Evaluation of thermally regulated promoter systems for use in *Escherichia coli* and *Bacillus subtilis*

by

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DECLARATION

I declare that the dissertation, which I hereby submit for the degree M.Sc (Microbiology) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signed: …….. Date: ……..

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This dissertation is dedicated in loving memory to my grandmother, Ouma Bettie

SUMMARY

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Regulated expression of heterologous genes is important for both basic research and practical applications. In addition to *Escherichia coli*, *Bacillus subtilis* is regarded as an appropriate host for heterologous gene expression and has been used for the production of industrial enzymes in large-scale fermentation processes. Although various inducible gene expression systems are available for *E. coli*, there are fewer regulated promoters available for Gram-positive bacteria, such as *B. subtilis*, due to their stringent control of promoter usage. In this investigation, two thermally regulated promoters, designated Pro2 and Pro3, were evaluated in *E. coli* and *B. subtilis*, and subsequently used to produce an enzyme of industrial importance in *B. subtilis*.

To enable evaluation of the Pro2 and Pro3 promoters, which are both regulated by the bacteriophage P1 temperature-sensitive C1 repressor, plasmid-based expression vectors were constructed based on the *E. coli-Bacillus* shuttle vector pNW33N. Using transcription fusions to the *lacZ* reporter gene to monitor gene expression, the strength, basal expression and induced expression of the respective promoters were evaluated. The production of β-galactosidase driven by the promoters was higher at 42°C than at 30°C, both in *E. coli* and *B. subtilis*. In *E. coli*, the Pro3 promoter exhibited low basal expression and, under inducing conditions, gave a high level of expression (138-fold induction). In contrast, the Pro2

promoter showed higher expression strength in *B. subtilis* (12-fold induction). These results were verified by making use of a second, different reporter gene. Although transcription fusions to the *sapS* reporter gene yielded lower induction factors, these may be ascribed to the different enzyme characteristics of the respective reporters.

The utility of the thermally regulated promoter systems was subsequently evaluated in *B. subtilis*. For this purpose, controlled overproduction of a nucleoside phosphorylase from *B. halodurans* was investigated. Nucleoside phosphorylases are key enzymes for the synthesis of nucleosides and nucleoside analogues, which are used as antiviral and anticancer reagents. In agreement with the above results, the highest enzyme activity was measured when the gene was cloned under the control of the Pro2 promoter (4 U/mg). To optimize the expression of the heterologous enzyme, the effects of different induction temperatures and the duration of thermal induction were investigated. The results indicated that the enzyme activity was increased 3.4-fold by growing the cells at 46°C for 4 h (13.5 U/mg). The specificity of the *B. halodurans* enzyme to different purine and pyrimidine nucleosides was determined by thin layer chromatography, the results of which indicated that the enzyme is a purine nucleoside phosphorylase.

In conclusion, the versatility of two thermally regulated promoters was demonstrated by fusing the promoters to two different reporter genes and by overexpression of a purine nucleoside phosphorylase enzyme through modulation of the promoter systems. The data of this study demonstrated that these promoters may be suitable for controlled expression of heterologous genes in *B. subtilis* and *E. coli* and thus could have potential as an industrial application.

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LIST OF ABBREVIATIONS

%	percent
°C	degrees Celsius
µF	microFaraday
µg	microgram
µl	microlitre
5'-MU	5'-methyluridine
A	absorbance
abacavir	6-cyclopropylamino-2',3'-didehydro-2'3'-dideoxyguanosine
ATP	adenosine triphosphate
Avg.	average
AZT	3'-azido-3'-deoxythymidine
BGSC	*Bacillus* Genetic Stock Centre
BLAST	Basic Local Alignment Search Tool
bp	base pairs
BSA	bovine serum albumin
C	carboxy
ca.	approximately
cat	chloramphenicol acetyltransferase gene
CAT	chloramphenicol acetyltransferase
cladribine	2'-chloro-2'-deoxyadenosine
cm	centimetre
cytarabine	1-β-D-arabinofuranosylcytosine
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside-5'-triphosphate
DTT	dithiothreitol
e.g.	*exempli gratia* (for example)
EDTA	ethylenediamine tetracetic acid
ELISA	enzyme-linked immunosorbent assay
et al.	*et alia* (and others)
FDG	fluorescein-di-β-D-galactopyranoside
Fig.	figure
fludarabine	2-fluor-9-β-D-arabinofuranosyladenine
gemcitabine	2',2'-difluorodeoxycytidine
GFP	green fluorescent protein

gfp	green fluorescent protein gene
h	hour
HIV	human immunodeficiency virus
i.e.	*id est* (that is)
IPTG	isopropyl-β-D-thiogalactoside
kb	kilobase pair
kDa	kilodalton
KOAC	potassium acetate
kV	kilovolt
L	litre
lacZ	β-galactosidase gene
lamivudine	2'-deoxy-3'-thiacytidine
LB	Luria-Bertani
M	molar
MCS	multiple cloning site
mg	milligram
min	minute
ml	millilitre
mM	millimolar
mRNA	messenger RNA
MTAP	5'-deoxy-5'-methylthioadenosine phosphorylase
mU	milliunit
MUG	methylumbelliferyl-β-D-galactoside
N	amino
NaOAC	sodium acetate
ng	nanogram
nm	nanometre
no.	number
NP-I	nucleoside phosphorylase-I
NPases	nucleoside phosphorylase enzymes
NP-II	nucleoside phosphorylase-II
nt	nucleotide
OD	optical density
ONPG	*o*-nitrophenyl-β-D-galactoside
ORF	open reading frame
ori	origin of replication
PCR	polymerase chain reaction

PEG	polyethylene glycol
pmol	picomole
p-NP	*p*-nitrophenol
PNP	purine nucleoside phosphorylase
p-NPP	*p*-nitrophenyl phosphate
PyNP	pyrimidine nucleoside phosphorylase
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA
s	second
sapS	*Staphylococcus aureus* acid phosphatase gene
SD	standard deviation
stavudine	2',3'-didehydro-3'-deoxythymidine
SURE	subtilin-regulated gene expression system
TLC	thin layer chromatography
TP	thymidine phosphorylase
Tris	Tris(hydroxymethyl)aminomethane
Tris-OAc	Tris-acetate
U	units
UP	uridine phosphorylase
UTR	untranslated region
UV	ultraviolet
V	volt
v.	version
v/v	volume per volume
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
XO	xanthine oxidase
zalcitabine	2',3'-dideoxycytidine
α	alpha
β	beta
λ	lambda
μM	micromolar
σ	sigma
Φ	phi
Ω	ohm

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CHAPTER ONE

LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

The classical way of protein purification starting from a large number of cells producing the protein using its authentic expression signals has largely been superseded by recombinant technology, where the protein of interest is overproduced in a regulated way. This often means that the gene of interest is fused to a controllable promoter, which is activated by addition of an inducer to initiate transcription. In most cases, the inducer is a small molecule that is either taken up by the cells or diffuses through the cytoplasmic membrane, such as isopropyl-β-D-thiogalactoside (ITPG). Alternatively, the inducer can be a stress factor, such as a sudden increase or decrease in growth temperature. Ideally, the expression system should provide tight regulation of the promoter. This means a very low transcription rate in the absence of the inducer and a high expression rate after addition of the inducer, thus resulting in the high-level synthesis of the recombinant protein (Baneyx, 1999; Schumann and Ferreira, 2004).

The overproduction of recombinant proteins is typically a two-step process that starts with a growth regimen to obtain a high cell density, followed by the expression phase during which the cells are induced. If the protein does not contain post-translational modifications, then production of the recombinant protein is often carried out using prokaryotic expression hosts. In addition to *Escherichia coli*, which is usually the principal bacterium of choice for cloning genes and expression of recombinant proteins (Baneyx, 1999; Samuelson, 2011), several Gram-positive bacteria are also being used as production organisms. Amongst these, *Bacillus subtilis* is regarded as an attractive host for recombinant protein production (Harwood, 1992; Wong, 1995). Not only has *B. subtilis* been a paradigm for the genetics of Gram-positive bacteria for over 50 years, but its genome has been fully sequenced and the mechanisms for gene expression and protein secretion have been studied extensively (Meima *et al.*, 2004; Hecker and Völker, 2004; Tosato and Bruschi, 2004). Moreover, *B. subtilis* has been used widely for the production of enzymes in large-scale fermentation processes (Jan *et al.*, 2001).

Enzymes catalyze a vast number of reactions that are necessary for the synthesis, modification and degradation of organic molecules that make up living organisms. Amongst these is the reversible phosphorolysis of purine and pyrimidine nucleotides, which provides an alternative to the *de novo* purine and pyrimidine synthetic pathways (Neuhard, 1983; Nygaard, 1983). Based on structural studies, all of the nucleoside phosphorylase enzymes (NPases) that

catalyze the phosphorolysis of nucleotides have one of two distinct protein folds and has thus provided the basis for defining two families of nucleoside phosphorylases (Pugmire and Ealick, 2002). The first family, NP-I, includes enzymes that share a common single-domain subunit, with either a trimeric or a hexameric quaternary structure, and accept a range of both purine and pyrimidine nucleoside substrates. Members of the second family, NP-II, have a common two-domain subunit fold and dimeric quaternary structure, and are specific for pyrimidine nucleosides. In addition to their important cellular functions, the NPases have been exploited as tools for the enzymatic synthesis of nucleoside analogues with antiviral and anticancer activities (Elizabeth *et al*., 2000; Van Rompay *et al*., 2003; De Clercq and Field, 2006; Dienstag *et al*., 2009). Consequently, overexpression of different NPases in suitable expression hosts may be beneficial to the industrial production of nucleosides and therapeutic nucleoside analogues.

In this review of the literature, information pertinent to high-level expression of recombinant proteins in Gram-negative (*E. coli*) and Gram-positive (*B. subtilis*) bacteria will be discussed, including different promoter systems that allows for controlled gene expression in these bacterial hosts. Different reporter genes that can be used to assess promoter activity will also be highlighted. The section is concluded with a discussion of nucleoside phosphorylases, the expression of which is closely aligned with the aims of the investigation.

1.2 RECOMBINANT PROTEIN EXPRESSION

The objective of gene cloning for biotechnological purposes is often the expression of the cloned gene in a selected host organism. Although *Escherichia coli* is usually the bacterium of choice for cloning genes and expressing recombinant proteins, it is unlikely that a single bacterium, such as *E. coli*, will be suitable for every application and the choice of host organism is thus primarily influenced by the application at hand. Consequently, many different bacteria have been developed as alternative hosts to *E. coli*, most notably *B. subtilis*, but the availability of tools for their genetic manipulation has generally dictated the extent of their utilization. In this part of the Literature Review, emphasis will be placed on prokaryotic gene expression and aspects that are considered to be of importance for the successful overproduction of recombinant proteins will be highlighted.

1.2.1 Transcriptional regulation

1.2.1.1 Importance of sigma factors

The term gene expression refers to the entire process whereby the information encoded in a particular gene is decoded into a particular protein. Although it being a complex process involving many different steps, transcription initiation through promoter clearance and release from the RNA polymerase is the most important control point in determining whether or not most genes are expressed (Reznikoff *et al*., 1985). However, before genes can be transcribed from specific DNA promoter sequences, the bacterial core RNA polymerase (with a subunit composition of $\alpha_2\beta\beta'\omega$) must combine with a dissociable sigma (σ) subunit to form a RNA polymerase holoenzyme (Burgess *et al*., 1969; Helmann and Chamberlin, 1988; Deighan *et al*., 2011). The reversible binding of various different alternative σ factors, which are expressed under specific conditions or may be sequestered by complementary anti-sigmas until needed (Arthur and Burgess, 1998), allows the formation of different holoenzymes able to distinguish groups of promoters required for different cellular functions (Stragier and Losick, 1990; Gross *et al*., 1992; Blattner *et al*., 1997). In addition to double-stranded DNA promoter recognition and binding, σ proteins also play a role in promoter melting (de Haseth and Helmann, 1995; Wösten, 1998; Fenton *et al*., 2000), inhibition of non-specific transcription initiation and they are often targets for activators (Gribskov and Burgess, 1986; Helmann and Chamberlin, 1988; Gross *et al*., 1998).

Although multiple σ factors have been documented in both *E. coli* and *B. subtilis*, the principal σ factor is σ^{70} in *E. coli* (Lonetto *et al*., 1992) and σ^{A} in *B. subtilis* (Haldenwang, 1995). The respective σ factors have similar consensus promoter sequences (see below) and they are also responsible for directing the transcription of most of the *E. coli* and *B. subtilis* genes. These σ factors are therefore present in much greater quantities than other minor σ factors in the bacterial cells and thus transcription of housekeeping genes is not limited because of a shortage of available σ factor (Haldenwang, 1995; Record *et al*., 1996). Consequently, the majority of expression systems developed for high-level expression of homologous and heterologous genes in *E. coli* and *B. subtilis* include promoters recognized by σ^{70}- or σ^{A}-RNA polymerase holoenzymes (Goldstein and Doi, 1995; Haldenwang, 1995).

1.2.1.2 Promoters

The minimum requirement for an effective gene expression system is the presence of a promoter sequence upstream of the cloned gene. Over the years, a large number of σ^{70}-dependent promoters from *E. coli* have been analyzed and compared that resulted in the formulation of a consensus promoter sequence (Hawley and McClure, 1983; Lisser and Margalit, 1993; Goldstein and Doi, 1995). Typically, the promoter consists of a hexanucleotide sequence located 35 base pairs (bp) upstream of the transcription initiation base (-35 region; 5'-TTGACA-3') and is separated by a short 17-bp spacer sequence from another hexanucleotide sequence (-10 region; 5'-TATAAT-3'). In general, the *in vivo* strength of a promoter correlates with its qualitative homology with the consensus sequence and the favoured spacing of 17 bp between the -35 and -10 sequences (Reznikoff *et al*., 1985; Typas and Hengge, 2006; Singh *et al*., 2011). Although the optimal spacer length is 17 ± 1 nucleotide (nt), functional promoters with a spacing between 15 and 20 non-conserved nt have also been reported (Hawley and McClure, 1983; de Haseth and Helmann, 1995; Lin *et al*., 2004). The role of the spacer length is to orientate the positions of the -35 and -10 hexamers for recognition by the RNA polymerase holoenzyme (Record *et al*., 1996). The level of gene transcription may also be affected by sequences located upstream of the core promoter elements. Some *E. coli* promoters appear to lack the -35 sequence and mutational studies have indicated that in these cases the sequence immediately upstream of the -10 region affects promoter activity. The optimal sequence for these "extended" -10 sequences appears to be T_nTG_n, followed by the -10 region (Record *et al*., 1996; Typas and Hengge, 2006).

In the case of the *E. coli rrnB* P1 promoter, an A+T-rich region located at -40 to -60 relative to the transcriptional start site, the upstream (UP) element, has been shown to increase promoter activity, possibly by increasing the rate of transcription initiation *in vivo* (Gourse *et al*., 1986; Rao *et al*., 1994; Ross *et al*., 1998). The UP element contains two conserved regions, an 11-bp distal region (-57 to -47: 5'-AAA(a/t)(a/t) TTT-3') and a 4-bp proximal region (-44 to -41: 5'-AAAA-3'). Each region can function independently, but the proximal region confers a higher degree of transcription activation on the core promoter (>100-fold) than the distal region (15-fold) (Ross *et al*., 1998). Interestingly, the UP element may also function as an independent promoter module, because when it is fused to other promoters it stimulates transcription (Ross *et al*., 1993; Rao *et al*., 1994).

Considering that the composition of the core RNA polymerase in *B. subtilis* resembles that of *E. coli*, it is not surprising that analyses of many σ^A-dependent *Bacillus* promoters indicate that they contain the -35 and -10 sequences found in *E. coli* promoters (Haldenwang, 1995). However, in contrast to some *E. coli* promoters that lack the -35 region, this never occurs in *B. subtilis* (Helmann, 1995). In *B. subtilis*, many promoters contain an essential TGTG motif (-16 region) upstream of the -10 region and mutagenesis of this region may reduce promoter strength (Helmann, 1995; Voskuil and Chambliss, 1998). The promoters also have conserved polyA and polyT tracts upstream of the -35 region, which contributes to increased promoter strength.

1.2.2 Transcriptional terminators

In prokaryotes, transcription termination is effected by either a Rho-dependent or Rho-independent mechanism (Richardson, 1993). Rho-dependent transcription termination depends on the hexameric protein Rho, which causes the release of the nascent RNA transcript from the DNA template. In contrast, Rho-independent termination depends on signals encoded in the template. These comprise of a region of dyad symmetry that encodes a hairpin or stem-loop structure in the nascent RNA, and a second region that is rich in dA and dT and is located 4 to 9 bp distal to the dyadic sequence (Richardson, 1993; Wilson and von Hippel, 1995). Efficient transcription terminators are indispensable elements of expression vectors, because they serve several important functions. Transcription through a promoter may inhibit its function, a phenomenon known as promoter occlusion (Adhya and Gottesman, 1982). This interference can be prevented by the proper placement of a transcription terminator downstream of the coding sequence to prevent continued transcription through another promoter. Similarly, a transcription terminator placed upstream of the promoter that drives expression of the gene of interest minimizes background transcription (Nishihara *et al.*, 1994). In addition, transcription terminators enhance mRNA stability and can thus substantially increase the level of protein production (Hayashi and Hayashi, 1985; Vasquez *et al.*, 1989).

