGAATTCATGCTATATTTGCAGGAACTGCTTATCTTGTAA449-Digrev primer
CTTATTTGTTACAAATTAATATACCTGGAAGTGCAGCTTTTGGTGGACCTTTCTTAAGCTTTATATTTAA
TGGAATTATGCAATCAGATAAAGGTTCTCACTGGATATTTGTTCCTATAGTAGGTGTAATATATTTTTGT509rev primer
TTATACTATTTCAGTTTCAAATTTGCAATAAAAAAATGGGATTTAAAAACTCCAGGACGAGAACTTGAAG
AAGATTCAGAAGAAATTTCAGTAGTTTCATCAAGTAATACAATAATTAATGATATAGTCGATGCACTTGG
AGGAAAAAATAATATTAAAAGTGTAGATGCCTGCTTTACAAGATTGAGGGTTTCTGTTAATGATATGTCT
ATGGTTAAAGATGATAATATATGGAAGAAACTTGGTGCTAATGGAGTTGTTAAGGTTAAAGATGGTGTTC
AGGTCATCTATGGAGCAAAAGCTGATGTTTATAAAACTCAAGTAAGAGATTTACTTGGAATGGAATAGGT
GATTATATATGTTTAGTAAATTATTTAAGAAAAAAAATGATATTGTAATATATTCTCCGATAAAGGGGAA
AATTGTTGATATTTCCGAAGTCCCAGATCCAGTGTTTTCTGATAAAATTATGGGTGAAGGTATTGCAGTA450-Digfwd primer
ATTCCTGAAGATAATATTATATGCTCACCAGTAAATGGTTATGTAGCTCAAATTTTTAAAACTAAGCATG
CAATTTTACTAAAGAGTAGTGATGATTTAGAAATAATAATTCATATTGGATTAGAAACAGTTAATTTAAA
TGGTGAAGGTTTTGAAGTGTTAATAAATGAAGGTGACGAAGTAACTACAGGTAAAAAACTTATAAAGTA
GACTTTGAGTTCATGAAAAATAAAGGGATTAATACAATAATTCCAGTTGTCATAATTAATCATGCGGATA
GAAATATTATTAAATATTTTGGAGACAAGCAAACTGGAGCTGAGATAATGAAAATCTCACAATAGCCAAT451-Digrevprimer
TAATATGGATAATAAAGGGAATAGTGTGCAATTCAAGTCTAATAATAAGATTTGAAATTATAGACCT 433rev primer

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Figure 5.2: Sequence of the genes *cbei* 4984, *cbei* 4983, *cbei* 4982 and annealing positions of primers used in this project. The sequence of the positive strand of the chromosome is shown.

■ Amplification primers for gene cloning (*cbei* 4984/ *cbei* 4983 / *cbei* 4982)

■ Primers for Dig-labelling of hybridization probes for genes (*cbei* 4984/ *cbei* 4983/*cbei* 4982)

■ Primer 455 reverse primer 281 for gene *cbei* 4983

■ Primer 508 forward primer for gene *cbei* 4983

■ Primer 509 reverse primer 181 for gene *cbei* 4983

■ Primers 437forward primer and 438 reverse primer (*glcX*) for gene knockout for (*cbei* 4983/*cbei* 4982)

Appendix II. Protein sequences

Sequences of PTS proteins used in construction of phylogenetic tree

phosphotransferase system IICB, N-acetylglucosamine- specific [*E. coli*]

LOCUS WP_000317748

```
  1 tlimfknafa nlqkvgkslm lpvsvlpiag illgvgsanf swlpavvshv maeaggsvfa
 61 nmplifaigv algftnndgv salaavvayg imvktmavva plvlhlpaee iaskhladtg
121 vlggiisgai aaymfnrfyr iklpeylgff agkrfvpiis glaaiftgvv lsfiwppigs
181 aiqtfsqwaa yqnpvvafgi ygfierclvp fglhhiwnvp fqmqigeytn aagqvfhgdi
241 prymagdpta gklsggflfk myglpaaaia iwhsakpenr akvggimisa altsfltgit
301 epiefsfmfv apilyiihai laglafpici llgmrdgtsf shglidfivl sgnssklwlf
361 pivgigyaiv yytifrvlik aldlktpgre datedakatg tsemapalva afggkenitn
421 ldacitrlrv svadvskvdq aglkklgaag vvvagsgvqa ifgtksdnlk temdeyirnh
```

phosphotransferase system EIICBA glucose-specific [*B. subtilis*]