1.2.3 Translational regulation

1.2.3.1 Translational issues

Putting a cloned gene under the control of a promoter, although essential, may not be sufficient to maximize the yield of the cloned gene product. Other factors, such as the efficiency of translation and the stability of the newly transcribed mRNA, may also affect the amount of product. Because of the close coupling between transcription and translation in prokaryotes, engineering of the translation initiation region is considered a powerful tool for modulating gene expression in a promoter-independent fashion (Ringquist *et al*., 1992). Initiation of translation of prokaryotic mRNAs requires a Shine-Dalgarno (SD) sequence complementary to the 3' end of the 16S rRNA, followed by an initiation codon, which is most commonly AUG (Shine and Dalgarno, 1974; Gualerzi and Pon, 1990). Although the optimal spacing between these two features is 8 nt, translation initiation is severely affected if the distance is reduced below 4 nt or increased above 14 nt (Ringquist *et al*., 1992; Chen *et al*., 1994). Stable mRNA secondary structures encompassing the SD sequence and/or the initiation codon have been reported to dramatically reduce gene expression by interfering with ribosome binding (Hall *et al*., 1982; de Smit and van Duin, 1990). It is believed that occlusion of the SD region and/or the AUG codon by a stem-loop structure precludes the accessibility of the 30S ribosomal subunits and inhibits translation (Ramesh and De Nagaraja, 1994). This problem can be circumvented by increasing the homology of SD regions to the consensus (Coleman *et al*., 1985; Stanssens *et al*., 1985) and by raising the number of A residues in the initiation region through site-directed mutagenesis (Olsen *et al*., 1989; Chen *et al*., 1994).

Interestingly, it has been demonstrated that *E. coli* ribosomes can support protein synthesis by mRNA from Gram-negative and Gram-positive bacteria, whereas ribosomes from *B. subtilis* recognized only homologous mRNA (Stallcup *et al*., 1974). It was subsequently reported that the selectivity of the *B. subtilis* ribosomes can be ascribed to a lack of a counterpart of the largest *E. coli* ribosomal protein, S1 (Higo *et al*., 1982; Roberts and Rabinowitz, 1989). Notably, other Gram-positive bacteria, such as *Staphylococcus*, *Streptococcus*, *Clostridium* and *Lactobacillus*, also lack an S1-equivalent protein and they too exhibit mRNA selectivity (Vellanowth, 1993). The role of the S1 ribosomal protein is believed to be to bind RNA non-specifically and bring it to the decoding site of the 30S subunit where proper positioning of the SD sequence and initiation codon signals can occur. This is reflected in a more extensive

complementarity between the SD sequence and the 3' end of the 16S rRNA than found in bacteria that do have ribosomal protein S1 (Vellanoweth, 1993).

1.2.4 mRNA stability

The process of mRNA degradation provides a major control point of gene expression in virtually all organisms (Ross, 1995). Prokaryotic mRNAs are rather unstable, with half-lives ranging between 30 s and 20 min. The major enzymes involved in mRNA degradation are two 3'→5' exonucleases (RNase II and polynucleotide phosphorylase) and the endonuclease RNase E (Carpousis *et al*., 1999; Coburn and Mackie, 1999). The catalytic activity of RNase E is located at the amino (N) terminus, whereas the carboxy (C) terminus serves as a scaffold for the assembly of a highly efficient "degradosome" involving PNPase, the RNA helicase RhlB and endolase. Two classes of protective elements are known to stabilize mRNAs in bacteria and may subsequently be used to prolong the half-life of heterologous mRNAs (Duvoisin *et al*., 1986; Chen *et al*., 1991; Emory *et al*., 1992). One class consists of sequences in the 5' untranslated regions (UTRs) of mRNAs, and the other class consists of sequences in the 3' UTR sequences that can form stem-loop structures, thereby blocking exonucleolytic degradation of the transcripts from the 3' terminus (Wong and Chang, 1986). In either case, stable secondary structures in the 5' UTR of certain transcripts, as well as 3' Rho-independent terminators can both increase mRNA stability and consequently, increase the level of protein production (Ehretsmann *et al*., 1992; Carpousis *et al*., 1999).

1.2.5 Other factors of importance

1.2.5.1 Metabolic load

The introduction and expression of foreign DNA in an expression host often changes the metabolism of the organism in ways that may impair normal cellular functioning. This phenomenon, which is a multifaceted biological response, is due to a metabolic load that is imposed upon the host by the foreign DNA. A metabolic load can occur as the result of a variety of conditions (Glick and Whitney, 1987; Glick, 1995). For example, increasing plasmid copy number and/or size requires increasing amounts of cellular energy for plasmid replication and maintenance, and the limited amount of dissolved oxygen in the growth medium is often insufficient for both host cell metabolism and plasmid maintenance and expression. In such cases, plasmid-bearing cells grow more slowly than untransformed cells, often resulting in the loss of the recombinant plasmid or a portion of the plasmid DNA. Since

cells growing in the presence of a metabolic load generally have a decreased level of energy available for a variety of cellular functions, the cell's energy-intensive metabolic processes such a protein synthesis are invariably adversely affected by a metabolic load (Glick and Whitney, 1987). Moreover, overproduction of foreign proteins may deplete the pools of some aminoacyl-tRNAs and/or drain the host cell of its energy in the form of ATP or GTP. This may result in the initiation of a cellular stress response, which includes increased synthesis of cellular proteases so that the overexpressed recombinant protein is rapidly degraded (Glick, 1995).

1.2.5.2 Plasmid copy number and maintenance

To achieve high gene dosage, the genes to be expressed are typically cloned into plasmids that replicate in a relaxed fashion and are present at high copy numbers, ranging from 15 to a few hundred copies per cell. Under laboratory conditions, such multicopy plasmids are randomly distributed during cell division and, in the absence of selective pressure, are lost at low frequency, primarily as a result of multimerization (Summers, 1998). However, plasmid-loss can increase in the case of very high copy number plasmids, when plasmid-borne genes are toxic to the host or otherwise significantly reduce its growth rate, or when cells are cultivated at high density or in continuous processes (Summers, 1998).

The simplest way to address this problem is to take advantage of plasmid-encoded antibiotic resistance markers and supplement the growth medium with antibiotics to kill plasmid-free cells. The problems with the approach are loss of selective pressure as a result of antibiotic degradation or inactivation and the contamination of the product or biomass by antibiotics that may be unacceptable in the production of human therapeutic proteins (Glick and Whitney, 1987). A number of alternative strategies have therefore been developed to ensure that plasmid-free cells will not overtake a culture. In most cases, cloning vectors are engineered to carry gene(s) that cause cell death upon plasmid-loss (Williams *et al.*, 1998). Alternatively, the heterologous genes may be directly inserted within the chromosome of the host organism (Chopin *et al.*, 1989; Olson *et al.*, 1998; Hinds *et al.*, 1999). In this case, problems associated with plasmid instability may be overcome and the transformed host cell will also not waste its resources synthesizing unwanted and unneeded antibiotic resistance marker gene products.

1.3 REGULATABLE PROMOTER SYSTEMS FOR RECOMBINANT PROTEIN PRODUCTION

As indicated previously, the minimum requirement for an effective gene expression system is the presence of a promoter sequence located upstream from the cloned gene. In order to be suitable for high-level protein synthesis, such a promoter should have several properties (Goldstein and Doi, 1995). The promoter must be strong, resulting in the accumulation of protein making up to 10 to 30% or more of the total cellular protein. The promoter should exhibit a minimal level of basal transcription activity as large-scale gene expression usually employs cell growth to high density and minimal promoter activity, followed by induction or derepression of the promoter. The tight regulation of a promoter is also essential for the synthesis of proteins that may be detrimental to the host cell (Wülfing and Plückthun, 1993; Suter-Crazzolara and Unsicker, 1995; Bowers *et al*., 2004). Moreover, incomplete repressed expression systems may cause plasmid instability, a decrease in cell growth rate and consequently, may prevent the efficient production of recombinant protein (Chen *et al*., 1991; Mertens *et al*., 1995). Since the use of regulated promoter systems for heterologous protein expression is a focus of this study, they will be discussed in greater detail and emphasis will be placed on those systems for use in Gram-positive bacteria, such as *B. subtilis.*

1.3.1 Inducible promoter systems for use in *E. coli*

Many promoters used to transcribe heterologous genes in *E. coli* have been constructed from *lac*-derived regulatory elements. Although the *lac* promoter is rather weak and rarely used for high-level production of recombinant proteins, they are valuable tools to achieve graded expression of toxic proteins (Hashemzadeh-Bonehi *et al*., 1998). The synthetic *tac* and *trc* promoters, which consist of the -35 region of the *trp* promoter and the -10 region of the *lac* promoter, differ by 1 bp only in the length of the spacer domain separating the two hexamers (Friesen and An, 1983; de Boer *et al.*, 1983). Both promoters are quite strong and routinely allow the accumulation of proteins to about 15-30% of the total cell protein. Although all of the above promoters can be induced with IPTG and have been used widely for basic research, the use of IPTG for the large-scale production of human therapeutic proteins is undesirable due to its toxicity and high cost (Figge *et al*., 1988; Kosinski *et al*., 1992). Consequently, other promoters have been characterized that provide alternative options for high-level gene expression systems in *E. coli.* For example, the nutritionally inducible *trp* and *araBAD* promoters, which are induced by tryptophan limitation and the inexpensive sugar L-arabinose,

respectively, have also been used, but they are weaker than the *tac* promoter (Newman and Fuqua, 1999; Guzman *et al*., 2005; Merino *et al*., 2008).

The P_L promoter of the *E. coli* phage λ is one of the promoters responsible for transcription of the λ DNA genome. The P_L promoter is a very strong promoter recognized by the *E. coli* RNA polymerase, which is subverted by phage λ into transcribing the phage DNA. The promoter is repressed by the product of the phage λ *cI857* gene (Giladi *et al*., 1995). Expression vectors that harbor the P_L promoter are used with a mutant *E. coli* host that synthesizes a temperature-sensitive mutant version of the CI857 protein. At low temperatures (less than 30°C) the mutant CI857 protein is able to repress the λ P_L promoter, but at higher temperatures (42°C) the protein is inactivated and results in transcription of the cloned gene (Gupta *et al*., 2004).

For biotechnological applications, it is important that the promoter be induced in a simple and cost-effective manner. In this regard, it is interesting to note that protein synthesis is increased when *E. coli* is cultured at 10-20°C as opposed to culturing at 37°C, suggesting that cold-responsive promoters occur naturally in *E. coli* (Jones *et al*., 1987). Subsequent studies have shown that cold-responsive promoters facilitate efficient gene expression at reduced temperatures (Goldstein *et al*., 1990; Qoronfleh *et al*., 1992; Giladi *et al*., 1995; Vasina and Baneyx, 1997). The rationale behind the use of cold-responsive promoters for gene expression is based on the supposition that the rate of protein folding will be only slightly affected at lower temperatures, whereas the rates of transcription and translation (being biochemical reactions) will be substantially decreased. This, in turn, will provide sufficient time for protein refolding, yielding active proteins and avoiding the formation of inactive protein aggregates, *i.e.* inclusion bodies, without reducing the final yield of the target protein (Giladi *et al*., 1995; Qing *et al*., 2004).

1.3.2 Inducible promoter systems for use in *B. subtilis*

The development of regulated promoter systems for high-level controlled expression of heterologous genes in *Bacillus* spp. is not as well advanced as it is in *E. coli*, possibly due to the more stringent control of promoter usage in Gram-positive bacterial species than in Gram-negative species (Wong, 1995; Westers *et al*., 2004). Initially, regulated promoter systems for use in *Bacillus* spp. were developed that are entirely of Gram-negative origin or that contain

elements of both Gram-positive and Gram-negative origin. Such promoter systems have been used for successful high-level controlled synthesis of heterologous proteins and include the hybrid P25/O promoter system, consisting of the phage T5 promoter P_{N25} and the *E. coli lac* operator (Williams *et al*., 1981; Osbourne *et al*., 1985; Le Grice *et al*., 1986), and the hybrid *spac-1* promoter system, consisting of the *B. licheniformis* penicillinase promoter and the *E. coli lac* operator (Yansura and Henner, 1984). Both these promoter systems were reported to exhibit no expression without the addition of the inducer, while high levels of expression (10 to 15% of the total protein) were observed after IPTG induction. Subsequently, several inducible promoter systems have been developed and are described below.

A popular promoter system that has frequently been used for heterologous gene expression in *B. subtilis* is the *xylA* system (Kim *et al*., 1996). In this system, a gene of interest is cloned downstream of the xylose-inducible *xylA* promoter and is integrated into the *amyE* locus of the *B. subtilis* chromosome. Using this system, it has been reported that a very high transcription activity is generated upon xylose addition, whereas the basal level of expression is low (Kim *et al*., 1996). However, regulation of the *xylA* promoter is subject to glucose repression, which results in lower levels of expression in glucose-containing culture media. As a consequence, high protein production levels can be achieved only by using media with extremely low glucose levels, which, in turn, increases promoter leakage and can be a problem when the recombinant protein has detrimental effects on the expression host (Bhavsar *et al*., 2001). Moreover, since this system depends on chromosomal integration of the promoter and gene of interest, high-level expression may furthermore be limited due to the low copy number (Bhavsar *et al*., 2001). More recently, plasmid-based alternatives to xylose-inducible systems have been reported in which promoters that are inducible by maltose (P_{glv}) or sucrose (P_{sacB}) are used. Although the maltose-inducible expression system was not characterized in any great detail (Ming-Ming *et al*., 2006), a direct comparison of the P_{sacB}- and P_{xylA}-inducible promoter systems has been described (Biedendieck *et al*., 2007). The results indicated that although the *xylA* promoter exhibits lower basal levels of expression than the *sacB* promoter, the P_{sacB} promoter generates higher levels of expression following induction. However, since these P_{glv} and P_{sacB} systems are derived from the sugar-fermenting capacities of *B. subtilis*, it can be expected that these promoters are likely also under glucose repression control as observed for the P_{xylA} system.

An alternative to sugar-inducible promoter systems, which is based on the promoter of the *acoABCL* operon of *B. subtilis*, has been reported (Silbersack *et al.*, 2006). This operon encodes the acetoin dehydrogenase complex, which is the major enzyme system responsible for the catabolism of acetoin in *B. subtilis* (Huang *et al.*, 1999). It was shown that transcription of reporter gene fusions with the *acoA* promoter of the operon is strongly repressed by glucose, but induced by acetoin as soon as the preferred carbon source glucose is exhausted. During glucose limitation, the utilization of the alternate energy source acetoin keeps the protein synthesis machinery of *B. subtilis* active and thus allows for long-lasting *acoA*-controlled expression of recombinant proteins. Both as a single chromosomal fusion and as a multicopy plasmid-based fusion, the *acoA* promoter was shown to have a very low basal activity, but was strongly induced during glucose limitation if acetoin was available (Silbersack *et al.*, 2006).

A promising system for regulated gene expression in *B. subtilis* is the so-called subtilin-regulated gene expression (SURE) system (Bongers *et al.*, 2005). This system is based on the regulatory module involved in cell-density-dependent control of the production of the lantibiotic subtilin. In *B. subtilis*, production of subtilin is subjected to quorum sensing control that depends on sensing of subtilin by a dedicated sensor kinase (SpaK) and its subsequent signal transduction to the corresponding response regulator (SpaR). Upon phosphorylation, SpaK binds to *spa* boxes in the promoter regions upstream of the *spaB*, *spaI* and *spaS* genes located in the subtilin biosynthesis gene cluster, thereby triggering promoter activation (Stein *et al.*, 2002; Kleerebezem *et al.*, 2004). Amongst these promoters, the *spaS* promoter was reported to drive the highest level of transcription activation upon induction with subtilin (Stein *et al.*, 2003). Consequently, in the SURE system, *spaR*- and s*paK*-dependent signal transduction is used to control P_{spaS}-driven gene expression. This regulated gene expression system relies on the use of an expression vector harbouring the *spaS* promoter-derived subtilin response promoter elements, and a *spaRK*-expressing production host, which is obtained by integrating the essential sensor-regulator couple *spaRK* into the *amyE* locus of the *B. subtilis* chromosome. The *spaS* promoter was reported to exhibit very low levels of basal expression, while very high levels of expression (10-fold increase) were observed upon induction with subtilin. Moreover, the level of expression depended directly on the amount of inducer used (Bongers *et al.*, 2005).

An interesting promoter system for regulated expression in *B. subtilis* is based on a glycine riboswitch. Riboswitches are regulatory elements located within the 5' UTR of some mRNAs (Mandal and Breaker, 2004; Winkler and Breaker, 2005). They form secondary structures that serve as binding sites for metabolites, such as vitamins and amino acids, and often control expression of genes involved in the biosynthesis or transport of the metabolite sensed. In bacteria, riboswitches control either transcription elongation or translation initiation. While most metabolites prevent gene expression by interaction with their cognate riboswitch, binding of glycine to its riboswitch leads to transcription attenuation in *B. subtilis* (Mandal *et al*., 2004). Specifically, glycine leads to activation of the tricistronic operon *gcvT-gcvPA-gcvPB* involved in the degradation of this amino acid if present at high concentrations. Based on this finding, an expression system has been constructed that can be induced by the addition of glycine (Phan and Schumann, 2007). Although the promoter was induced to a lesser extent as compared to the xylose-inducible *xylA* promoter, the yield of recombinant protein was comparable.

In addition to the above inducible promoter systems, a limited number of temperature-induced expression systems has also been described. Thornewell *et al*. (1993) described the construction of a heat-inducible expression system, based on a derivative of the *B. subtilis* phage Φ105. The system comprises an integrative expression vector, in which the gene of interest is cloned downstream of a strong phage Φ105 promoter, and a *B. subtilis* MU331 strain containing a defective prophage Φ105. The defective MU331 prophage carries a deletion in a region needed for cell lysis and contains a temperature-sensitive mutation in the phage repressor of the promoter present in the expression vector, thus allowing induction by a shift in temperature without concomitant cell lysis. Consequently, upon heat induction (50°C for 5-10 min), the Φ105 repressor protein is inactivated, the promoter derepressed and transcription commences. Although the Φ105-MU331 prophage system shows very tight control of gene expression, it is a technically demanding and cumbersome system to use. A simplified regulated expression vector that does not require lysogenic *B. subtilis* strains was more recently described (Serrano-Heras *et al*., 2005). The expression vector carries the P_R promoter and the *cI857* gene, which encodes a temperature-sensitive transcriptional repressor of the P_R promoter from the *E. coli* phage λ. Using this system, regulated expression of the *gfp* reporter gene was demonstrated in *B. subtilis* and resulted in an 8-fold increase in the amount of synthesized green fluorescent protein (GFP) upon heat induction at 40°C or 45°C.

In an effort to seek new temperature-inducible promoters, Li *et al*. (2007) constructed a promoter library via a promoter trapping vector and identified two temperature-sensitive promoters. These promoters, which both contained prokaryotic promoter conserved regions, were designated P2 and P7, respectively. The P7 promoter preceded a gene coding for YdhI of which the function is not known, while the P2 promoter controls transcription of a non-protein coding RNA. Both of these promoters were shown to be induced upon incubation at 45°C and production of a β-galactosidase reporter protein was 10-fold higher than that produced by incubation at 37°C. The P2 promoter was subsequently cloned into an *E. coli-B. subtilis* shuttle vector to generate a heat-inducible expression vector (Li *et al*., 2007).

Rather than using a temperature upshift, it is also possible to overproduce recombinant proteins at low temperatures, provided that the gene of interest is fused to a cold-inducible promoter. In *B. subtilis*, the *des* gene codes for the enzyme desaturase, which introduces *cis* double bonds into a wide variety of saturated fatty acids (Aguilar *et al*., 1998). Expression of the *des* gene depends on a two-component signal transduction system, which consists of the sensor kinase DesK and the response regulator DesR (Aguilar *et al*., 2001). When the sensor kinase senses a temperature downshift through changes in the physical state of the cytoplasmic membrane, it undergoes autophosphorylation with subsequent transfer of the phosphate group to the response regulator. Phosphorylated DesR binds to two adjacent DNA-binding sites, leading to transcription activation of the *des* promoter. Two expression vectors have been constructed, based on this cold-inducible promoter, which respectively allowed for intra- and extracellular synthesis of recombinant protein upon a temperature downshift from 37°C to 25°C (Le and Schumann, 2007).

In contrast to *B. subtilis*, the ability to selectively regulate gene expression is lacking for many pathogenic Gram-positive bacteria. To redress this imbalance, Schofield *et al*. (2003) described a series of promoters that are regulated by a bacteriophage P1 temperature-sensitive C1 repressor. The promoters exhibited very low basal expression in *Enterococcus faecium, Enterococcus faecalis* and *Staphylococcus aureus*. However, under inducing conditions (incubation at 42°C), the promoters yielded high levels of expression (100- to 1000-fold induction). Although the functionality of the thermally regulated promoter systems was demonstrated in the above clinically relevant Gram-positive bacteria, the promoters were not tested in other Gram-positive species such as *B. subtilis*. Based on the very high induction ratio achieved with these promoter systems, they may have great potential for the production

of industrially important proteins. During the course of this study, the promoter systems developed by Schofield *et al.* (2003) were evaluated for use in *B. subtilis* and a detailed description of their features is provided in the relevant Chapter (Chapter 2, this dissertation).

1.4 REPORTER GENES TO EVALUATE PROMOTER ACTIVITY

Reporter genes are commonly used in the construction of transcriptional fusions to regulatory elements of interest, and have facilitated greatly the analysis of gene expression and the study of individual promoters and their regulation (Naylor, 1999; Lu *et al.*, 2004; Mijakovic *et al.*, 2005). The results from these analyses have found application in both basic and applied sciences. In molecular and cellular biology, reporter genes have contributed to improved understanding of the cellular events associated with gene expression (Tsien and Miyawaki, 1998; Koga *et al.*, 2006). In biotechnology, reporter genes have been used for the identification and/or characterization of novel promoters that can be used to establish improved expression systems for high-level production of recombinant proteins (Cagnon *et al.*, 1991; Karunakaran et *al.*, 2005; Serrano-Heras *et al.*, 2005; Miksch *et al.* 2006). The choice of a reporter gene is determined by a number of criteria. These include the absence of activities similar to those of the reporter protein in the host organism, and the availability of simple, rapid and sensitive methods for the qualitative and quantitative assay of reporter protein activity. These methods should preferably allow assaying of the reporter protein activity in the presence of cellular components, thus obviating the need for purification steps prior to assay (Naylor, 1999; Mijakovic *et al.*, 2005). A number of different reporter genes have been used for analysis of different aspects of gene expression and these will be discussed briefly in the following sections.

1.4.1 Antibiotic resistance genes

A number of antibiotic resistance genes have been used as reporter genes in a variety of Gram-negative and Gram-positive bacteria (Forsman and Jaurin, 1987; Sibakov *et al.*, 1991; Labes *et al.*, 1997). The gene conferring chloramphenicol (*cat* or *cm*^{r}) resistance upon its host has been particularly useful, owing to the fact that the expression of these reporter genes can be selected for in growth medium, and can be also quantified via enzyme assays for chloramphenicol acetyltransferase (CAT) activity (Shaw, 1975). The *cat* gene has thus been employed to monitor qualitative and quantitative gene expression in a wide variety of bacteria

(Osbourne *et al*., 1987; Cao *et al*., 2001; Kaur *et al*., 2007). CAT is fairly stable in cells growing at pH values between 4.0 and 7.5 and it has been reported that CAT, at least in a number of streptococci, has a short half-life, thereby enabling the assay of temporal gene expression (Burne and Chen, 2000). CAT activity may be measured by various methods, including kinetic assays and ELISAs with anti-CAT antibodies. However, assays for CAT activity are time-consuming and expensive (Naylor, 1999).

1.4.2 Green fluorescent protein genes

The *gfp* gene, encoding green fluorescent protein (GFP), has been isolated and cloned from the jellyfish *Aequorea victoria* (Prasher *et al*., 1992). Through an autocatalytic reaction, GFP forms a cyclic peptide that is highly fluorescent and stable (Cody *et al*., 1993). An advantage of GFP over other reporters is the fact that no other energy source or substrate addition is required, other than oxygen during initial formation of the chromophore (Chalfie *et al*., 1994; De Weger *et al*., 1994). In addition, GFP is stable in the presence of many denaturants and proteases, and persists at high temperatures (65°C) and pH values (6-12) (Ward *et al*., 1998). Consequently, GFP has become one of the most frequently used reporters in both prokaryotic and eukaryotic cells (Tsien, 1998; Southward and Surette, 2002). A potential disadvantage of using *gfp* as a reporter gene is the extreme stability of the GFP protein (Tambolini *et al*., 1997). This may be problematic in studies regarding temporal changes in gene expression since, once the GFP reporter protein is synthesized, it may persist. To overcome this problem, various unstable variants of GFP have been constructed that are more susceptible to degradation by ClpXP-type proteases and therefore have shorter half-lives (Cormack *et al*., 1996; Keiler *et al*., 1996; Andersen *et al*., 1998). Moreover, various groups have obtained GFP mutants exhibiting diverse spectral properties that may allow simultaneous gene expression from a number of different promoters (Delgrave *et al*., 1995; Mena *et al*., 2006). Depending on the nature of the study, GFP fluorescence can be monitored by fluorometric detection (Burlage *et al*., 1996; Karunakaran *et al*., 2005), confocal laser scanning microscopy (Eberl *et al*., 1997) or flow cytometry (Tambolini *et al*., 1997). Numerous *gfp*-based reporter systems have been developed for studying gene expression in Gram-positive (Chen *et al*., 2003; Chary *et al*., 2005; Serrano-Heras *et al*., 2005) and Gram-negative bacteria (Karunakaran *et al*., 2005; Miksch *et al*., 2006).

1.4.3 Genes encoding chromogenic substrate cleavage enzymes

A number of genes encoding metabolic enzymes that are capable of cleaving chromogenic substrates have been described and used as reporter genes, *e.g. xylE* encoding catechol 2,3-dioxygenase (Curcic *et al*., 1994), *phoA* encoding alkaline phosphatase (Reuber *et al*., 1991), *celB* encoding β-glucosidase (Sessitsch *et al*., 1996) and *amyL* encoding α-amylase (Ugorcakova *et al*., 2000). The β-galactosidase (*lacZ*) gene of *E. coli* was one of the first genes of this class to be used as a reporter gene (Drahos *et al*., 1986), and it is still one of the most widely used reporter genes (Talukder *et al*., 2005). Its popularity has been due to the ease with which the *lacZ* gene activity can be quantitatively assayed using a variety of relatively inexpensive chromogenic (*o*-nitrophenyl-β-D-galactoside [ONPG]) and fluorescent substrates (fluorescein-di-β-D-galactopyranoside [FDG] and methylumbelliferyl-β-D-galactoside [MUG]). The primary advantages of using the *lacZ* metabolic marker are that rapid visual screening is possible and the enzyme activity assay can be performed in cuvettes or in microtiter plates, and the samples can be read on an ELISA plate reader, spectrophotometer, luminometer or fluorimeter, depending on the product of the reaction (Miller, 1992; Schenborn and Groskreutz, 1999). Alternatively, epifluorescence or confocal laser scanning microscopy can be used, in combination with fluorescent substrates, to study expression of the reporter gene at a single-cell level (Davies and Geesey, 1995). Although *lacZ* is a versatile reporter, its utility may be hindered by the presence of endogenous microbial β-galactosidases (Bronstein *et al*., 1994) and thus necessitates deletion of the native *lacZ* gene prior to its use. Further disadvantages of using *lacZ* are the stability of the enzyme, preventing the study of temporal gene expression, and the denaturation of the enzyme at pH values below neutrality (Burne and Chen, 2000).

In addition to the above, bacterial non-specific acid phosphatases have also been exploited as reporters (Thaller *et al*., 1998; Du Plessis *et al*., 2007). Bacterial non-specific acid phosphatases dephosphorylate a broad array of substrates and have optimal catalytic activity at an acid to neutral pH (Rossolini *et al*., 1998). A novel class C non-specific acid phosphatase, designated SapS, that is capable of hydrolyzing *p*-nitrophenyl phosphate (*p*-NPP) was identified and characterized from the culture supernatant of a *Staphylococcus aureus* strain isolated from vegetables (Du Plessis *et al*., 2002). The 30-kDa protein was active over a range of temperatures, varying from 20-65°C, but displayed optimum activity at 40°C and pH 5. Based on its size and the ease with which enzyme activity assays could be

performed, the SapS acid phosphatase was subsequently evaluated as a reporter in both Gram-negative and Gram-positive bacterial species (Du Plessis *et al*., 2007). The results indicated that the *sapS* gene could be used as a reliable reporter gene to characterize and evaluate a range of heterologous promoters in *E. coli*, *B. subtilis* and *B. halodurans* Alk36.

1.5 NUCLEOSIDE PHOSPHORYLASES (NPases)

Nucleoside phosphorylases (NPases) catalyze the reversible phosphorolysis of the glycosidic bond of purine and pyrimidine nucleosides, in the presence of inorganic phosphate, to yield the free base and ribose-1-phosphate (Lewkowicz and Iribarren, 2006). The biochemical significance of glycosidic bond cleavage in purines and pyrimidines by purine nucleoside phophorylase (PNP) and pyrimidine nucleoside phosphorylase (PyNP), respectively, is most apparent in the salvage pathway (Neuhard, 1983; Nygaard, 1983). The biosynthesis of purine and pyrimidine nucleotides, which are critical compounds in nearly all biochemical reactions in cells, can proceed through either the *de novo* pathway, which uses amino acids and other low-level precursors to produce nucleotides, or the salvage pathway, which makes use of preformed nucleobases and nucleosides as precursors in the synthesis of nucleotides. The salvage pathway thus allows the cell to circumvent the energy-costly *de novo* pathway when appropriate precursors are available and the by-products released in the salvage pathway also provide a source of carbon, nitrogen and energy to the cell. In addition to their key role in nucleotide metabolism and their requirement for normal cellular function, NPases have been exploited as tools for enzymatic synthesis of nucleoside analogues that are used as antiviral (Iribarren *et al*., 1990; Van Rompay *et al*., 2003; Dienstag *et al*., 2009) and anticancer agents (Sorscher *et al*., 1994; Secrist *et al*., 1999; Elizabeth *et al*., 2000).

Despite NPases having been identified more than 100 years ago (Levene *et al*., 1906; Levene and Medigreceanu, 1911), insights regarding their distribution (Bzowska *et al*., 2000), properties (Lewkowicz and Iribarren, 2006) and structure (Pugmire and Ealick, 2002) have only more recently been obtained. Based on the results of structural studies, all of the enzymes that catalyze the phosphorolytic cleavage of the glycosidic bond in nucleosides can be grouped into one of two distinct families. Consequently, in the following sections, current knowledge on these enzymes will be presented and their use in industrial applications will be highlighted.

1.5.1 The nucleoside phosphorylase-I (NP-I) family

Since its initial characterization by Kalckar (1947), PNP is the most thoroughly studied member of the NP-I family (Bzowska *et al*., 2000). PNP (EC 2.4.2.1) has been isolated from a variety of mammalian sources, *e.g*. bovine brain (Lewis and Glantz, 1976) and calf spleen (Price *et al*., 1955), and bacterial sources, *e.g*. *E. coli* and *Salmonella typhimurium* (Jensen and Nygaard, 1975), *Sulfolobus solfataricus* (Cacciapuoti *et al*., 1994) and *B. cereus* (Gilpin and Sadoff, 1971), and more recently from the yeast *Saccharomyces cerevisae* (Lecoq *et al*., 2001). These studies revealed the existence of two forms of PNP, *i.e.* a trimeric PNP, which has a subunit molecular mass of *ca*. 31 kDa and is specific for guanine and hypoxanthine 2'-deoxyribonucleosides, and a hexameric PNP, which has a subunit molecular mass of *ca.* 26 kDa and accepts adenine, as well as guanine and hypoxanthine 2'-deoxyribonucleosides. Mammalian species generally possess only the trimeric form of PNP, whereas bacterial species have largely been shown to possess only the hexameric form. However, in some bacterial species, such as *B. stearothermophilus* (Hori *et al*., 1989a, 1989b), both a trimeric and hexameric form have been identified.

In addition to PNP, other members of the NP-I family include uridine phosphorylase (UP; EC 2.4.2.3) and 5'-deoxy-5'-methylthioadenosine phosphorylase (MTAP; EC 2.4.2.28). UP, which is specific for uridine nucleotides but also accepts 2'-deoxypyrimidine nucleosides in higher organisms (Krenitsky *et al*., 1965), has been isolated from *S. solfataricus* (Cacciapuoti *et al*., 1994) and mammalian species (Ferro *et al*., 1979; Della Ragione *et al*., 1990). MTAP is reported to function as a trimer in all species, except in *S. solfataricus*, in which it appears to function as a hexamer. MTAP consists of identical subunits, each with a molecular mass of *ca.* 30 kDa.

Sequence comparisons of all known members of the NP-I family indicated that there appears to be obvious identity among sequences with a known trimeric quaternary structure, and also among sequences with a known hexameric quaternary structure. However, little sequence similarity exists between trimeric and hexameric NP-I subfamilies (Pugmire and Ealick, 2002). X-ray crystallographic studies have provided crystal structures of various members of the NP-I family (Morgunova *et al*., 1995; Mao *et al*., 1997; Narayana *et al*., 1997; Appleby *et al*., 1999, 2001). Despite species-dependent differences in the quaternary structures of members of the NP-I family, the subunit fold is highly conserved (Pugmire and Ealick, 2002).

The main feature of the common subunit fold of NP-I enzymes consists of a central β-sheet that forms a distorted β-barrel, surrounded by several α-helices. The active site consists of adjacent phosphate- and nucleoside-binding pockets. These binding sites are formed by residues from the central β-sheet and the interconnecting loops, and residues from an adjacent subunit. Notably, all of the β-strands are structurally conserved among the structures of the NP-I family. The central β-sheet motif, where the nucleoside and phosphate bind at the C-terminal end of the strands, is completely conserved, despite divergence in the sequences among the structures (Pugmire and Ealick, 2002). This suggests a structural motif that is important in binding nucleosides and phosphate, and in catalyzing the phosphorolysis reaction (Pugmire and Ealick, 2002). The conserved helices are also likely to play an important role in the structure-function relationship of the NP-I family. Four conserved helices are distributed on both sides of the central β-sheet and contribute to the overall stability of the α/β-fold (Pugmire and Ealick, 2002). The structure and topology of trimeric and hexameric NP-I enzymes are shown in Fig. 1.1.

Several different mechanisms have been reported to explain the catalytic activity of members of the NP-I family from different species (Bzwoska *et al*., 2000; Lewkowicz and Iribarren, 2006). Early kinetic data indicated that catalysis occurs through a ternary complex of enzyme, phosphate and nucleoside, and that the reaction is sequential (*i.e.* all substrates bind to the enzyme before any product is released) (Krenitsky, 1967; Kim *et al*., 1968). However, what is not clear is whether the reaction proceeds via ordered or random binding. For example, studies with PNP from bovine thyroid indicated an ordered mechanism with phosphate binding before the nucleoside (Carlson and Fischer, 1979), while the reverse order has been reported for the enzyme from bovine brain (Lewis and Glantz, 1976). Interestingly, a study reporting kinetic data from bovine spleen PNP indicated an ordered sequential mechanism in the phosphorolytic direction with phosphate binding before the nucleoside, and a random-sequential mechanism in the reverse synthetic direction (Porter, 1992). Kinetic studies of UP from different species indicated both a random-sequential mechanism (Krenitsky, 1976) and an ordered-sequential mechanism in which phosphate is the first substrate to bind and uracil is the first product to be released (Kraut and Yamada, 1971).