LOCUS NP_389272

```
  1 mfkalfgvlq kigralmlpv ailpaagill aignamqnkd miqvlhflsn dnvqlvagvm
 61 esagqivfdn lpllfavgva iglangdgva giaaiigylv mnvsmsavll angtipsdsv
121 erakfftenh payvnmlgip tlatgvfggi ivgvlaallf nrfytielpq ylgffagkrf
181 vpivtsisal ilglimlviw ppiqhglnaf stglveanpt laafifgvie rslipfglhh
241 ifyspfwyef fsyksaagei irgdqrifma qikdgvqlta gtfmtgkypf mmfglpaaal
301 aiyheakpqn kklvagimgs aaltsfltgi teplefsflf vapvlfaihc lfaglsfmvm
361 qllnvkigmt fsgglidyfl fgilpnrtaw wlvipvglgl aviyyfgfrf airkfnlktp
421 gredaaeeta apgktgeagd lpyeilqamg dqenikhlda citrlrvtvn dqkkvdkdrl
481 kqlgasgvle vgnniqaifg prsdglktqm qdiiagrkpr pepktsaqee vgqqveevia
541 eplqneigee vfvspitgei hpitdvpdqv fsgkmmgdgf ailpsegivv spvrgkilnv
601 fptkhaiglq sdggreilih fgidtvslkg egftsfvseg drvepgqkll evdldavkpn
661 vpslmtpivf tnlaegetvs ikasgsvnre qedivkiek
```

glucose-specific PTS system enzyme II, factor IIA [*Staphylococcus carnosus*]

LOCUS YP_002634092

```
  1 mwkkffgqlq rigkalmlpv ailpaaglll algnafqgda lqslmpfika egfqnvakmm
 61 egaggiifdn laiifalgva iglasgdgva aiaafvgfiv lnktmgmflg vtpekaadaa
121 tgfanvlgip tlqtgvfggi iigalaawcy nkfynislps ylgffagkrf vpimmatcsf
181 ilafpmaiiw psiqgglnaf segllasntg lavflfgfik rllipfglhh ifhapfwfef
241 gsyknaagqi ihgdqrifie qirdnvplta gkfmqgefpv mmfglpaaal aiyqtakken
301 kkvvaglmls galtsfltgi teplefsflf vapllffiha vldglsflil ylldlhlgyt
361 fsggfidffl lgilpnktqw wlvipvglvy aaiyyiifrf livkfnfktp gredkevkss
421 nvaaselpfk vldamggkan ikhldacitr lrvevndkak vdvqelkdlg asgvlevgnn
481 mqaifgpksd qikhdmqqim dgkitspeet tvteegdket aeiaaagggv vyapikgevv
541 disevpdkvf sekmmgdgia ikpetgevva pfdgvvkmvf ptkhaigles kdgiellihf
601 gletvklegk gfdilvkend nivlgqplmk vdldyikeha dstitpivvt nlngrtmevl
661 qhgevkqgdk vilvk
```

glucose-specific PTS system enzyme II, factor IIB [*Staphylococcus carnosus*]

LOCUS YP_002634093

```
  1  mknllkkffg qlqrigkalm lpvailpaag illtfgnamh neqilhfapw mqhhyiqlis
 61  qimeasgqvi fdnlpllfam gtalglaggd gvagiaalvg ylimsatmgk iagitiddif
121  syadgaktlg qsakdpahal vlgiptlqtg vfggiiigal aawcynkfyn iqlpqflgff
181  agkrfvpiit slvaivtgiv lsfvwppvqd glnnlsnfll gknlalttfi fgiierslip
241  fglhhifyap fwfefghyvn esgnlvrgdq riwmaqyqdg vpftagaftt gkypfmmfgl
301  paaafaiyrq akperrkvvg glmlsaalts fltgiteple fsflfvapil yvahvilagt
361  sflimhllhv qigmtfsggf idyilyglls wdrsnallvi pvgiayaliy yflftflikk
421  lnlktpgred keveskdvsv selpfevlea mgnkdnikhl dacitrlrve vrdkglvdve
481  klkqlgasgv levgnnmqai fgpksdqikh dmqqimdgki tspaettvte dgdvetaeiv
541  aeggaviyap itgeavdlse vpdkvfsakm mgdgiaikpe tgevvapfdg kvkmifptkh
601  aigleskdgi ellihfglet vkldgegfei lvkendnivl gqplmkvdln yikehaddti
661  tpiiitnags anievlhtgk veqgeklllv nn
```

Phosphotransferase system EIICBA glucosamine-specific[*B. subtilis*]

LOCUS NP_388117

```
  1  mfkkafqilq qlgralmtpv avlpaagllll rfgdkdllni piikdaggvv fdnlplifav
 61  gvaiglagge gvaglaavig yliltvtldn mgkllglqpp yegaehlidm gvfggiiigl
121  laaylykrfs sielhpvlgf fsgkrfvpii tsvsslvigv ifsfvwpliq nginaassli
181  adstvglffy atiyrllipf glhhifytpf yfmmgeytdp stgntvtgdl trffagdpta
241  grfmmgdfpy mifclpaval aiihtarpek kkmisgvmis aaltsmltgi tepvefsflf
301  vapvlylins ilagvifvvc dlfhvrhgyt fsgggidyvl nyglstngwv vipvgivfaf
361  iyyylfrfai lkwnlktpgr etdedgqnee kapvakdqla fhvlqalggq qnianldaci
421  trlrvtvhqp sqvckdelkr lgavgvlevn nnfqaifgtk sdalkddikt imaggvpata
481  aaldtvtdkp lkpdsdetfi ypikgetvsl gdvpdqvfse kmmgegfaii psegkvvapa
541  dgeivsifpt khaigfmsag gteilihvgi dtvklngegf eahvtsgqav kqgelllltfd
601  lnyikqhaas aitpviftnt seedlkhiqm k
```

phosphotransferase IIABC transporter subunit [*C. acetobutylicum* ATCC 824]

LOCUS NP_347209

```
  1  mgnkifavlq kigkslmlpv svlpaagilll rlgqpdllnm pyvqaagnai fnnlplifav
 61  gvaigfsgge gvaalaavig elileavekt agdtaaaala ktaaashhmt laafqktqey
121  snivtkttis mgvfggiiig ltaailynkf hdikmpqvlg ffggkrfvpi itsisaliia
181  tigvniwlpi qaginslaaf attspigpam yaggkrllip lglhhiyypl flyqfghfvs
241  ngvtyvgdta ryfhgdptag nfmaaeypil mfglpgaala miaaakkekr kemagmmisa
301  afvafvtgit epiefsfifv apvlfvfhvl aafasglits ylhirlgytf sasfidyvlg
361  fkyaghplli wlvgigffvl yfvvfyftik amniktpgre dddaegvkki nvkgkakaak
421  vleaiggkdn ikvldacitr lrlnlndpsl vdkatlkalg aagvmtaeds vqvvfgteae
481  rikddikaii qnggyvedds dkeeevqedk qiskgahell spadgevvgi esvpdstfae
541  kmlgdgfavi psgnevhspa dgevsvlfpt khafaitteg glellihvgi dtvalngegf
601  tahvkqgdkv kkgdliltld tefikskgkn litpvivtnm dvvgnidvkl gnvknsekaa
661  dvtvk
```

phosphotransferase system EIIC maltose- and glucose-specific [*E. coli*]

LOCUS PTOCB_ECOLI

```
  1 mtaktapkvt lweffqqlgk tfmlpvalls fcgimlgigs slsshdvitl ipvlgnpvlq
 61 aiftwmskig sfafsflpvm fciaiplgla renkgvaafa gfigyavmnl avnfwltnkg
121 ilpttdaavl kanniqsilg iqsidtgilg aviagiivwm lherfhnirl pdalaffggt
181 rfvpiisslv mglvglvipl vwpifamgis glghminsag dfgpmlfgtg erlllpfglh
241 hilvalirft daggtqevcg qtvsgaltif qaqlscptth gfsesatrfl sqgkmpaflg
301 glpgaalamy hcarpenrhk ikgllisgli acvvggttep leflflfvap vlyvihallt
361 glgftvmsvl gvtigntdgn iidfvvfgil hglstkwymv pvvaaiwfvv yyvifrfaitn
421 rfnlktpgrd sevassieka vagapgksgy nvpailealg gadnivsldn citrlrlsvk
481 dmslvnvqal kdnraigvvq lnqhnlqvvi gpqvqsvkde maglmhtvqa
```

phosphotransferase IIABC transporter subunit [*E. faecalis*]

LOCUS NP_814695

```
  1 mkkmfsfefw qkfgkalmvv vavmpaaglm isigkslpli dpnlgllvtt ggvlesigwa
 61 iignlhllfa laiggswakd raggafaagi sfvlinritg aifgvtneml adeqafthtl
121 fgtkimvkgf ftsvleapal nmgvfvgiia gfvgamaynk yynyrklpda lsffngkrfv
181 pfvvilwsti vsialaliwp niqaginnfg lwiaqsqdsa pilapflygt lerlllpfgl
241 hhmltipiny tqlggtyeil sgaqagtqvf gqdplwlawa tdlvnlkgag dmskyqfvle
301 nwtparfkvg qmigssgilm gmalamyrnv dadkkakyks myfsaalavf ltgvteplef
361 mfmfaavply viyaviqgaa famadilplr vhsfgniell trtplaikag lggdlinfvl
421 mviifgvvty flanflikkf nyatpgrngn ydndnseeia sgaagsgvvd qqiaqivyll
481 ggkqnikevd acmtrlrvsv kdrekvgsee awkragamgl ivkdngvqav ygpkadvlks
541 diedllasgv dipepviaes tagvpttnfl gkkkdfvava tgevipmaqv ndpvfsqkmm
601 gdgfavkple gevvapisgk vlsvfpskha iglqteegie vlvhmgidtv ematpafesf
661 vkegqslkag tklakmnldv ieqagketti ivaftnsdkv eqvvinqlgt ttagtvigqi
721 ei
```

phosphotransferase system IIABC glucose-specific transporter [*S. mutans*]