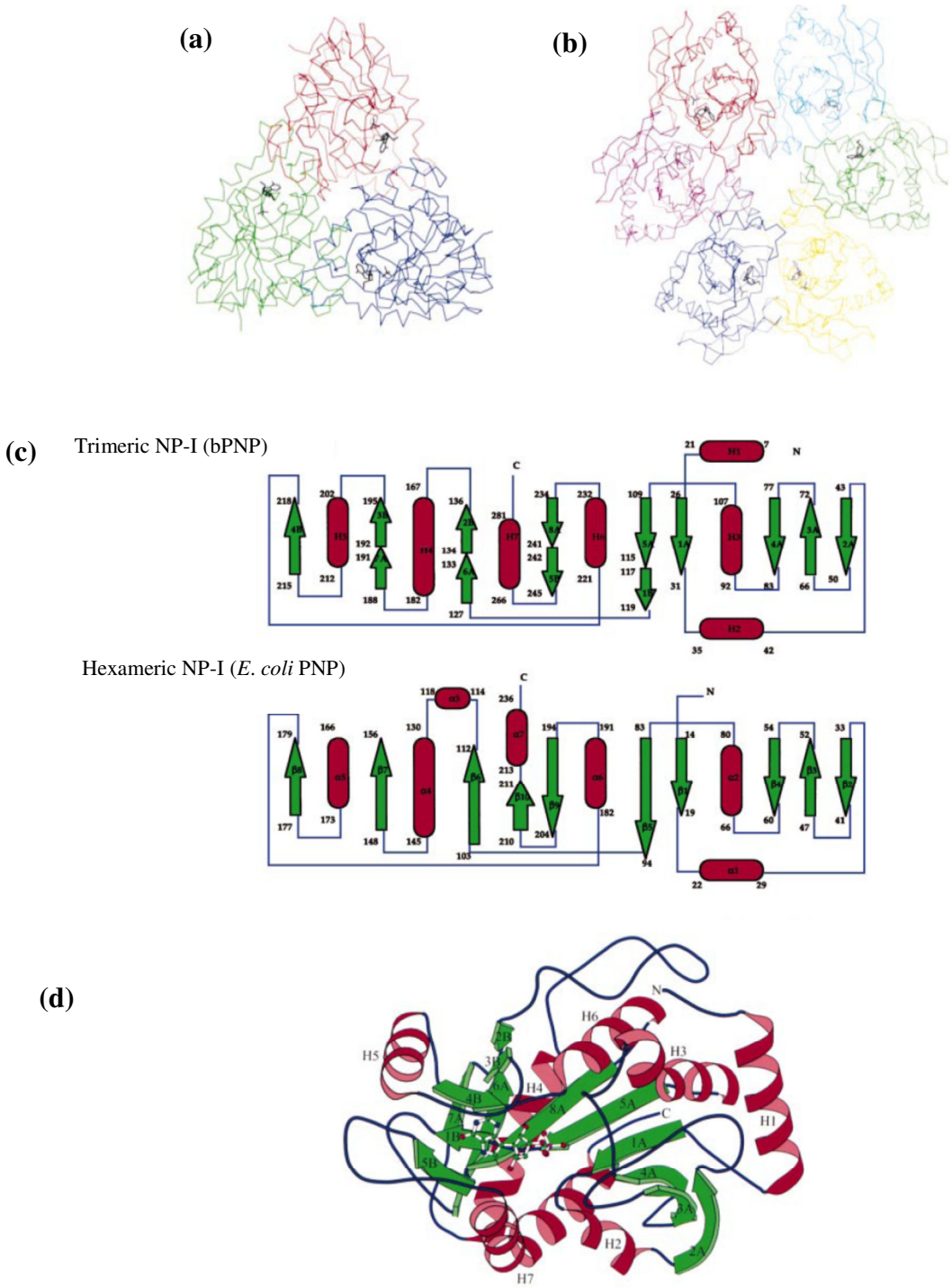


Fig. 1.1: Structure and topology of NP-I enzymes. (a) The trimeric structure of bovine PNP (bPNP). (b) Hexameric structure of *E. coli* PNP. In both (a) and (b), the active-site locations are indicated by the substrate positions, which are drawn as stick models. (c) Topologies from the trimeric and hexameric subfamilies of NP-I. Trimeric topology of bPNP [α-helixes are indicated as red cylinders and labeled H1-H7; β-strands are shown as green arrows, where the strands that make up sheet A are labeled 1A-8A and those making up sheet B are labeled 1B-5B]. Hexameric topology of *E. coli* PNP [α-helixes are labeled α1-α7 (red), and β-strands are labeled β1-β10 (green)]. (d) Ribbon drawing of a subunit of bPNP in which the secondary structural elements have been labeled. The substrates are represented as ball-and-stick models to indicate the position of the active site (Pugmire and Ealick, 2002).

Based on structural and kinetic analyses of a human PNP (hPNP), a catalytic mechanism has been proposed for hPNP (Erion *et al*., 1997) that is suggested to be similar for all members of the NP-I family (Pugmire and Ealick, 2002). The proposed mechanism suggests that the PNP-substrate complex is in a high energy conformation such that it produces steric strain, which encourages glycosidic cleavage. The glycosidic bond is weakened further as electrons flow from O-4' of the ribose to the purine ring. The phosphate ion binds on the α-side of the ribose ring, where it is positioned to participate in a nucleophilic attack at the C-1 position. The flow of electrons from the glycosidic bond to the purine ring is probably stabilized by active site residues (Pugmire and Ealick, 2002). A schematic diagram of the proposed catalytic mechanism is shown in Fig. 1.2.

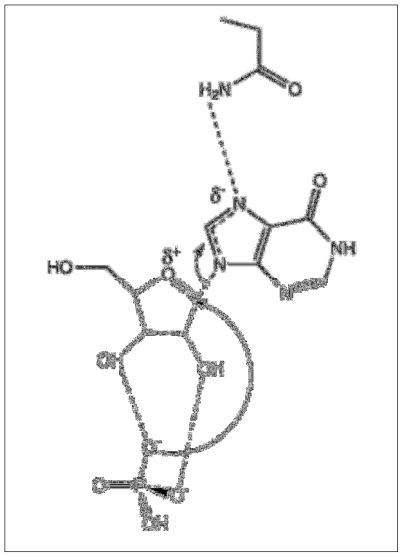


Fig. 1.2: The proposed catalytic mechanism for hPNP, representative of a likely mechanism for all NP-I family enzymes (Pugmire and Ealick, 2002).

1.5.2 The nucleoside phosphorylase-II (NP-II) family

Following the initial discovery that a distinct enzyme is responsible for the phosphorolysis of pyrimidines, subsequent studies have indicated the existence of two separate PyNPs, *i.e.* one specific for thymidine (TP; EC 2.4.2.4) and one specific for uridine (UP; EC 2.4.2.3) (Paege and Schlenk, 1952; Laster and Blair, 1963; Krenitsky *et al*., 1965). In contrast to UP that functions as a 165-kDa hexamer and is thus classified as a member of the NP-I family (see above), TP functions as a dimer, ranging in molecular mass from 80 to 110 kDa (Desgranges *et al*., 1981; Avraham *et al*., 1990). A discriminating feature between UP and TP is the high specificity of TP for the 2'-deoxyribose moiety. Likewise, the TPs from rat liver (Yamada,

1968), horse liver (Friedkin and Roberts, 1954), *E. coli* (Razzell and Khorana, 1958) and *Lactobacillus casei* (Avraham *et al*., 1990) have all shown specificity for the 2'-deoxyribose moiety. Moreover, the 5'-position of the pyrimidine ring was less important in determining specificity than was the 2'-position of the ribose moiety. However, *B. stearothermophilus* (Hamamoto *et al*., 1996) and *Haemophilus influenza* (Scocca, 1971) have been reported to contain a single PyNP that does not discriminate at the 2'-position of the ribose. Consequently, enzymes that accept both thymidine and uridine as substrates are referred to as PyNPs.

Both PyNPs and TPs show a high degree of sequence identity (>30%) (Pugmire and Ealick, 2002) and detailed structural information on the NP-II family was provided by the crystal structures of *B. stearothermophilus* PyNP (Pugmire and Ealick, 1998) and *E. coli* TP (Walter *et al*., 1990). The three-dimensional structures revealed a S-shape homodimer in which each subunit of the dimer contains a large mixed α-helical and β-sheet domain (the α/β-domain) that is separated from a smaller α-helical domain (the α-domain) by a large cleft. Three loop regions connect the two domains (Fig. 1.3). The active site of each subunit consists of a pyrimidine-binding site in the α-domain and a phosphate-binding site across the cleft in the α/β-domain. The large distance between the phosphate- and pyrimidine-binding sites suggests that rigid-body domain movement, where the loop regions connecting the two domains act as hinges, may be required to close the cleft and enable catalysis (Pugmire *et al*., 1998; Pugmire and Ealick, 2002).

The catalytic mechanism for the NP-II family has not been studied to the same extent as for PNP. Nevertheless, kinetic studies with *E. coli* TP and rabbit muscle TP have shown that the enzyme follows a sequential mechanism whereby phosphate is the first substrate to bind and 2'-deoxyribose-1-phosphate is the last product to leave (Krenitsky and Tuttle, 1982). A catalytic mechanism has been proposed that begins with weakening of the glycosidic bond due to the high energy conformation of the bound pyrimidine nucleoside. The glycosidic bond is weakened further as electrons flow from O-4' of the ribose moiety to the pyrimidine ring, and are stabilized through interactions with positively charged lysine and arginine residues. The phosphate ion, which binds to the α-side of the ribose ring, is then positioned to attack the C-1 position of the ribose ring to yield 1-phosphate and the free pyrimidine base (Pugmire and Ealick, 2002). The proposed catalytic mechanism for the NP-II family is presented in Fig. 1.4.

Fig. 1.3: Ribbon drawing of the dimeric structure of *E. coli* TP in an open conformation. Crystallographically observed thymine and phosphate are shown as ball-and-stick models to indicate the positions of the binding sites in each of the subunits (Pugmire and Ealick, 2002).

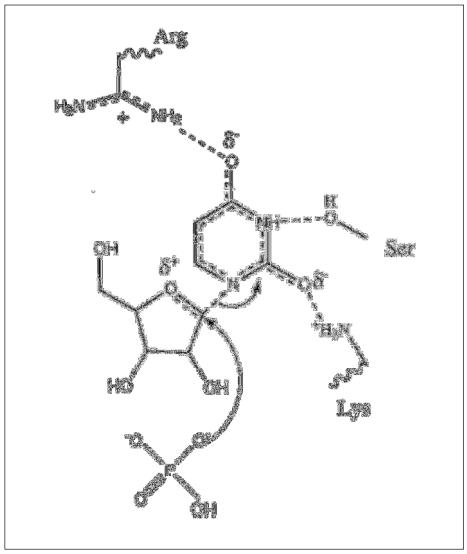


Fig. 1.4: The proposed catalytic mechanism for the NP-II family, analogues to that of the NP-I family (Pugmire and Ealick, 2002).

1.5.3 Industrial applications of nucleoside phosphorylases (NPases)

The NPases have been exploited as tools for the enzymatic synthesis of nucleosides and nucleoside analogues that are difficult to prepare or are obtained in low yields by chemical synthesis. With specific reference to the need for nucleosides, it has been suggested that the need for 2'-deoxyribonucleosides will grow in the near future due to increasing demand in the medical and biotechnological fields (Lewkowicz and Iribarren, 2006). This is due to 2'-deoxyribonucleosides being used as building blocks of antisense drugs for cancer therapy, as synthetic intermediates of antiviral agents and they are also precursors of the 2'-deoxyribonucleotides used for polymerase chain reaction (PCR) applications.

Since the development of the first antiviral agents over 40 years ago, nucleoside analogues have been used extensively in antiviral and anticancer therapies. As antiviral agents, nucleoside analogues block replication of the viral genome, whereas anticancer compounds inhibit cellular DNA replication and repair (Plunkett and Ghandi, 1996; Van Rompay *et al*., 2003). These molecules exert their action through different mechanisms, such as the inhibition of polymerase enzyme activity or by acting as chain terminators during RNA or DNA synthesis (Balzarini *et al*., 2001; Holy, 2001). Since the discovery of the anti-HIV activity of 3'-azido-3'-deoxythymidine (AZT; zidovudine) during the late 1980s (Ishii *et al*., 1989), many nucleoside analogues have been designed as antiviral drugs against targets including herpes simplex virus, hepatitis virus, HIV and cytomegalovirus (Tan *et al*., 1999; Tanm *et al*., 2004; Zoulim, 2004; Caroll and Olsen, 2006; Meerbach *et al*., 2006). Nucleosides such as 2',3'-dideoxyinosine (didanosine), 2',3'-dideoxycytidine (zalcitabine), 2'-deoxy-3'-thiacytidine (lamivudine), 2',3'-didehydro-3'-deoxythymidine (stavudine) and 6-cyclopropylamino-2',3'-didehydro-2',3'-dideoxyguanosine (abacavir), are all used in antiviral treatments (De Clercq, 2002a; De Clercq and Field, 2006). Representative examples of anticancer nucleoside analogues include 2-chloro-2'-deoxyadenosine (cladribine), 2-fluor-9-β-D-arabinofuranosyladenine (fludarabine) and 2',2'-difluorodeoxycytidine (gemcitabine) (De Clercq, 2002a; Galmarini *et al*., 2002; De Clercq, 2009). The structure of selected nucleoside analogues are depicted in Fig. 1.5. Since treatments relying on the use of nucleoside analogues may lead to the acquirement of resistance and side-effects such as non-specific cytotoxicity (Balint, 2001; Galmarini *et al*., 2008), new compounds possessing more potent and broad activities are continually being designed and developed (De Clercq, 2002b; De Clercq and Field, 2006).

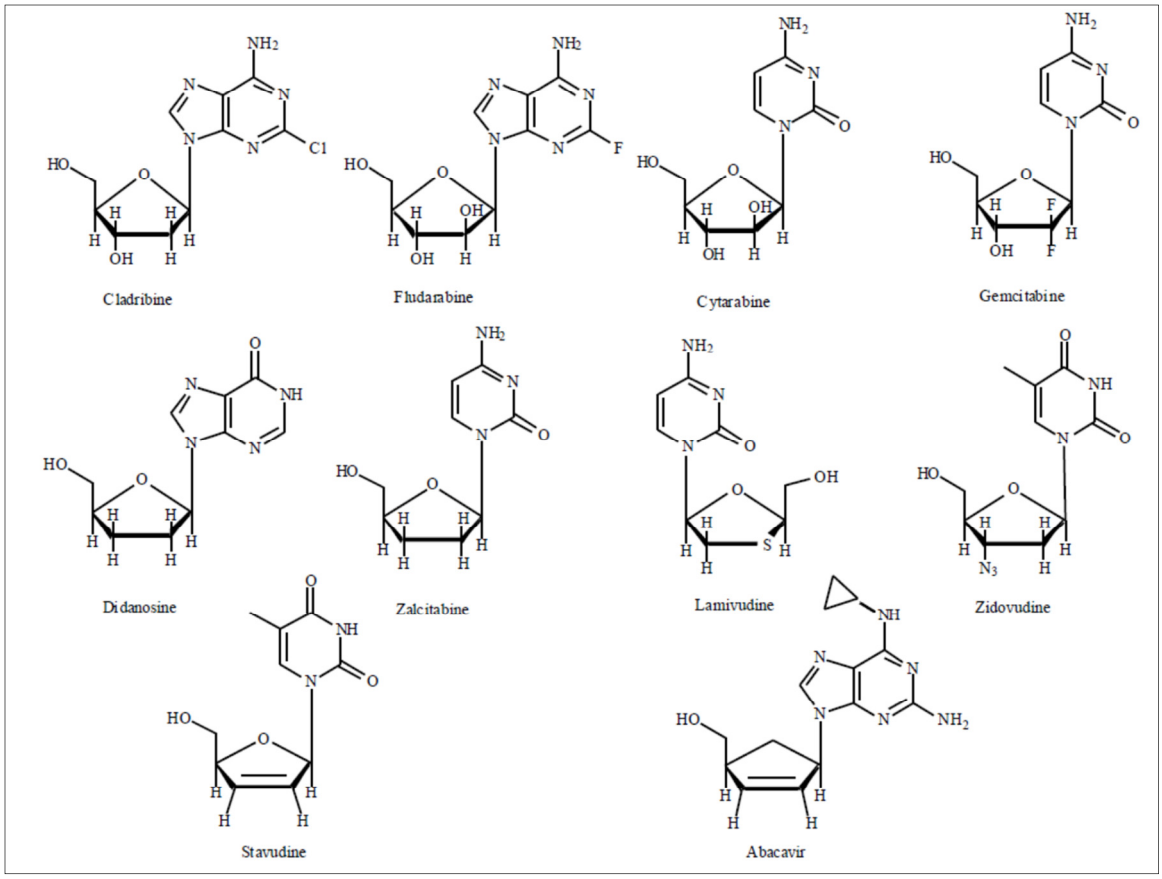


Fig. 1.5: Antiviral and anticancer nucleoside analogues (Lewkowicz and Iribarren, 2006).

Traditionally, nucleosides and nucleoside analogues are prepared by various chemical methods, which often involve difficult, inefficient and time-consuming multi-step processes, and give low yields and regio- and stereoisomers of the products (Ichikawa and Kato, 2001). In contrast, the enzymatic synthesis of both natural and unnatural nucleosides is not only cost-effective and simpler to perform, but highly regio- and stereo-controlled reactions can occur (Krenitsky *et al*., 1981; Lewkowicz and Iribarren, 2006). Due to their broader substrate specificity and greater thermal stability, the NPases from prokaryotic microorganisms rather than those from mammalian sources have been favoured in these enzymatic synthesis reactions (Mao *et al*., 1997). The reactions have been performed by making use of whole-cells or immobilized enzymes and, more recently, whole-cells of recombinant bacterial strains or purified overexpressed recombinant NPases have been used as biocatalysts (Okuyama *et al*., 1996; Hamamoto *et al*., 1996; Rocchietti *et al*., 2004).

Several review articles dealing with the biocatalytical synthesis of nucleosides and nucleoside analogues using NPases have been published previously (Hanrahan and Hutchinson, 1992; Prasad *et al*., 1999; Bzowska *et al*., 2000; Lewkowicz and Iribarren, 2006), and will thus not be discussed in this Literature Review. However, the general procedure for such enzymatic synthesis involves the use of a PyNP to release the pentose-1-phosphate from uridine or thymidine, followed by its coupling to the desired purine base by PNP. The use of a pyrimidine nucleoside as the donor of the pentose-1-phosphate is dictated in part by the expense of the latter, and is also useful for the synthesis of purine nucleosides with pentose rings other than ribose or deoxyribose, the pentose-1-phosphate of which are not readily accessible (Bzwoska *et al*., 2000). A generalized scheme for the synthesis of a purine nucleoside is indicated in Fig. 1.6.