LOCUS NP_722340

```
  1 mknykqlfsf efwqkfgkcl mvviavmpaa glmvsignsl alidpkstll vtvaniiaqi
 61 gwgvinnlhi lfavaiggsw akeraggafa aalafilinl itgnfygisl emiadktsyv
121 hnifggkmhv adyfinvlgq palnmgvfvg iisgfvgata ynkyynfrkl pdvlsffngk
181 rfvpfvvivr stivalilsv fwpivqsgin gfgmwiassq htapflapfl ygtlerlllp
241 fglhhmltip mnytqlggty vvltgaqagk hvlgqdplwl awvqdlihlk gaghmsqyhh
301 lltsvtparf kvgqmigssg ilmgltlamy rnvdpdkkek ykgmflsaav avfltgvtep
361 leymfmfaal plylvyavvq glafasadli hlrvhsfgni efltrtpmai kaglamdivn
421 fivvsvvfgv amyfitnfmi kkfnlatsgr ngnydtgdda sdetasnsna gtanansqiv
481 kiinllggke nisdvdacmt rlritvtdva kvgdeaawkk agamglivkg ngvqavygpk
541 advlksdiqd lldsgvdipk tdvtaleedk tadvsfkgvt eeiatvadgq vlpitqvhdp
601 vfsqkmmgdg favepengni yspvaglvts vfptkhalgl ltddglevlv hvgldtvaln
661 gapfsakvkd gqrvalgdll lvadleaiks adrettviva ftntaelksv tlektgqqaa
721 ktvvakvel
```

phosphotransferase system β-glucoside-specific transporter II [*S. mutans*]

LOCUS NP_721375

```
  1 makdytelaq divshvggkd nivklvhcvt rlrftlkdes kadddylkqr egivtvvkag
 61 gqyqvvignh vpdvydtvlk vsgitgeggi dvdegdipkg nlfdrfialv sglfqpmlga
121 lsaagmikgl vailaavgvs ktdgayvvln aagdgffqfl plilaltaak rfkmeqftal
181 aigfalvypn iaasftvkhp lytlfqgtli espiystffg ipiifpassy lqtvlpivva
241 iwagskietf fkkiipdvvk vfvvpfftil itvplsflvi gpvmswasdl vgaiftgiyg
301 fnpviygivl gamwqvlvmf glhwglvpla ilelqkgpgv ilvatiaicf aqagsllnim
361 mrtkenkvrq lsipafisal fgvtepaiyg itlpmrvpfi mtcvsgaisg aylalfnvkm
421 qvmggmglfa ipsfidpkns milihfliai amnfvlgfvl tqfikipyly geptstdsdv
481 ddkeepvkel keikqeiiss pligkvvkle nvpdevfasg amgkgiaidp ddgivvaptk
541 gevtlvfptk havglrteng aeilihigmd tvslagkgfk sfvkvgdhve agqtllefdv
601 naikaaglpv itpvivtnsd qfedvlttqe rtveagdyll tavk
```

PTS system IIABC sucrose-specific transporter [*S. mutans*]

LOCUS NP_722158

```
  1 mdyskvasev itavgkdnlv aaahcatrlr lvlkddskvd qkaldknadv kgtfktdgqy
 61 qviigpgdvn fvydeiikqt gltevstddl kkiaasgkkf npimalikll sdifvpiipa
121 lvaggllmal nnfltseglf gtkslvqqfp iikgssdmiq lmsaapfwfl pilvgisaak
181 rfganqflga sigmimvapg aaniiglaan apiskaatig aytgfwnifg lhvtqasyty
241 qvipvlvavw llsilekffh krlpsavdft ftpllsviit gfltfivigp vmkevsdwlt
301 ngivwlydtt gflgmgvfga lyspvvmtgl hqsfpaietq lisafqngtg hgdfifvtas
361 manvaqgaat faiyfltkdk kmkglssssg vsallgitep alfgvnlkyr fpffcaligs
421 asaaaiagll qvvavslgsa gflgflsika ssipfyvvce lisfaiafav tygygktkav
481 dvfaaeaave eaieevqeip eeaasaanka qvtdevlaap lageavelts vndpvfssea
541 mgkgiaikps gntvyapvdg tvqiafdtgh aygiksdnga eilihigidt vsmegkgfeq
601 kvqadqkikk gdvlgtfdsd kiaeagldnt tmfivtntad yasvetlass gtvavgdsll
661 evkk
```

phosphotransferase system IIABC trehalose-specific transporter [*S. mutans*]

LOCUS NP_722334

```
  1 mgkfekdakv lldaiggken vsavthcatr mrfvlndesk advktieaip avkgtftnag
 61 qfqiiigndv plfyndftav sgiegvsket aksaakshqn piqrvmtlla eiftpiipai
121 ivgglilgfr nvidqvqwgf ldgqtiaqhs qfwsgissfl wlpgeaifqf lpvgivwsvs
181 rkmgtsqilg ivlgiclvsp qllnaynvas tpaaeiakkw vwdfgfftvr rigyqgqvip
241 allaglslsy leifwrkkip evvsmifvpf lslipalila htvlgpigwt igqwistivy
301 agltgpvkwl fgaifgalya pfvitglhhm tnaidtqlva dtggtglwpm ialsniaqgs
361 avfayylmnr heereaqvsl patisaylgv tepalfgvni kyvyplvagm igssiagfls
421 vtfnvtanai gigglpgils ikvkymlpff iimlvaiavp mvltvffrri nfltksedea
481 hqektetaei sasektnepa vgttfeiksp lkgqvkplse avdpvfaqgv mgqgvlidps
541 eglltspvng vvsvlfptkh alglvtdqgl ellihigmdt vnlegkgfet yvkqgdtikv
601 gdklirfdid aihqagytte tpvivtnqnd yqpdvvgslp reiergevll taski
```

phosphotransferase transmembrane protein [*Streptomyces coelicolor*]