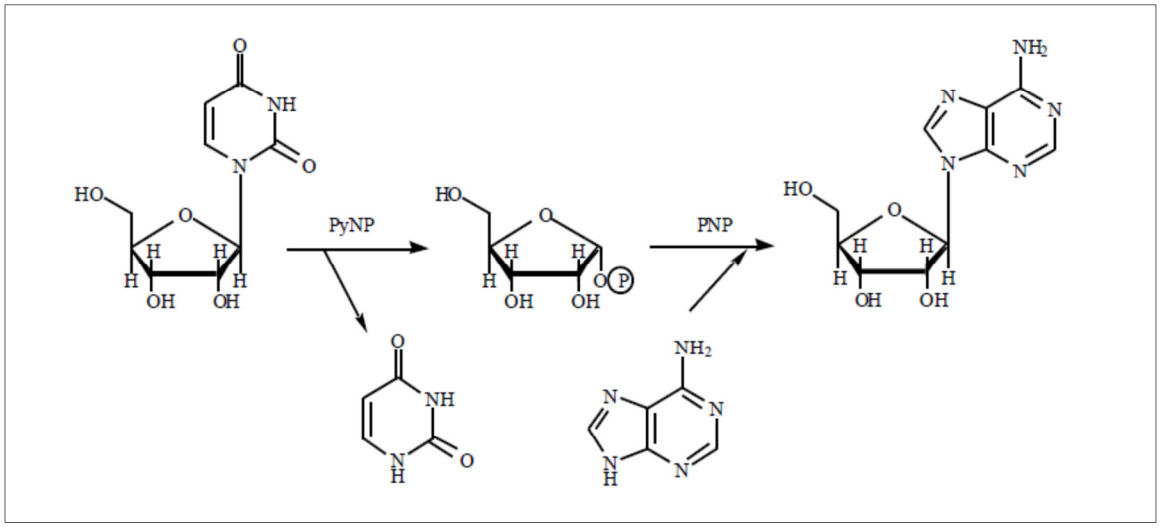


Fig. 1.6: Synthesis of purine nucleosides from pyrimidine substrates (Lewkowicz and Iribarren, 2006).

1.6 AIMS OF THIS STUDY

Nucleoside phosphorylases (NPases) catalyze the reversible phosphorolysis of purine and pyrimidine nucleotides, and are widely distributed in prokaryotes and eukaryotes. Not only do these enzymes play a key role in the nucleotide salvage pathway (Neuhard, 1983; Nygaard, 1983), but they have been exploited as tools for the enzymatic synthesis of nucleosides and therapeutic nucleoside analogues (Hanrahan and Hutchinson, 1992; Bzowska *et al*., 2000; Lewkowicz and Iribarren, 2006). Compared to chemical synthesis, the enzymatic synthesis of

these molecules has the advantages of reducing the cost of production, shortening synthetic routes and providing complete stereo-control of the synthesized products. Frequently, whole microbial cells are used as biocatalysts for nucleoside biosynthesis (Yokozeki *et al*., 1982; Trelles *et al*., 2004; Medici *et al*., 2006). However, this approach is limited by the low enzymatic activity of the wild-type strains, thus resulting in low yields of the products. Moreover, substrates such as guanosine and thymine are relatively insoluble and the most effective method of solubilizing them is in hot aqueous solutions. Therefore, it would be preferable to use thermostable enzymes to catalyze the synthesis of nucleosides and nucleoside analogues. Recently, a purine nucleoside phosphorylase (PNP) from the moderately thermophilic bacterium *B. halodurans* Alk36 was characterized in terms of its physical and kinetic properties (Visser *et al*., 2010). Based on its thermostability, it was proposed that the enzyme may potentially be used in nucleoside biosynthesis. The substrate specificity of the PNP was, however, not determined. It can be envisaged that overexpression of the thermostable PNP in a suitable expression host may provide a means to overcome the above-mentioned impediments.

Despite *E. coli* having been used extensively as a host for the expression of foreign genes (Baneyx, 1999; Samuelson, 2011), it may not necessarily be the expression host of choice for the expression of all heterologous proteins. Consequently, alternative expression hosts have been developed, including the Gram-positive bacterium *B. subtilis* (Harwood, 1992; Schumann and Ferreira, 2004). In order to produce homologous or heterologous proteins, a limited number of systems for inducible gene expression in *B. subtilis* have been developed. The most widely used induction systems in *B. subtilis* are mediated by the *spac* (Yansura and Henner, 1984) and *xylA* (Kim *et al*., 1996) promoters, since the expression can be readily regulated by varying the concentration of the inducer IPTG and xylose, respectively. However, the toxicity of IPTG (Kosinski *et al*., 1992) and the cost of IPTG and xylose are restrictive to the use of these promoter systems in especially industrial applications. In contrast, temperature-sensitive promoters provide a simple an inexpensive means of induction for target protein production (Li *et al*., 2007; Le and Schumann, 2007). Recently, Schofield *et al*. (2003) developed thermally regulated promoter systems for use in pathogenic Gram-positive bacterial species. The promoter systems showed high induction ratios in these bacterial species and thus suggest that they may have great potential in the overproduction of recombinant proteins. Despite having been evaluated in a number of clinically relevant Gram-positive bacterial species, the promoter systems were not evaluated in *B. subtilis*.

Based on the above, the aims of this study were:

- To evaluate two thermally regulated promoter systems, previously developed for use in pathogenic Gram-positive species, for use in *E. coli* and *B. subtilis*.

- To assess the utility of the thermally regulated promoter systems for the overexpression of a purine nucleoside phosphorylase in *B. subtilis*.

CHAPTER TWO

EVALUATION OF THERMALLY REGULATED PROMOTER SYSTEMS IN *Escherichia coli* AND *Bacillus subtilis*

2.1 INTRODUCTION

High-level production of recombinant proteins is often a prerequisite for their purification and characterization. Consequently, various different biological expression systems have been developed that greatly facilitate the production of large quantities of proteins in heterologous hosts (Werner *et al*., 1998; Porro *et al*., 2005; Jarvis, 2009; Zerbst *et al*., 2009). Among the many systems that are available for heterologous protein production, the Gram-negative bacterium *Escherichia coli* has remained one of the most attractive due to its ability to grow rapidly and at high density on inexpensive substrates, its well-characterized genetics and the availability of a large number of expression vectors and mutant host strains (Baneyx, 1999; Schumann and Ferreira, 2004; Samuelson, 2011). In addition to *E. coli*, many other bacteria have also been evaluated as expression hosts (de Vos *et al*., 1997; Terpe, 2006; Morello *et al*., 2008). Gram-positive bacteria, such as *Bacillus* spp., are increasingly regarded as attractive hosts for recombinant protein production (Tosato and Bruschi, 2004; Schumann, 2007; Pohl and Harwood, 2010). Indeed, *B. subtilis* is generally considered to have great potential for the production of proteins of clinical interest (Olmos-Soto and Contreras-Flores, 2003; Airaksinen *et al*., 2003; Kakeshita *et al*., 2011) and enzymes of industrial interest (Lam *et al*., 1998; Jan *et al*., 2001; Ho and Lim, 2003). Not only is *B. subtilis* capable of high-cell-density growth, but it is non-pathogenic and does not synthesize endotoxins. Furthermore, *B. subtilis* is able to secrete proteins directly into the growth medium, which facilitates isolation and purification of the proteins during downstream processing (Pohl and Harwood, 2010).

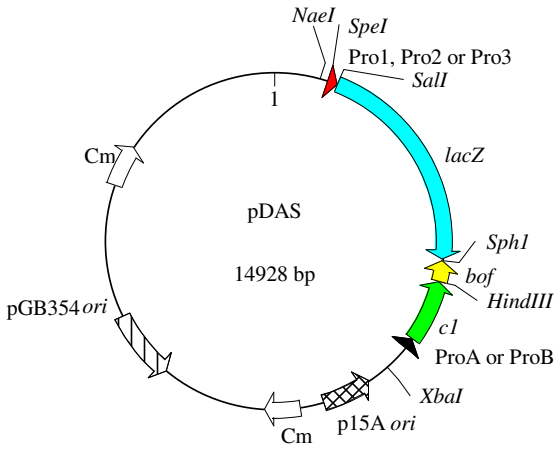
In order to produce homologous or heterologous proteins, several systems for inducible gene expression in both *E. coli* (Friesen and An, 1983; de Boer *et al*., 1983; Donovan *et al*., 1996) and *B. subtilis* (Kim *et al*., 1996; Bongers *et al*., 2005; Silbersack *et al*., 2006; Li *et al*., 2007) have been developed. However, the development of engineered systems regulating gene expression in *B. subtilis* is not as well advanced as it is for *E. coli* (Wong, 1995; Westers *et al*., 2004). This may be due to more stringent control of promoter usage in Gram-positive bacterial species than in Gram-negative bacterial species. Multiple conserved regions, in addition to the -35 and -10 hexamers, have been identified in promoters of Gram-positive bacterial species (Helmann, 1995; Voskuil and Chambliss, 1998). Consequently, well-characterized promoters from Gram-negative bacterial species, such as the *tac* and *trc* promoters, are inactive in Gram-positive hosts despite containing consensus -35 and -10 hexamers (Poyart and Trieu-Cuot, 1997). Notably, a bacteriophage P1-derived promoter, in

conjunction with the temperature-sensitive C1 repressor protein, has been used to regulate gene expression in Gram-negative bacterial species (Schofield *et al*., 2001). This thermally regulated promoter system was subsequently modified through the use of synthetic promoters to control gene expression in the pathogenic Gram-positive species *Enterococcus faecium*, *E. faecalis* and *Staphylococcus aureus* (Schofield *et al*., 2003). Since this thermally regulated expression system was used extensively throughout this study, a more detailed description is provided below.

Towards developing a thermally regulated promoter system for use in pathogenic Gram-positive bacterial species, Schofield *et al*. (2003) performed a compilation analysis of promoters from Gram-positive species and subsequently identified three promoters (Pro1, -2 and -3) with conserved elements. The conserved elements consisted of the -35 and the -10 hexamers, an adenine tract and thymine 5' of the -35 hexamer, a TG dinucleotide 5' of the -10 hexamer, and two adenine nucleotides 3' of the -10 hexamer. The promoters differed by a single nucleotide within the -10 hexamer (Pro1 versus Pro2) or by the addition of TG nucleotides (Pro2 versus Pro3). The promoters were furthermore designed to contain two partially overlapping C1 operator sites. To allow for the synthesis of differing amounts of C1 repressor protein, the *c1* gene was placed under the transcriptional control of two promoters (ProA and ProB), which have consensus -35 and -10 hexamers but differ in their spacer sequences (Fig. 2.1). To enhance binding of the C1 repressor to its operator, a *bof* gene was cloned 3'of the *c1* gene. The Bof protein does not bind to C1 alone, but binds to the C1 operators by forming a C1-Bof-operator DNA ternary complex. At the permissive temperature (30°C), C1 binds to its operator sites and prevents transcription, while at the non-permissive temperature (42°C), C1 is thermally unstable thereby allowing transcription to proceed (Schofield *et al*., 2003).

Results presented by Schofield *et al*. (2003) suggested that the thermally regulated promoter systems show great potential for the controlled expression of recombinant proteins. However, the promoter systems were not evaluated in frequently used expression hosts, including *B. subtilis.* For this system to be applied in the production of heterologous proteins, the promoter strength and regulation would therefore need to be assessed in different expression hosts. Reporter genes have been used frequently for this purpose (Pedraza-Reyes *et al*., 1994; Wang *et al*., 2004; Koga *et al*., 2006). Despite its popularity, each reporter gene has its own

(a)



(b)

Pro1	CTAGTAAAAAGCTGTTGACATATTGCTCTAATAAATTTATTAGAGCAAT GATCAT T TTTCGACAACTGTATAACGAGATTATTTAAATAATCTCGTTA
Pro2	CTAGTAAAAAGCTGTTGACATATTGCTCTAATAAATTTATTATAGCAAT GATCATTTTTCGACAACTGTATAACGAGATTATTTAAATAATATCGTTA
Pro3	CTAGTAAAAAGCTGTTGACATATTGCTCTAATAATGTTATTATAGCAAT GATCATTTTTCGACAACTGTATAACGAGATTATTACAATAATATCGTTA
ProA	GATCCAAAAAGCTGTTGACATAATCGAATTATTATGTTATAATAACATC
ProB	GATCCAAAAAGCTGTTGACATTGTGAGCGGATAACAATATAATAACATC

Fig. 2.1: Temperature-sensitive C1-regulated promoter systems evaluated during the course of this study. (a) Map of the pDAS plasmid and its relevant features. The *lacZ* reporter gene was placed under transcriptional control of a C1-regulated promoter (Pro1, -2, or -3). To control gene expression, and aid in the binding of the repressor to its operator site, the C1 repressor and Bof modulator were cloned 3' of the *lacZ* gene and placed under transcriptional control of either ProA or ProB. (b) Topography and sequence of the promoters. The synthetic promoters (Pro1, -2, and -3) consist of two partially overlapping C1 operators (top and bottom strands; underlined sequences). Pro2 differs from Pro1 by a single nucleotide in the -10 hexamer (G versus the consensus T). Pro 3 differs from Pro2 by two nucleotide changes in the spacer region (AT versus the consensus TG). ProA and ProB, which drive *c1* gene expression, differ in the nucleotide spacer sequence between the -35 and -10 hexamers (Schofield *et al*., 2003).

advantages and disadvantages that may limit its usefulness in specific host organisms and in specific types of studies (Naylor, 1999). Since no single reporter gene is universally applicable, it is therefore recommended to have a number of reporter genes available (Janatova *et al*., 2003; Perez-Arellano and Perez-Martinez, 2003). A widely used reporter gene is the *lacZ* gene encoding β-galactosidase (Talukder *et al*., 2005). Its popularity is derived from the availability of a large number of well-established methods for the qualitative and quantitative assay of β-galactosidase activity (Miller, 1972; Biran *et al*., 1999). Recently, a novel class C non-specific acid phosphatase secreted by *S. aureus* strain 154 was isolated and characterized (Du Plessis *et al*., 2002). This enzyme, designated SapS, and encoded by the *sapS* gene, has subsequently been developed and evaluated for the characterization of promoters and signal sequences in both Gram-negative (*E. coli*) and Gram-positive (*B. subtilis* and *B. halodurans*) bacterial hosts (Du Plessis *et al*., 2007). The acid phosphatase activity could be detected easily with a plate screen assay and quantitatively measured by a simple enzyme assay.

Based on the above, the aims of this part of the study were to evaluate the thermally regulated promoter systems developed by Schofield *et al*. (2003) in *E. coli* and *B. subtilis*. For this purpose, the promoter systems comprising of the Pro2 and Pro3 promoters were selected for use since they displayed higher induced expression levels compared to the Pro1 promoter system. The activity of these two prmoter systems in the respective expression hosts was assessed by making use of *lacZ* and *sapS* reporter genes.

2.2 MATERIALS AND METHODS

2.2.1 Bacterial strains, plasmids and culturing conditions

The bacterial strains and plasmids used in this part of the study are indicated in Table 2.1. *E. coli* and *B. subtilis* strains were cultured in Luria-Bertani broth (LB: 0.5% [w/v] yeast extract, 1% [w/v] tryptone, 1% [w/v] NaCl; pH 7) at 37°C with shaking at 175 rpm, and maintained at 4°C on LB agar (LB broth containing 1.2% [w/v] bacteriological agar) or at -70°C as glycerol cultures. For plasmid DNA selection and maintenance, the *E. coli* growth medium was supplemented with ampicillin (100 µg/ml) or chloramphenicol (20 µg/ml), and *B. subtilis* growth medium was supplemented with chloramphenicol (5 µg/ml). All antibiotics were purchased from Roche Diagnostics.

Table 2.1: Bacterial strains and plasmids used in this part of the study

Strains or Plasmids	Relevant properties	Source or Reference
Strains:		
E. coli DH10B	F⁻ *mrc*A Δ(*mrr-hsdRMS-mcrBC)* ΔlacX74 *end*A1 *rec*A1 *deo*R Δ(*ara-leu*)7697 *ara*D139 *gal*U *gal*K *nup*G *rps*L λ⁻	Invitrogen
E. coli JM110	*rps*L (Str^r) *thr leu thi-1 lacY galK galT ara tonA tsw dam⁻ dcm⁻ supE44 Δ(lac-proAB)*	Invitrogen
E. coli CU1867	BL21(DE3) strain with the chromosomal acid phosphatase *appA* gene disrupted	Du Plessis *et al.* (2007)
B. subtilis 1A46	*rec*E4 *thr*-5 *trp*C2	BGSC[a]
S. aureus strain 154	SapS acid phosphatase producer	Du Plessis *et al.* (2002)
Plasmids:		
pBluescript II SK (+)	*E. coli* cloning vector, ColE1 *ori*, Amp^r , LacZα-peptide	Stratagene
pNW33N	*E. coli-Bacillus-Geobacillus* shuttle vector, Chl^r, Amp^r *E. coli* pBR322 *ori*, *Bacillus* pBC1 *ori*	BGSC[a, b]
pDAS113	C1-regulated reporter vector, *lacZ* reporter gene cloned under transcriptional control of the Pro2 promoter, ProB promoter driving *c1* and *bof*, p15A *ori*, pGB354 *ori*, Chl^r	Schofield *et al.* (2003)
pDAS122	C1-regulated reporter vector, *lacZ* reporter gene cloned under transcriptional control of the Pro3 promoter, ProA promoter driving *c1* and *bof*, p15A *ori*, pGB354 *ori*, Chl^r	Schofield *et al.* (2003)
pNW13*lacZ*	pNW33N containing the transcription cassette of pDAS113	This study
pNW22*lacZ*	pNW33N containing the transcription cassette of pDAS122	This study
pSK*sapS*	pBluescript II SK (+) containing the acid phosphatase reporter gene	This study
pDS13*sapS*	pDAS113 containing the acid phosphatase reporter gene under transcriptional control of the Pro2 promoter	This study
pDS22*sapS*	pDAS122 containing the acid phosphatase reporter gene under transcriptional control of the Pro3 promoter	This study
pNW13*sapS*	pNW33N containing the transcription cassette of pDS13*sapS*	This study
pNW22*sapS*	pNW33N containing the transcription cassette of pDS22*sapS*	This study

[a] BGSC - *Bacillus* Genetic Stock Centre, Columbus, Ohio, USA

[b] GenBank accession no. AY237122

2.2.2 Genomic DNA extraction

Genomic DNA was isolated from *Staphylococcus aureus* strain 154, as described by Lovett and Keggins (1979) with the following modifications. The bacterial cells from 200 ml of an overnight culture were harvested by centrifugation at 7000 rpm for 15 min, rinsed once with TES buffer (30 mM Tris, 5 mM EDTA, 50 mM NaCl; pH 7.5) and suspended in 50 ml of the same buffer. Lysozyme was added to the cell suspension to a final concentration of 10 mg/ml, followed by incubation at 37°C for 25 min. The suspension was subsequently diluted with an equal volume of TES buffer, and Proteinase K was added to a final concentration of 100 µg/ml and Sarkosyl to a final concentration of 0.8% (w/v). Following incubation at 37°C for 30 min, the DNA sample was deproteinized by repeated phenol/chloroform (1:1) extractions. The genomic DNA was precipitated from the final aqueous phase by addition of two volumes of ice-cold 96% ethanol and incubation at -20°C overnight. The precipitated genomic DNA was spooled out on a hooked Pasteur pipette, air-dried and suspended in 5 ml of TE buffer (10 mM Tris, 1 mM EDTA; pH 8).

2.2.3 DNA amplification

2.2.3.1 Primers

Primers used for PCR amplification of the *S. aureus* strain 154 acid phosphatase gene (*sapS*) were designed based on the previously determined nucleotide sequence of the gene (Du Plessis *et al*., 2002; GenBank accession no. AY061973). The *sapS* forward primer was designed to anneal 22 bp upstream from the start codon (ATG) of the gene, thus excluding the promoter sequence. To facilitate cloning of the amplicon, unique restriction endonuclease recognition sequences were incorporated at the 5' terminus of the respective primers. The primers, indicated in Table 2.2, were obtained from Inqaba Biotechnical Industries.

Table 2.2: Primer set used for the amplification of the *S. aureus* strain 154 acid phosphatase gene

Primers[a]	Sequence	Relevant properties
sapS forward	5'-GGGTCGACCATGAGGTGATAAGATG-3'	*Sal*I site incorporated
sapS reverse	5'-GGGCATGCTTATTTAACTTCGCCTGT-3'	*Sph*I site incorporated

[a] Relevant restriction endonuclease sites are underlined

2.2.3.2 Polymerase chain reaction (PCR)

The PCR reaction mixture (100 µl) contained 15 ng of the genomic DNA as template, 1 × PCR buffer (75 mM Tris-HCl [pH 8.8], 16 mM $(NH_4)_2SO_2$, 0.1% [v/v] Tween-20), 2 mM $MgCl_2$, 200 µM of each deoxynucleotide triphosphate (dNTP), 0.5 µM of each of the forward and reverse primers and 1 U of Biotaq DNA polymerase (Bioline, Inc.). The PCR reaction mixture was placed in a Progene thermocycler (Techne). Following incubation at 94°C for 4 min, the sample was subjected to 35 cycles of denaturation at 94°C for 1 min, primer annealing at 56°C for 1 min and elongation at 72°C for 1.5 min. After the last cycle, a final extension step was performed at 72°C for 5 min to complete synthesis of all DNA strands. For control purposes, a reaction mixture containing all reagents except template DNA was included. The PCR reaction mixture was analyzed by electrophoresis on an 8% (w/v) agarose gel in the presence of an appropriate DNA molecular weight marker.

2.2.4 Agarose gel electrophoresis

DNA was analyzed on horizontal 0.8% (w/v) agarose slab gels supplemented with ethidium bromide (0.5 µg/ml) in order to allow visualization of the DNA on a UV transilluminator (Sambrook and Russel, 2001). The agarose gels were electrophoresed at 90 V in 1 × TAE buffer (40 mM Tris-HCl, 20 mM NaOAc, 1 mM EDTA; pH 8.5). The DNA was sized according to their migration in the gel as compared to that of a standard DNA molecular weight marker (phage λ DNA digested with both *Eco*RI and *Hind*III, MassRuler™ High Range DNA Ladder [Fermentas] or Gene Direct 1-kb Ladder [Genedirex]).

2.2.5 Purification of DNA fragments from agarose gels

DNA fragments were purified from agarose gels with the Geneclean™ kit (Bio101, Inc.) according to the instructions of the manufacturer. The DNA fragment of interest was excised from the agarose gel with a scalpel blade and mixed with 3 volumes of a 6 M NaI solution. The agarose gel was dissolved by incubating the gel slice at 55°C for 3-5 min, after which 5 µl of glassmilk® was added to the sample. Following incubation on ice for 5 min, the silica-bound DNA was pelleted by centrifugation at 15 000 rpm for 30 s and washed three times with ice-cold NEW wash (50 mM NaCl, 10 mM Tris [pH 8], 50% [v/v] ethanol). The DNA was eluted from the silica at 55°C for 5 min in a final volume of 12 µl of TE buffer. An aliquot (1 µl) of the eluate was analyzed by electrophoresis on a 0.8% (w/v) agarose gel to assess both its purity and concentration.

2.2.6 Cloning of DNA fragments into plasmid vectors

2.2.6.1 Ligation of DNA fragments to vector DNA

For cloning of PCR amplicons, T-tailed plasmid pBluescript II SK (+) was prepared according to the procedures of Marchuk *et al*. (1990) and ligation reactions were performed with the FAST-Link DNA ligation kit (Epicentre). The gel-purified amplicon (20 ng) and T-tailed vector DNA (10 ng) were ligated at room temperature for 1 h in a reaction mixture that contained 1 × FAST-Link ligation buffer (33 mM Tris-OAc [pH 7.8], 66 mM KOAc, 10 mM $MgCl_2$, 0.5 mM DTT, 10 mM ATP) and 1 U of DNA ligase in a final reaction volume of 15 µl. For all other ligation reactions, the DNA fragments of interest and restricted vector DNA were ligated at 16°C overnight in a 30-µl reaction volume containing 1 × DNA ligase buffer (66 mM Tris-HCl [pH 7.5], 1 mM DTT, 5 mM $MgCl_2$, 1 mM ATP) and 1 U of T4 DNA ligase (1 U/µl; Fermentas). The ratio of insert to vector DNA was typically in excess of 2:1.

2.2.7 Transformation of *E. coli*

2.2.7.1 Preparation of electro-competent *E. coli* cells

Electro-competent *E. coli* cells were prepared according to the method of Armitage *et al.* (1998) with the following modifications. An aliquot (500 µl) of an overnight *E. coli* culture was inoculated into 100 ml of pre-warmed (37°C) sterile LB broth and the culture was incubated at 37°C until an OD_{600} of 0.6 was reached. The culture was subsequently incubated on ice for 30 min and the cells were then harvested by centrifugation at 5000 rpm for 15 min in a Sorvall RC-5B centrifuge. The cells were rinsed twice with 100 ml of ice-cold 10% (v/v) glycerol and suspended in 0.4 ml of ice-cold GYT medium (10.5% [v/v] glycerol, 0.125% [w/v] yeast extract, 0.245% [w/v] tryptone; pH 7.3). Aliquots (100 µl) of the cells were pipetted into 1.5-ml Eppendorf tubes and either used immediately or stored at -70°C until further use.

2.2.7.2 Electroporation

DNA was introduced into the electro-competent *E. coli* cells by electroporation according to the method described by Tung and Chow (1995). For this purpose, an aliquot of plasmid DNA (50 ng) or ligation reaction mixture (2 µl) was added to 100 µl of the competent cells, and then transferred to an ice-cold electroporation cuvette with a 0.1-cm electrode gap (BioRad). The cells were exposed to a single electrical pulse using a BioRad Gene-Pulser™

set at 1.8 kV, 25 µF and 100 Ω. Immediately following the electrical discharge, 1 ml of SOC recovery medium (0.5% [w/v] yeast extract, 2% [w/v] tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM $MgSO_4$, 20 mM glucose; pH 7) was added to the cells. The transformation mixtures were incubated at 37°C for 1 h with shaking and the transformed cells were selected by plating the cells onto LB agar containing the appropriate antibiotic. The agar plates were incubated at 37°C overnight. When appropriate, the cells were plated together with 10 µl of IPTG (100 mM stock solution) and 50 of µl X-gal (2% [w/v] stock solution) to allow for blue/white colour selection, based on insertional inactivation of the *lacZ'* marker gene in the pBluescript SK II (+) vector.

2.2.8 Transformation of *B. subtilis* 1A46

2.2.8.1 Preparation of protoplasts

Protoplasts were prepared according to the method of Chang and Cohen (1979). A single colony of *B. subtilis* 1A46 was inoculated into 60 ml of LB broth and the culture was incubated at 37°C overnight. The overnight culture was used to inoculate 60 ml of LB broth to an OD_{540} of 0.06, and the flask was incubated at 37°C until an OD_{540} of 0.3 was reached. The cells were then harvested by centrifugation at 6000 rpm for 10 min and the cell pellet suspended in 5 ml of SMMP medium. The SMMP medium was prepared by mixing 50 ml of 2 × SMM buffer (34% [w/v] sucrose, 0.46% [w/v] maleic acid, 3.98 mM $MgCl_2$; pH 6.5) with an equal volume of 4 × PAB (0.3% [w/v] beef extract, 0.3% [w/v] yeast extract, 1% [w/v] peptone, 0.2% [w/v] glucose, 0.7% [w/v] NaCl, 4.19 mM K_2HPO_4, 1.98 mM KH_2PO_4; pH 7). To obtain protoplasts, lysozyme was added to a final concentration of 2 mg/ml and the suspension was incubated at 37°C for 45 min with gentle shaking (40 rpm). Following incubation, the protoplasts were harvested by centrifugation at 4000 rpm for 20 min, washed once with 5 ml of SMMP medium and gently suspended in 4 ml of the SMMP medium.

2.2.8.2 Transformation

The prepared protoplasts were transformed utilizing a polyethylene glycol (PEG) method, as described by Chang and Cohen (1979). Prior to transformation, an equal volume of 2 × SMM buffer was added to the plasmid DNA preparations and then mixed with 500 µl of the prepared protoplast suspension. Following addition of 1.5 ml of 30% (w/v) PEG-4000 (prepared in 1 × SMM buffer), the suspensions were incubated at room temperature for 2 min. Subsequently, 5 ml of SMMP medium was added and the protoplasts were collected by

centrifugation at 4000 rpm for 20 min. The collected protoplasts were suspended in 1 ml of SMMP medium and incubated at 30°C for 90 min with gentle shaking (40 rpm). The transformed cells were selected by plating 200-µl aliquots of the suspension onto DM3 agar (13.5% [w/v] sodium succinate, 1% [w/v] casamino acids, 0.5% [w/v] yeast extract, 0.5% [w/v] glucose, 10 mM K_2HPO_4, 5.5 mM KH_2PO_4, 21.5 mM $MgCl_2$, 0.6% [w/v] agar), supplemented with 2% (w/v) filter-sterilized bovine serum albumin (BSA) and 5 µg/ml chloramphenicol. The agar plates were incubated at 37°C until colonies become visible.

2.2.9 Plasmid DNA extractions

2.2.9.1 Small-scale plasmid extractions

Plasmid DNA was extracted with the alkaline lysis method, as described by Sambrook and Russel (2001). Single colonies were each inoculated into 5 ml of LB broth supplemented with the appropriate antibiotic and incubated at 37°C overnight. The cells from 2 ml of the overnight cultures were harvested by centrifugation at 12 000 rpm for 10 min, and the cell pellets were suspended in 200 µl of ice-cold Solution 1 (50 mM glucose, 25 mM Tris-HCl [pH 7.5], 10 mM EDTA, 100 µg/ml RNase A). The cells were lysed by addition of 400 µl of Solution 2 (0.2 N NaOH, 1% [w/v] SDS) and after incubation on ice for 5 min, 300 µl of ice-cold Solution 3 (2.55 M KOAc; pH 4.8) was added. After incubation on ice for 10 min, the insoluble aggregate that formed was removed by centrifugation at 10 000 rpm for 10 min. The plasmid DNA was precipitated from the recovered supernatant by addition of 450 µl of isopropanol and collected by centrifugation at 10 000 rpm for 10 min. The plasmid DNA was rinsed once with 70% ethanol, air-dried and suspended in 20 µl of TE buffer.

2.2.9.2 Large-scale plasmid extractions

Large-scale plasmid DNA extractions were performed with the Qiagen Plasmid Midi kit, according to the manufacturer's instructions. The bacterial cells from 100 ml of an overnight culture were harvested by centrifugation at 15 000 rpm for 15 min, and the cell pellet was suspended in 10 ml of Resuspension Buffer P1 (50 mM Tris-Cl [pH 8], 10 mM EDTA, 100 µg/ml RNase A), followed by addition of 10 ml of Lysis Buffer P2 (200 mM NaOH, 1% [w/v] SDS). Following incubation at room temperature for 5 min, 20 ml of Neutralization Buffer P3 (3 mM KOAc, pH 5.5) was added and the suspension was incubated on ice for 30 min. The lysate was subsequently added to a DNA-binding column, washed twice with 30 ml of Buffer QC (1 M NaCl, 50 mM MOPS [pH 7], 15% [v/v] isopropanol) and the DNA was

eluted in 15 ml of Buffer QF (1.25 M NaCl, 50 mM Tris-HCl [pH 8.5], 15% [v/v] isopropanol). Plasmid DNA was precipitated by addition of 10.5 ml of isopropanol and recovered by centrifugation at 12 000 rpm for 10 min. The DNA pellet was rinsed with 70% ethanol, air-dried and suspended in 200 µl of TE buffer.

2.2.10 Restriction endonuclease digestions

Restriction endonuclease digestions were performed in Eppendorf tubes in a final volume of 15-20 µl. The reactions contained the appropriate concentration of salt (using the 10 × buffer supplied by the manufacturer) for the specific enzyme and 5 U of enzyme per µg of DNA. The reaction mixtures were incubated at 37°C for 1 h, except for DNA digested with *Sma*I, which was incubated at 25°C. When digestion entailed the use of two different restriction enzymes, the plasmid DNA was first digested with the enzyme requiring a lower salt concentration, after which the DNA was purified with the Geneclean™ kit (Bio 101, Inc.) and then digested with the second enzyme. All restriction endonucleases were supplied by Fermentas. The digestion products were analyzed by agarose gel electrophoresis in the presence of an appropriate DNA molecular marker.

2.2.11 Nucleotide sequencing and sequence analysis

The nucleotide sequence of PCR-amplified insert DNA was determined by automated sequencing with the ABI PRISM®BigDye™ Terminator v.3.1 Cycle Sequencing Ready Reaction kit (Applied Biosystems) according to the manufacturer's instructions. The reaction mixtures (10 µl) contained 250 ng of purified plasmid DNA, 2 µl of BigDye™ Termination Mix, 1 × sequencing buffer and 3.2 pmol of either the pUC/M13 forward (5'-GTTTCCCAGTCACGAC-3') or pUC/M13 reverse (5'-GTAAAACGACGGCCAGT-3') sequencing primer. Cycle sequencing reactions were performed in a Perkin-Elmer GeneAmp® 2700 thermal cycler with 25 of the following cycles: denaturation at 96°C for 10 s, primer annealing at 50°C for 15 s, and elongation at 60°C for 4 min. The extension products were precipitated by addition of 1 µl of 3 M NaOAc (pH 4.6) and 25 µl of absolute ethanol. The tubes were incubated for 15 min at room temperature in the dark, centrifuged at 15 000 rpm for 30 min and the supernatant carefully aspirated. The extension products were resolved on an ABI PRISM® Model 3130 automated sequencer (Applied Biosystems).

The nucleotide sequences obtained were analyzed with the BioEdit v.7.0.4.1 (Hall, 1999) software package. The nucleotide and deduced amino acid sequences were compared against the entries in the GenBank database with respectively the BLAST-N and BLAST-P programmes (Altschul *et al*., 1997), available on the National Centre for Biotechnology Information web page (http://www.ncbi.nlm.nih.gov/BLAST).

2.2.12 Plasmid constructs

All molecular cloning techniques used in the construction of the recombinant plasmids were performed according to the procedures described in the preceding sections. All plasmid constructs were confirmed by restriction endonuclease digestion. The cloning strategies are indicated diagrammatically in Figs. 2.2 and 2.3.

- **pNW13*lacZ***

Plasmid pDAS113 was digested with both *Nae*I and *Xba*I, and the transcription cassette was recovered and cloned into the *E. coli-Bacillus* shuttle vector pNW33N that had been digested with both *Sma*I and *Xba*I to generate pNW13*lacZ.*

- **pNW22*lacZ***

Plasmid pDAS122 was digested with both *Nae*I and *Xba*I, and the excised transcription cassette was subsequently cloned into the *Sma*I and *Xba*I sites of pNW33N to generate pNW22*lacZ.*

- **pSK*sapS***

Primers *sapS* forward and *sapS* reverse were used with genomic DNA of *S. aureus* strain 154 as template DNA to PCR amplify the promoterless *sapS* gene. The amplicon was cloned into T-tailed pBluescript II SK (+) vector DNA to yield pSK*sapS.*

- **pNW13*sapS***

Plasmid pDAS113 was digested with both *Sal*I and *Sph*I to excise the *lac*Z reporter gene. The promoterless *sapS* gene, which had been recovered from plasmid pSK*sapS* by digestion with both *Sal*I and *Sph*I, was cloned into the deletion site of pDAS113 to generate pDS13*sapS*. To construct plasmid pNW13*sapS,* the transcription cassette was recovered from pDS13*sapS* by digestion with both *Nae*I and *Xba*I and cloned into the *Sma*I and *Xba*I sites of pNW33N.