LOCUS NP_627133

```
  1 mstatdtaap akkrgsglfq glqkvgrslq lpiavlpaag imvrlgqddi fgkdglgwdk
 61 vaavfnnagg altgslpilf cigvaigfak kadgstalaa vvgflvyskv leafpvteav
121 vqdgadvaat yndpgvlggi imgllaavlw qryhrkklvd wlgffngrrl vpiimafvgi
181 vvgvffglvw epigdgisnf gewmtglgsg gaalfggvnr alipvgmhqf vntvawfqlg
241 dftnsagdvv hgditrflag dpsagifqag ffpimmfglp aaalamahta rperrkavlg
301 mmislaatsf vtgvtepief sfmfiapvly vlhavltais maitwglgvh agfnfsagfi
361 dyalnwhlat kpwliipigl vfaaiyyvtf rfaivkfnlk tpgrepeeev edltka
```

protein-N-phosphohistidine sugar phosphotransferase II ABC, N-acetylglucosamine specific [*Ralstonia eutropha*]

LOCUS YP_724831

```
  1 mkmdllprvq rlgatlmlpi avlpvaglll rlgqpdvfdi klmaeagnav fanlallfai
 61 gvavgfardn ngaaalagti gylvlttvlk tidksldmgv lagivagava gglynryrnv
121 alppylgffs gkrfvpivta lcclllgivl ayawapvqag inaagawltt agsagalvfg
181 llnrlllvtg lhhlintlaw fvfgnyadpa tgaavsgdlh ryfagdpgag lfmtgffpvm
241 mfglpaacla myhetpparr alvggmlfsm altsfltgit epiefsfmfl apvlyglhal
301 mtglsmalch aldirlgftf sagaidyvlg yglssrgwla iplglayalv yyglfrffir
361 rfnlltpgrd evvpvaaagg aaqpaagsva qqyvealggp anlvvvdact trlrlnvadi
421 gavseprlka lgvrgvlkrp pnvvqvvigp qaeqvagdir avlqqapqat avvaaapava
481 tqasvpaaga fdpawwidal ggaaniasvg vvaltrlrvv vrerarvrad hlagsqlmwi
541 gddtahiafg haadghaaaf eralqampt
```

phosphotransferase C2-protein [*Streptomyces olivaceoviridis*]

LOCUS CAD_29623

```
  1 mavlqrigrs lmlpvavlpa aallvrlgna dmlgrpefpa fvtkiagfma aggnaildnm
 61 allfavgiai gfakksdgst alaavvgylv fknvlatftd knlpqvakav dgkvvmvdap
121 vdakvlggvv mglvvallyq rfyrtklpdw agffggrrlv pilsafaglv igivfgyiwp
181 vlgtglhnfg ewlvgsgavg agifgvanra lipigmhhll nsfpwfqage yegksgdiar
241 flagdptagq fmtgffpimm falpaaclai vhcarperrk vvggmmfsla ltsfvtgvte
301 pieftfmfia pvlyaihavl tgvsmaltwa lgmkdgfgfs agavdfflnl giasnpwgla
361 lvgvcfaaly yvvfrfaitk fnlptpgres deelaelrka egk
```

fused N-acetyl glucosamine specific Phosphotransferase enzyme: IICBA [*E.coli*]

LOCUS NP_415205

```
  1 mnilgffqrl gralqlpiav lpvaalllrf gqpdllnvaf iaqaggaifd nlalifaigv
 61 asswskdsag aaalagavgy fvltkamvti npeinmgvla giitglvgga aynrwsdikl
121 pdflsffggk rfvpiatgff clvlaaifgy vwppvqhaih aggewivsag algsgifgfi
181 nrlliptglh qvlntiawfq igeftnaagt vfhgdinrfy agdgtagmfm sgffpimmfg
241 lpgaalamyf aapkerrpmv ggmllsvavt afltgvtepl eflfmflapl lyllhalltg
301 islfvatllg ihagfsfsag aidyalmynl paasqnvwml lvmgviffai yfvvfslvir
361 mfnlktpgre dkedeivtee ansnteeglt qlatnyiaav ggtdnlkaid acitrlrltv
421 adsarvndtm ckrlgasgvv klnkqtiqvi vgakaesigd amkkvvargp vaaasaeatp
481 ataapvakpq avpnavsiae lvspitgdvv aldqvpdeaf askavgdgva vkptdkivvs
541 paagtivkif ntnhafclet ekgaeivvhm gidtvalegk gfkrlveega qvsagqpile
601 mdldylnana rsmispvvcs niddfsglii kaqghivagq tplyeikk
```


phosphotransferase system IIC transporter [*C. acetobutylicum*]

LOCUS NP_347981

```
  1 mgvtnkllaa cqklgkslmt piavlpaagl llrlgqpdll niswmmaagn gifnnlamif
 61 aigiavgfae enngvaglsa avgyfvltnv atsfnkhidm gvlggiivgi lagnlynkyk
121 strlpdflgf fggrrlvpil tslcslvlgl isglvwpaiq nvinafgnsv shagvvgsfi
181 ygllnrllip iglhhvlntl fwfqfgtfks asgkivtgdl hrffaldkta gtymtgffpi
241 mmfalpaacl amisaakken rkkvsgmllg iaftafltgv tepieflfmf lapvlyvvha
301 vltglsmait salgiksgft fsagfvdyim nfnistkpil livigilyai iyyflflfti
361 kkfnlptpgr mddlddlddl ddlddlddld eepentpkik sspsknstle ekavgileai
421 gnknniqsld acvtrirltv kdgskvdepk lkklgatgim klddknfqiv vgttadiiat
481 hikeiikk
```

phosphotransferase EIICB N-acetylglucosamine-specific [*B. subtilis*]

LOCUS NP_388651

```
  1 mlsflqklgk sfmlpiavlp avgiilalgr edvfnipfvy qagtavfdhl plifaigiai
 61 giskdsngaa glsgaisylm ldaatktidk tnnmavfggi iagliagyty nrfkdtklpe
121 ylgffsgrrl vpiltaiiti ilagifgvvw ppiqscinsf gewmlglggi gagifglfnr
181 lliplglhhv lnnifwfqfg eyngvtgdla rffakdptag tymtgffpim mfglpaacla
241 mvvtakpskr katagmmigf altafitgit epiefafmfl spllyavhav ltglslfivn
301 wlgirsgfsf sagaidyvls ygiaekplll llvgicyaav yfivfyvlik alnlktpgre
361 dddvdevlde ntvqdvneni mlkglggken lqtidhcatr lrltvkdtal vdeallkkag
421 akgvvksggq svqviigpnv efaaeelraa vk
```

phosphotransferase IIA, glucose subfamily [*C. beijerinckii* NCIMB 8052]

LOCUS "*Cbei_0751*"

```
  1 mkdkvfgvlq rvgrsfmlpi ailpvaglfl gigesftnkt mldtygitgl igpgtfvnal
 61 lsvmndagni vfenlplifa igvaigmskk erevaalaag iaflimhasi gamikihggt
121 eallsgaste vlgiislqmg vfggiivglg taalhnryyk ielpqvlsff ggtrfvpiic
181 sivylivgil mfyiwppvqg aiykvgnivl assyagtwvy glmerllipf glhhvfylpf
241 wqtavggtaq vgdkviegaq niffaelgtp githfsvsat rfmsgkfplm ifglpgaala
301 mykcakpekr kavgglllsa altsmltgit epieftflfv apvlygihcv laglaymlmh
361 mlgvgvgmtf sggfidlflf gilqgnakts wilivivgiv yfvvyyllft flikkldlkt
421 pgredsgevk lytrsdleak kndqnenade lsamicrglg gknnisdvdc cvtrlrctvh
481 nselvnegll kqtgasgifh kgvgvqimyg prvtviksnl edylvtapdk edtgyaiikk
541 esekdtekgi ekkvqgkvis tvilnspltg eakdlsevpd evfasrimgd gavvvpsdgn
601 viapadgvis fvfpskhalg ltttdglell ihigidtvkl dkkafetyve egdkvqagdk
661 ilsfdlefik nnapsiaspc ictalnsnqk vrllktgdik agealiavda fe
```

PTS system IIBC, glucose-like subunint [*C. beijerinckii* NCIMB 8052]

LOCUS "*Cbei_4983*"

```
  1 mtntsyfskf qqlgkvlmtp ililpiagil mgigsaflsp svmktmpflq mpffklffsl
 61 lkssgsivfn nlpaifaisi tigyakkekg iaalsaflgy mvmnvtmsal linlgkinpd
121 kllvgqsnil gvptvdtgvf ggiivgfliv ylhnkyynis lppvlsifsg tkfvpmvsii
181 gslilglals vvwpfiqgil ielsilikns gaygsmiygl aerallpfgl hhfvylpfff
241 tslggsmeig gkvvegavni yqaqlatpge mfnidvtrfa mngkviesmf glpgaalamy
301 kcakperkkv iggfflaavi paffsgitep iefaflfvap alygihaifa gtaylvtyll
361 qinipgsaaf ggpflsfifn gimqsdkgsh wifvpivgvi yfclyyfsfk faikkwdlkt
421 pgreleedse eisvvsssnt iindivdalg gknniksvda cftrlrvsvn dmsmvkddni
481 wkklgangvv kvkdgvqviy gakadvyktq vrdllgme
```

phosphotransferase system IIBC, N-acetylglucosamine [*C. beijerinckii* NCIMB 8052]

LOCUS "*Cbei_4532*"

```
  1 mmkylqklgk slmlpvaclp vasilmglgy wldptgwgan niasafmlka gsalidnmgi
 61 lfaigvgvgm sddndgtagl aglvswlmit tllstgavam fkgidvkeva pafaktqtqf
121 igilsgliga acynrfksvk lpdalgffsg krcvaivtaa ysivasivlf fawpliygal
181 vafgeaivst gavgsgiyaf fnrllipfgl hhalnsvfwf dvagindlgn fwsgkgtqgv
241 tgmymtgffp vmmfglpaga lamyhtakdk kkkavyglll aaaissfftg vteplefafm
301 flapglyvlh agltgisafv ctllpvragf nfsagfvdwf lsfkapmaen pimlipiglv
361 faviyyvtfr faitkfnlkt pgredddaee lnvklanndy tqvaavilkg vggkenvvsi
421 dncvtrlrle ikdqaavdek viksagvsgi irpgktsvqv vvgtqvqfva defkklck
```

Figure 5.3: Sequences of proteins used in alignments comparative in this project.