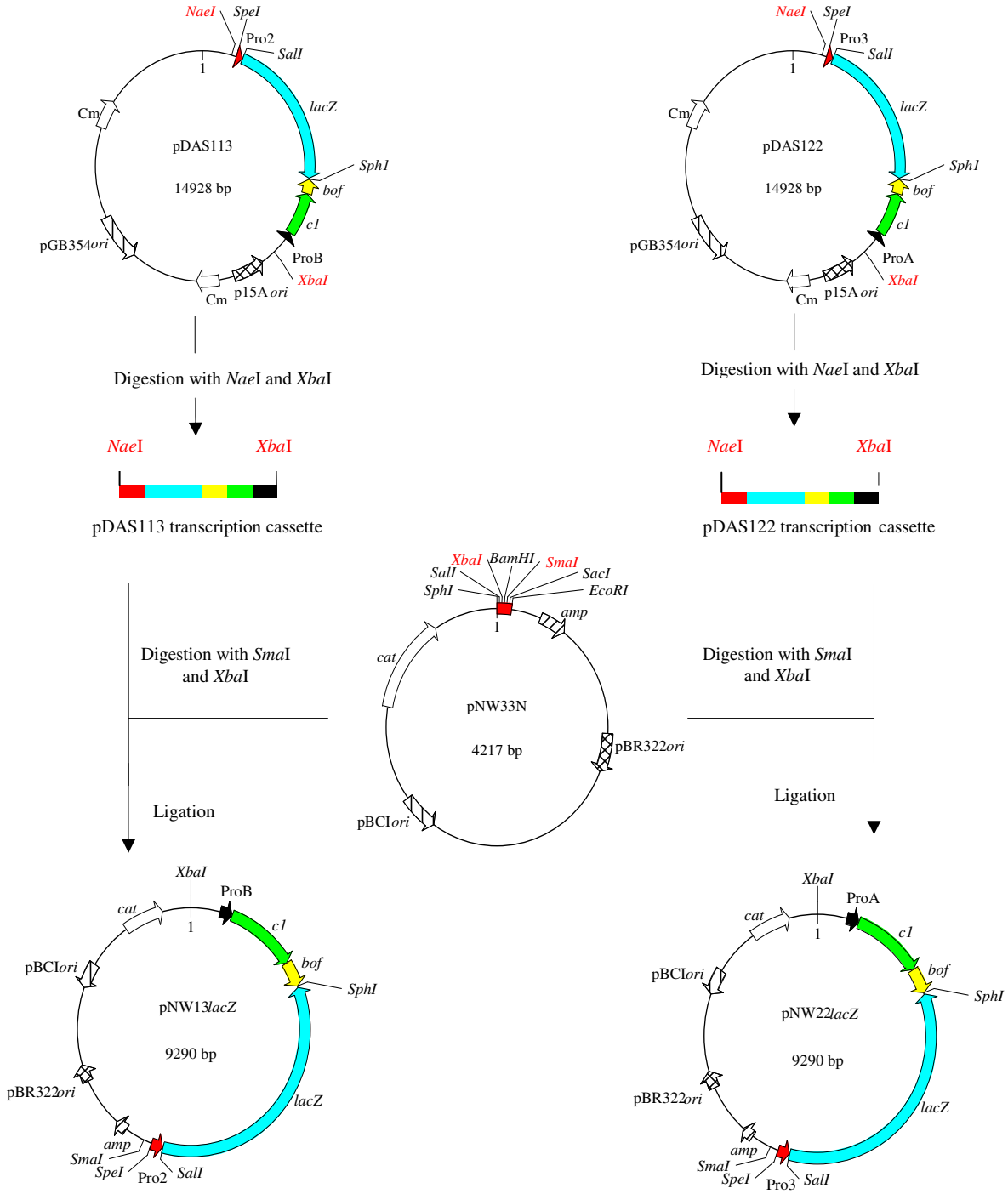


Fig. 2.2: Diagrammatic representation of the cloning strategies used to construct plasmids pNW13*lacZ* and pNW22*lacZ*.

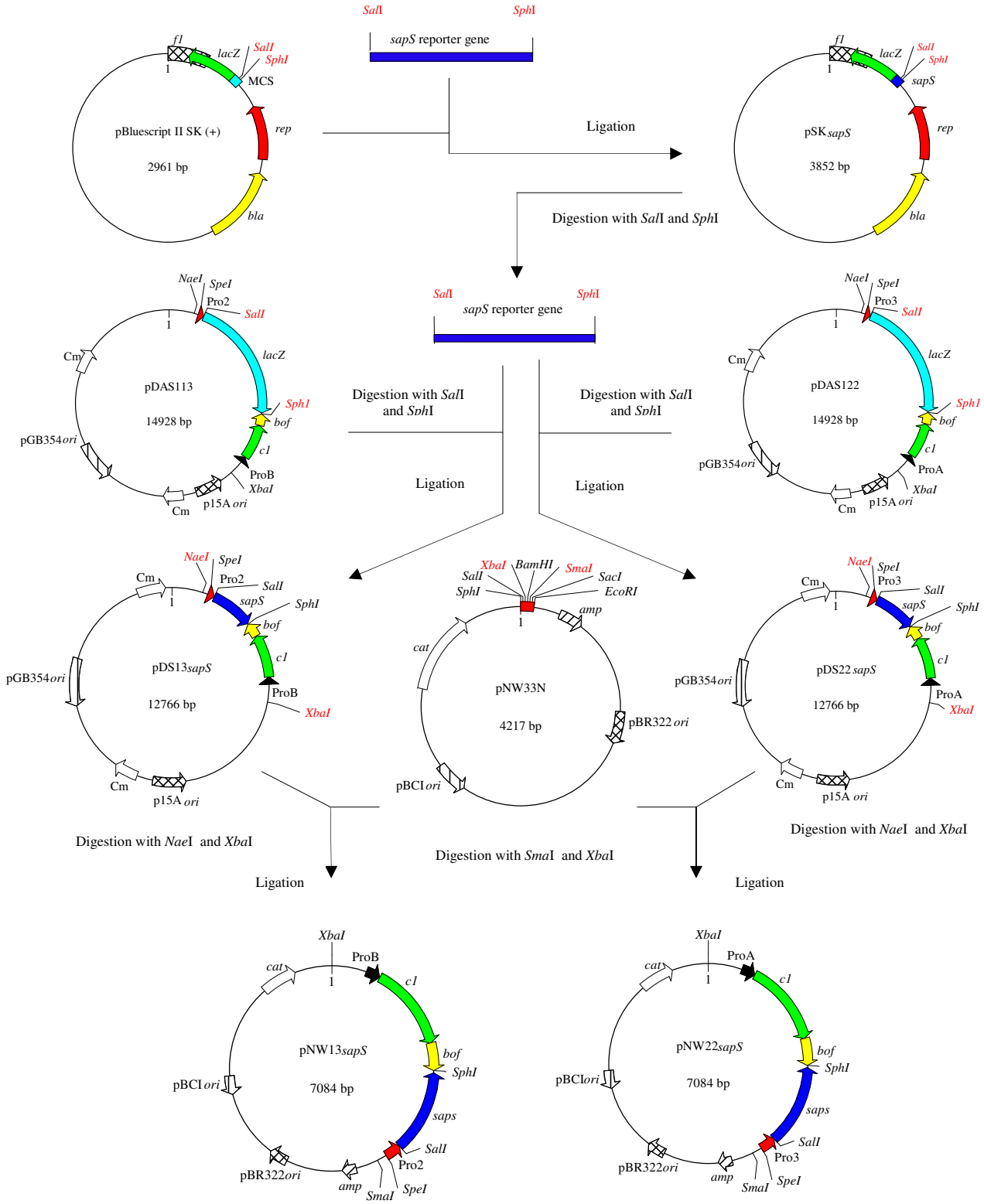


Fig. 2.3: Diagrammatic representation of the cloning strategies used to construct plasmids pNW13*sapS* and pNW22*sapS*.

- **pNW22*sapS***

Using a cloning strategy similar to that described above, the promoterless *sapS* gene was cloned into the *Sal*I and *Sph*I sites of plasmid pDAS122 to generate pDS22*sapS*. Subsequently, the transcription cassette was excised from pDS22*sapS* and the *Nae*I-*Xba*I restriction DNA fragment was cloned into the *Sma*I and *Xba*I sites of pNW33N to yield pNW22*sapS*.

2.2.13 β-galactosidase enzyme assays

2.2.13.1 Qualitative β-galactosidase plate screens

Transformed *E. coli* and *B. subtilis* strains, harbouring plasmids with promoter-*lacZ* transcriptional fusions, were streaked onto LB agar supplemented with the appropriate antibiotic and 40 µl of X-gal (20 mg/ml). The agar plates were incubated overnight at 30°C. To induce expression of the *lacZ* gene, the agar plates were incubated at 42°C for 3 h. β-galactosidase activity is evidenced by the streak cultures displaying a blue colour.

2.2.13.2 Quantitative β-galactosidase enzyme assay

- **Culturing conditions and preparation of crude enzyme extracts**

A single colony of the recombinant *E. coli* and *B. subtilis* strains was each inoculated into 50 ml of LB broth and cultured at 30°C overnight. Aliquots (4 ml) of the overnight cultures were inoculated into 420 ml of LB broth supplemented with the appropriate antibiotic. The cultures were incubated at 30°C until an OD_{600} of 0.1 was reached. Each of the cultures was then divided into two equal volumes (200 ml each) and one culture was incubated at 30°C, whereas the second culture was incubated at 42°C until an OD_{600} of 0.6 for the *E. coli* culture or 0.9 for the *B. subtilis* culture was reached. The bacterial cells from 10 ml of the recombinant *E. coli* and *B. subtilis* cultures were then harvested by centrifugation at 6000 rpm for 15 min and the cell pellets were each suspended in 5 ml of chilled Z-buffer (60 mM $Na_2HPO_4.7H_2O$, 40 mM $NaH_2PO_4.H_2O$, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol). The OD_{600} of the cell suspensions was determined spectrophotometrically, after being blanked against the same buffer. The bacterial cells were lysed by sonication on ice at 10-min intervals for 30 min with a Sonoplus Ultrasonic Homogenizer HD2070 (Bandelin Electronic). The cell lysates were subsequently used in enzyme assays, as described below.

- **β-galactosidase enzyme assay**

β-galactosidase activity was assayed using ONPG (Roche Diagnostics) as a chromogenic substrate according to the methods described by Miller (1972). Samples of the cell lysates (500 µl) were mixed with 500 µl of Z-buffer, after which 200 µl of the ONPG substrate (4 mg/ml in 0.1 M phosphate buffer) was added and mixed to initiate the color reaction. Following incubation at 28°C for 10 min, the reactions were terminated by addition of 500 µl of 1 M Na_2CO_3. The samples were centrifuged at 6000 rpm for 5 min to remove the cellular debris and hydrolysis of ONPG was quantified by measuring the A_{260} against a reagent blank with a WPA Model S2000 spectrophotometer (Labotec). Enzyme activity was calculated as the change in A_{420}/min/ml of cells/OD_{600}. All enzyme assays were performed in triplicate and the results are indicated as means ± standard deviation.

2.2.14 Acid phosphatase enzyme assays

2.2.14.1 Qualitative acid phosphatase plate screens

Transformed *E. coli* and *B. subtilis* strains, harbouring plasmids with promoter-*sapS* transcriptional fusions, were streaked onto LB agar supplemented with the appropriate antibiotic. The agar plates were incubated overnight at 30°C. To induce expression of the *sapS* gene, the agar plates were incubated at 42°C for 3 h. Acid phosphatase activity was detected by flooding the surface of the agar with 0.1 M NaOAc buffer (pH 5), containing 0.1% (w/v) α-naphthyl phosphate (Roche Diagnostics) and 0.2% (w/v) Fast Garnet GBC salt (Sigma-Aldrich) (Du Plessis *et al*., 2007). Acid phosphatase activity is evidenced by the streak cultures displaying a brown to black colour.

2.2.14.2 Quantitative acid phosphatase assay

- **Preparation of crude enzyme extracts**

Recombinant *E. coli* and *B. subtilis* strains were cultured, as described above (Section 2.2.13.2), after which the cells from 160 ml of the induced and non-induced cultures were harvested by centrifugation at 6000 rpm for 15 min. The cell pellets were each suspended in 5 ml of 0.1 M NaOAc buffer (pH 5) and sonicated on ice at 10-min intervals for 30 min with a Model HD 2070 Sonoplus Ultrasonic Homogenizer (Bandelin Electronic). The cell lysates were then clarified by centrifugation at 12 000 rpm for 15 min and the supernatants, considered the intracellular factions, were used in subsequent enzyme assays. The protein

concentration of the samples was determined according to the method of Bradford (1976), using the BioRad Protein Assay kit with bovine serum albumin (BSA) as standard.

- **Acid phosphatase enzyme assays**

Acid phosphatase activity in the extracts prepared from induced and non-induced cultures was quantified according to the method of Golovan *et al*. (2000). The assays were performed by incubating 200 µl of the enzyme preparation with 200 µl of *p*NPP substrate (Roche Diagnostics), at a final concentration of 25 mM, in 0.1 M NaOAc (pH 5). Following incubation at 37°C for 10 min, the reaction was terminated by addition of 1 ml of 1 M NaOH and the liberated *p*-nitrophenol (*p*NP) was measured at A_{405} against a reagent blank with a WPA Model S2000 spectrophotometer (Labotec). One unit of enzyme activity was defined as the amount of enzyme able to release 1 µmol of *p*NP per min under the assay conditions used. All enzyme assays were performed in triplicate, and the results are indicated as means ± standard deviation.

2.3 RESULTS

2.3.1 Construction of reporter vectors pNW13*lacZ* and pNW22*lacZ*

Towards evaluating the efficiency of the thermally regulated Pro2 and Pro3 promoter systems in both *E. coli* and *B. subtilis*, the promoter-*lacZ* transcriptional fusions were recovered from plasmids pDAS113 and pDAS122, respectively, and cloned into the *E. coli-Bacillus* shuttle vector pNW33N (De Rossi *et al*., 1991). This shuttle vector allows replication in *E. coli* from the pBR322 origin and in *Bacillus* spp. from the pBC1 origin, and encodes chloramphenicol acetyltransferase (CAT) that is expressed in both Gram-negative and Gram-positive bacterial hosts.

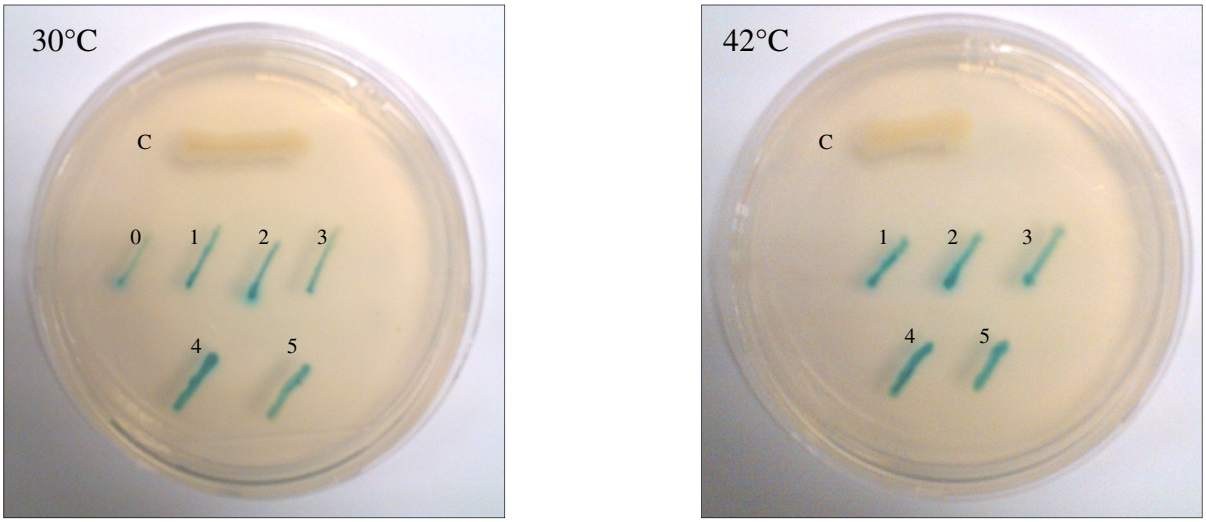
Since the *Nae*I restriction endonuclease used in the cloning strategy is sensitive to DNA methylation, the parental pDAS113 and pDAS122 vectors were first transformed into the methylation-deficient *E. coli* JM110 strain. The plasmid DNAs extracted from this bacterial host was subsequently digested with both *Nae*I and *Xba*I, and the 5.073-kb promoter-*lacZ* DNA fragments were cloned into the *Sma*I and *Xba*I sites of the pNW33N vector DNA. Following transformation of competent *E. coli* DH10B cells, randomly selected transformants were screened for β-galactosidase activity in order to identify transformants containing the

recombinant plasmid DNA. For this purpose, transformants were streaked onto selective medium (LB agar supplemented with chloramphenicol and X-gal) and the agar plates were first incubated at 30°C overnight and then at 42°C for 3 h. Following incubation, bacterial strains displaying a blue colour-phenotype were selected for further analysis (Fig. 2.4).