Appendix III. DNA molecular weight markers

Hyperladder I
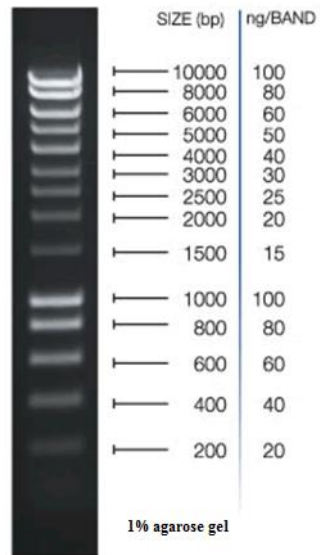
Figure 5.4: DNA molecular weight markers of Bioline Hyperladder I.

Appendix IV. Restriction map and sequence

(A)

```
                    lacZα ATG
   M13 Reverse Primer    |                          Hind III          Kpn I      Sac I  BamH I    Spe I
                         |                            |                 |           |     |         |
CAG GAA ACA GCT ATG ACC ATG ATT ACG CCA AGC TTG GTA CCG AGC TCG GAT CCA CTA
GTC CTT TGT CGA TAC TGG TAC TAA TGC GGT TCG AAC CAT GGC TCG AGC CTA GGT GAT
```

```
                           BstX I  EcoR I                                     EcoR I
                             |       |                                          |
GTA ACG GCC GCC AGT GTG CTG GAA TTC GCC CTT  PCR Product  AAG GGC GAA TTC TGC
CAT TGC CGG CGG TCA CAC GAC CTT AAG CGG GAA              TTC CCG CTT AAG ACG
```

```
   EcoR V              BstX I   Not I   Xho I           Nsi I Xba I        Apa I
     |                   |       |       |                |    |            |
AGA TAT CCA TCA CAC TGG CGG CCG CTC GAG CAT GCA TCT AGA GGG CCC AAT TCG CCC TAT
TCT ATA GGT AGT GTG ACC GCC GGC GAG CTC GTA CGT AGA TCT CCC GGG TTA AGC GGG ATA
                                                                               ↑
```

```
     T7 Promoter                    M13 Forward (-20) Primer
AGT GAG TCG TAT TAC AAT TCA CTG GCC GTC GTT TTA CAA CGT CGT GAC TGG GAA AAC
TCA CTC AGC ATA ATG TTA AGT GAC CGG CAG CAA AAT GTT GCA GCA CTG ACC CTT TTG
```

Figure 5.5: Sequence and restriction map of the pCR 2.1 TOPO vector.
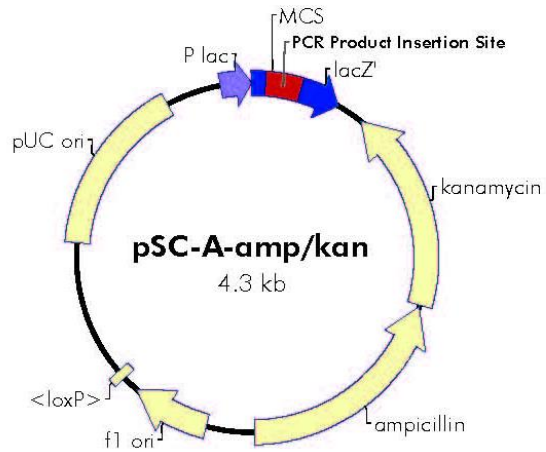
130

(B)
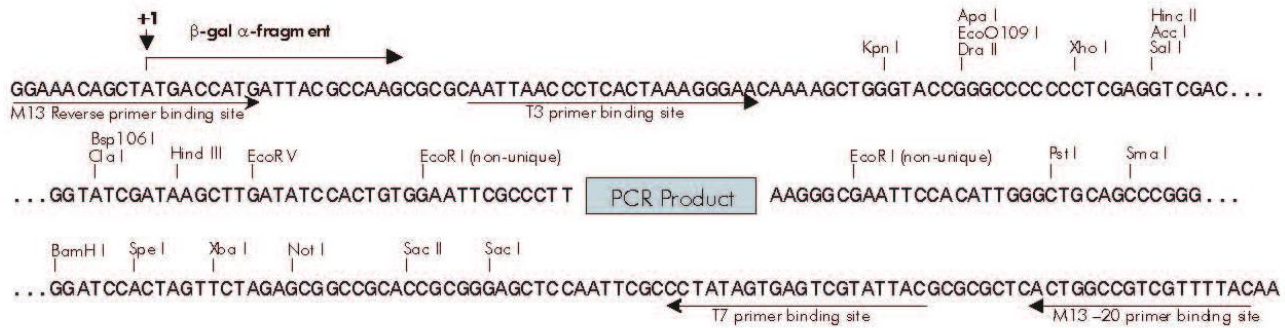
Figure 5.6: Sequence and restriction map of StrataClone PCR cloning vector pSC-A-amp/kan.
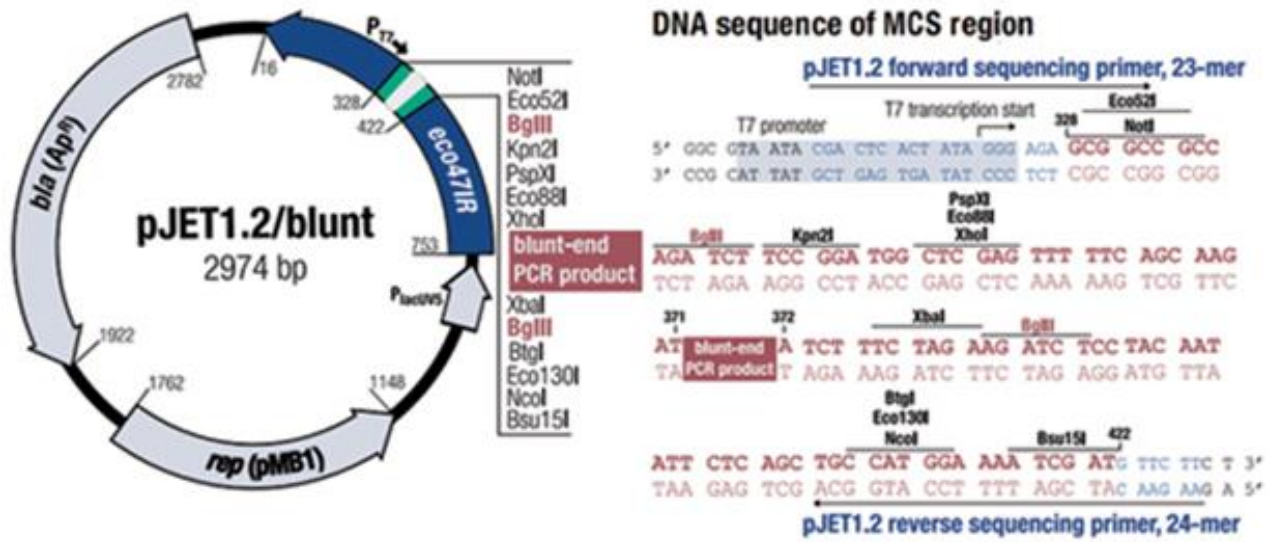
Figure 5.7: Sequence and restriction map of pJET1.2/blunt 2.1 Cloning Vector.

Figure 5.8: Sequence and restriction map of pUC18 vector.

Appnedix   V. Solutions

1) <u>20x saline sodium citrate (SSC) stock solution/1000 ml</u>

- 3 M sodium chloride (NaCl)

- 300 mM sodium acetate trihydrate ($CH_3COONa. 3H_2O$)

- pH 8.0

2) <u>2x saline sodium citrate (SSC) solution/1000 ml</u>

- 100 ml from 20x SSC stock solution

- 0.1 % sodium dodecyl sulfate (SDS)

3) <u>0.2x saline sodium citrate (SSC) solution/1000 ml</u>

- 10 ml from 20x SSC stock solution

- 0.1 % sodium dodecyl sulfate (SDS)

4) <u>DIG 1 buffer/ 1000 ml</u>

- 100 mM Tris HCl

- 1 M sodium chloride (NaCl)

- 0.2 % Tween 80

- pH 8.5

5) <u>DIG 4 buffer/1000 ml</u>

- 100 mM Tris HCl

- 1 M sodium chloride (NaCl)

- 0.2 % Tween 80

- pH 9.5

6) <u>Easy hybridization buffer/100</u>

- 6 M Urea

- 6x SSC stock solution (30 ml of 20x SSC)

- 1 % sodium dodecyl sulfate (SDS)

- 50 mM Tris HCl

- pH 7.5

7) <u>Blocking solution/100 ml</u>

   - 100 mM malic acid

   - 1 M sodium chloride (NaCl)

   - pH 8.0

   - 0.3 % Tween 80

   - 0.5 % casein

   - 0.1 % (DEPC)

8) <u>TAE buffer (1x)</u>

   - 40 mM Tris acetate

   - 20 mM glacial acetic acid

   - 1 mM EDTA

   - pH 8.0

9) <u>DEPC- treated water 1000 ml</u>

   - dH_2O: 0.1 % DEPC solution

10) <u>2x YTG/1000 ml</u>

   - Tryptone   16 g

   - Yeast extract 10 g

   - NaCl 5 g

   - 1 % Carbohydrate

   - pH 7.0

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