To confirm successful cloning of the promoter-*lac*Z transcriptional fusions, the plasmid DNA was extracted from putative recombinant transformants and characterized by restriction endonuclease digestions. Although both the *Nae*I and *Sma*I restriction endonucleases generate blunt ends so that the termini are compatible, the hybrid site, however, does not constitute a target site for either of the restriction endonucleases following ligation. Consequently, the plasmid DNA was first characterized by digestion with *Xba*I. Digestion of the recombinant plasmid DNA pNW13*lacZ* and pNW22*lacZ* with *Xba*I each yielded a single DNA fragment of *ca.* 9.3 kb, corresponding in size to the pNW33N vector DNA (4.217 kb) and the cloned insert DNA (5.073 kb) (Fig. 2.5, lanes 3 and 6). The successful cloning of the respective transcription cassettes was furthermore confirmed by digestion with both *Xba*I and *Spe*I, of which the recognition sequence of the latter is located downstream of the cloned insert DNA in the multiple cloning site (MCS) of the pNW33N vector DNA. Following agarose gel electrophoresis, the expected DNA fragments of *ca*. 4.2 and 5.0 kb, respectively, were observed in each case (Fig. 2.5, lanes 4 and 7).

Cumulatively, the above results confirmed that the thermally regulated Pro2 and Pro3 promoter systems, inclusive of the *lacZ* reporter gene, were cloned successfully into the *E. coli-Bacillus* shuttle vector pNW33N. The recombinant plasmid constructs were subsequently introduced into *B. subtilis* 1A46 by protoplast transformation. These strains, together with the recombinant *E. coli* DH10B strains, were used in subsequent assays.

(a)



(b)

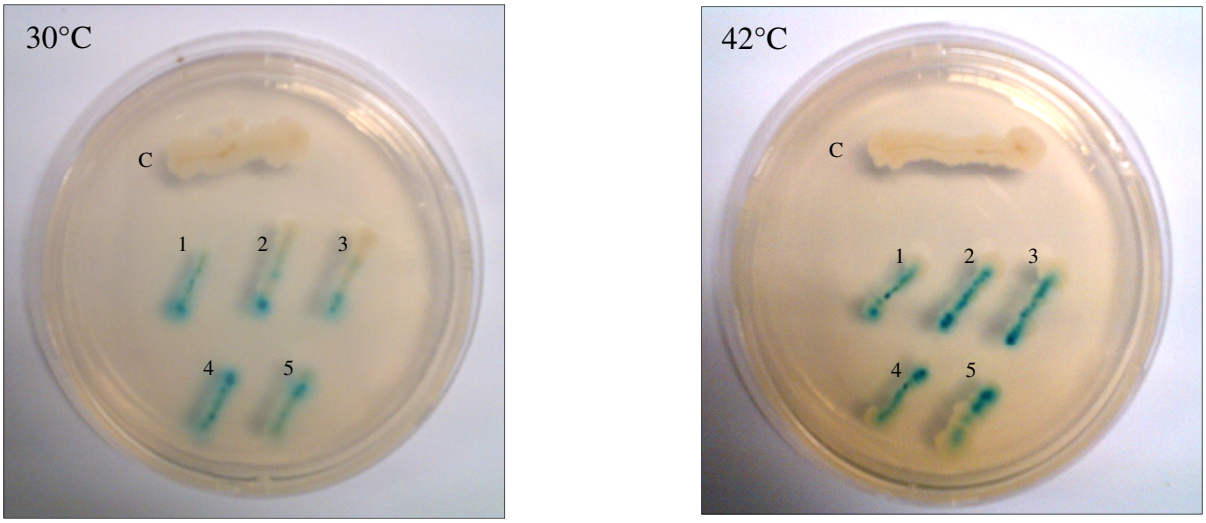


Fig. 2.4: Qualitative plate screen assays showing β-galactosidase enzyme activity. Randomly selected transformants were streaked onto selective LB agar medium and the agar plates were incubated overnight at 30°C and then at 42°C for 3 h to induce expression of the *lacZ* reporter gene. **Panel A:** Representative results are shown for *E. coli* DH10B harbouring the pNW13*lacZ* (4 and 5) or pNW22*lacZ* (0 through 3) constructs. As a control, *E. coli* DH10B transformed with the pNW33N vector was included (C). **Panel B:** Results obtained following transformation of *B. subtilis* 1A46 with pNW33N (C) and with the recombinant constructs pNW13*lacZ* (1 through 3) or pNW22*lacZ* (4 and 5) are shown for comparative purposes. The *E. coli* DH10B strain harbours a mutant *lacZ* gene, whereas the *B. subtilis* 1A46 strain lacks an endogenous *lacZ* gene. Due to their inability to produce a functional β-galactosidase enzyme, these bacterial strains are unable to hydrolyze the X-gal substrate and thus displayed a white phenotype. In contrast, the recombinant *E. coli* DH10B and *B. subtilis* 1A46 strains displayed a blue phenotype following incubation at 30°C and the phenotype was more intense after further incubation at 42°C. This therefore indicated that not only was the *lacZ* reporter gene expressed in both bacterial expression hosts, but also suggested that the Pro2 and Pro3 promoters are incompletely repressed at the non-permissive temperature of 30°C.

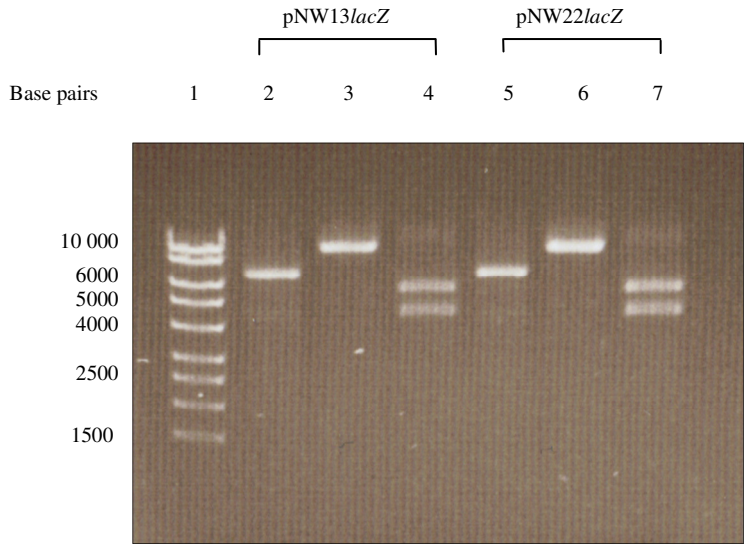


Fig. 2.5: Agarose gel electrophoretic analysis of the recombinant plasmids pNW13*lacZ* and pNW22*lacZ*. Lane 1, DNA molecular weight marker; lane 2, uncut pNW13*lacZ* plasmid DNA; lane 3, pNW13*lacZ* linearized by digestion with *Xba*I; lane 4, pNW13*lacZ* digested with both *Spe*I and *Xba*I; lane 5, uncut pNW22*lacZ* plasmid DNA; lane 6, pNW22*lacZ* linearized by digestion with *Xba*I; lane 7, pNW22*lacZ* digested with both *Spe*I and *Xba*I. The sizes of the DNA molecular weight marker, MassRuler™ High Range DNA Ladder (Fermentas), are indicated to the left of the figure.

2.3.2 Analysis of β-galactosidase activity in *E. coli* and *B. subtilis*

Since *E. coli* and *B. subtilis* are preferred hosts for heterologous protein production, it was therefore important to determine whether the thermally regulated Pro2 and Pro3 promoter systems are effectively repressed and induced in these expression hosts. Thus, to evaluate the functionality of the respective promoter systems, *E. coli* DH10B and *B. subtilis* 1A46 harbouring plasmids pNW13*lacZ* or pNW22*lacZ*, which contained Pro2-*lacZ* or Pro3-*lacZ* transcriptional fusions, respectively, were cultured in selective medium at 30°C and 42°C. The cells were subsequently harvested and the β-galactosidase activity was assessed using ONPG as substrate. The results of these assays are presented in Table 2.3.

Table 2.3: Basal and induced activities from *lacZ* fusions to C1-regulated promoters in *E. coli* DH10B and *B. subtilis* 1A46

Strain and plasmids	Promoter driving:		Avg. activity in Miller units (±SD)		Fold induction
	lacZ	*c1*	Basal (30°C)	Induced (42°C)	
E. coli					
pNW13*lacZ*	Pro2	ProB	230.14 (11.38)	542.67 (27.03)	2.4
pNW22*lacZ*	Pro3	ProA	4.12 (0.19)	570.07 (8.79)	138.4
B. subtilis					
pNW13*lacZ*	Pro2	ProB	53.62 (10.33)	640.87 (81.55)	12.0
pNW22*lacZ*	Pro3	ProA	7.48 (1.37)	61.68 (2.39)	8.2

In *E. coli*, quantitative β-galactosidase measurements indicated that the β-galactosidase activity driven from the Pro2 and Pro3 promoters at 42°C was respectively 2.4-fold and 138-fold greater than that at 30°C. This difference in the induction factors can be ascribed to the differences observed in the basal β-galactosidase levels. Although both the C1-regulated Pro2 and Pro3 promoters were capable of driving similar high levels of β-galactosidase activity at 42°C (543 and 570 Miller units, respectively), the β-galactosidase activity driven from the Pro2 promoter under non-inducing conditions (30°C) was *ca.* 56-fold greater than the β-galactosidase activity obtained with the Pro3 promoter (230 and 4 Miller units, respectively).

In *B. subtilis*, quantitative β-galactosidase measurements established that the Pro2 promoter displayed a 12-fold increase in activity at 42°C compared to the activity at 30°C, whereas the Pro3 promoter could be induced 8-fold under the same test conditions. Although the Pro2

promoter was capable of driving high levels of β-galactosidase activity (641 Miller units), a high level of basal β-galactosidase activity (54 Miller units) was also observed under non-inducing conditions. In contrast, comparatively lower levels of β-galactosidase activity were observed at 30°C and 42°C when the β-galactosidase activity was driven by the Pro3 promoter (7.5 and 62 Miller units, respectively).

Overall, the results suggest that the Pro2 and Pro3 promoters can be thermally regulated in *E. coli* and *B. subtilis*, albeit to differing extents. Interestingly, at the non-permissive temperature of 30°C, β-galactosidase activity from the Pro2 promoter was notably higher than that of the Pro3 promoter, suggesting that leakage of the Pro2 promoter appears to be due to ProB-dependent expression of C1.

2.3.3 Construction of reporter vectors pNW13*sapS* and pNW22*sapS*

The thermally regulated Pro2 and Pro3 promoter systems were also evaluated by making use of a different reporter gene, *i.e.* the *sapS* gene originating from *S. aureus* 154 that encodes a class C non-specific acid phosphatase (Du Plessis *et al*., 2002). The *sapS* gene was obtained by PCR amplification using genomic DNA of *S. aureus* 154 as template, together with primers *sapS* forward (containing a *Sal*I site) and *sapS* reverse (containing a *Sph*I site). The primers were designed to anneal downstream of the native promoter and at the 3'-end of the *sapS* gene, respectively. An aliquot of the reaction mixture was analyzed by agarose gel electrophoresis and a single amplicon of the expected size (905 bp) was observed. In contrast, no amplification products were observed in the negative control reaction in which template DNA was omitted (results not shown).

The amplicon was purified from the agarose gel and ligated into T-tailed pBluescript SK II (+) vector DNA. Following transformation of competent *E. coli* DH10B cells, recombinant transformants with a Lac-negative phenotype were selected from X-gal-containing indicator plates and cultured in LB broth supplemented with ampicillin. The extracted plasmid DNA was analyzed for the presence of cloned insert DNA by using restriction endonucleases of which the recognition sequences had been incorporated during the design of primers. The putative recombinant plasmid DNA was therefore digested with both *Sal*I and *Sph*I. Following agarose gel electrophoresis, DNA fragments of *ca.* 3.0 kb and 900 bp were observed that are in agreement with the expected size of the vector DNA (2.961 kb) and the

insert DNA (905 bp), respectively (Fig. 2.6, lane 2). A recombinant clone, designated pSK*sapS*, was selected and the integrity of the cloned insert DNA was verified by nucleotide sequencing prior to it being used in subsequent DNA cloning steps. The complete nucleotide sequence and deduced amino acid sequence of the cloned *sapS* gene is provided in the Appendix to this dissertation.

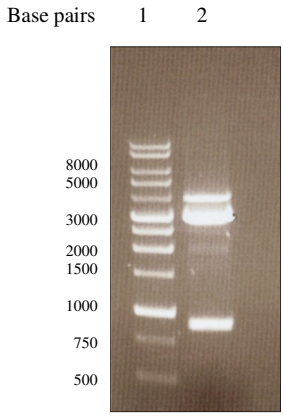


Fig. 2.6: Agarose gel electrophoretic analysis of the recombinant plasmid pSK*sapS*. Lane 1, DNA molecular weight marker; lane 2, recombinant plasmid pSK*sapS* digested with both *Sal*I and *Sph*I. The sizes of the DNA molecular weight marker, Gene Direct 1-kb Ladder (Genedirex), are indicated to the left of the figure.

Towards construction of the desired *sapS* reporter vectors, the *lacZ* reporter gene present in the pDAS113 and pDAS122 vector DNA was excised by digestion with both *Sal*I and *Sph*I, and the *sapS* gene was cloned as a *Sal*I-*Sph*I DNA fragment into the respective vectors. Following transformation of competent *E. coli* JM110 cells, a number of transformants were selected randomly and the extracted plasmid DNA was characterized by restriction endonuclease digestion. Recombinant plasmids from which the 905-bp *sapS*-specific insert was excised were selected for further use and designated pDS13*sapS* and pDS22*sapS*, respectively. To complete construction of the reporter vectors pNW13*sapS* and pNW22*sapS*, the respective promoter-*sapS* transcriptional fusions were recovered from the aforementioned plasmid DNAs by digestion with both *Nae*I and *Xba*I, and cloned into the *E. coli-Bacillus* shuttle vector pNW33N that had been prepared by digestion with *Sma*I and *Xba*I. Following transformation of competent *E. coli* CU187 cells, transformants were screened for acid phosphatase activity by plate screen assays. For this purpose, the bacterial strains were streaked onto LB agar supplemented with chloramphenicol and the agar plates were incubated at 30°C and then for 3 h at 42°C. Following incubation, the agar surface was flooded with

NaOAc containing α-naphtyl phosphate. In contrast to the acid phosphatase-deficient *E. coli* CU187 host strain, which showed no acid phosphatase activity after staining, recombinant strains containing the recombinant vector constructs stained dark brown and is indicative of acid phosphatase activity (Fig. 2.7).

To verify the presence of the respective promoter-*sapS* transcriptional fusions, the recombinant pNW13*sapS* and pNW22*sapS* plasmid DNA was characterized by agarose gel electrophoresis following restriction endonuclease digestion. Digestion of the recombinant plasmid DNA with *Xba*I each yielded a single DNA fragment of 7.084 kb, whereas digestion with both *Xba*I and *Spe*I yielded two DNA fragments of which the sizes are in agreement with those of the pNW33N vector DNA (4.217 kb) and cloned insert DNA (2.867 kb) (Fig. 2.8).

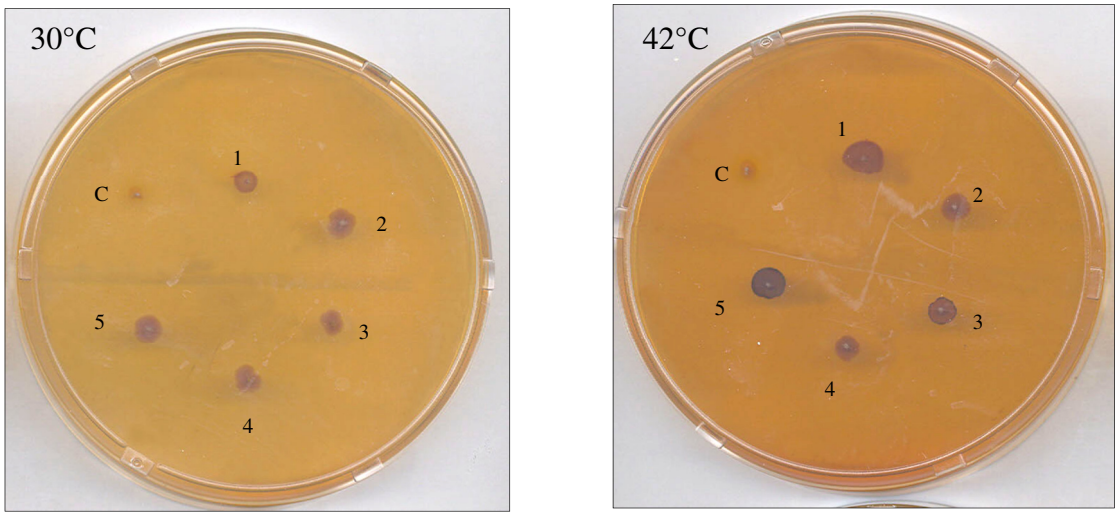
Having confirmed that the thermally regulated Pro2 and Pro3 promoter systems, inclusive of the *sapS* reporter gene, were cloned successfully into the *E. coli-Bacillus* shuttle vector pNW33N, the recombinant plasmid constructs were subsequently introduced into *B. subtilis* 1A46 by protoplast transformation. The derived strains were used together with the recombinant *E. coli* CU187 strains in subsequent assays.

2.3.4 Analysis of acid phosphatase activity in *E. coli and B. subtilis*

To furthermore characterize the functionality of the thermally regulated Pro2 and Pro3 promoters, the acid phosphatase activity of *E. coli* CU1867 and *B. subtilis* 1A46 strains harbouring plasmids pNW13*sapS* or pNW22*sapS*, which contained Pro2-*sapS* and Pro3-*sapS* transcriptional fusions, respectively, was determined. The acid phosphatase activity was determined quantitatively with *in vitro* enzyme assays, as described under Materials and Methods (Section 2.2.14.2).

Quantitative acid phosphatase measurements indicated that in *E. coli* the enzyme activity driven from the Pro2 and Pro3 promoters at 42°C was respectively 2-fold and 8-fold greater than that at 30°C (Table 2.4). This difference in the induction factors is most likely due to leakage of the Pro2 promoter, since the basal acid phosphatase activity levels were greater for the Pro2 promoter compared to that driven by the Pro3 promoter (48 and 16 mU/mg, respectively). In contrast to *E. coli*, much lower levels of enzyme activity were detected under non-inducing and inducing conditions for *B. subtilis*. Nevertheless, the results indicated

(a)



(b)

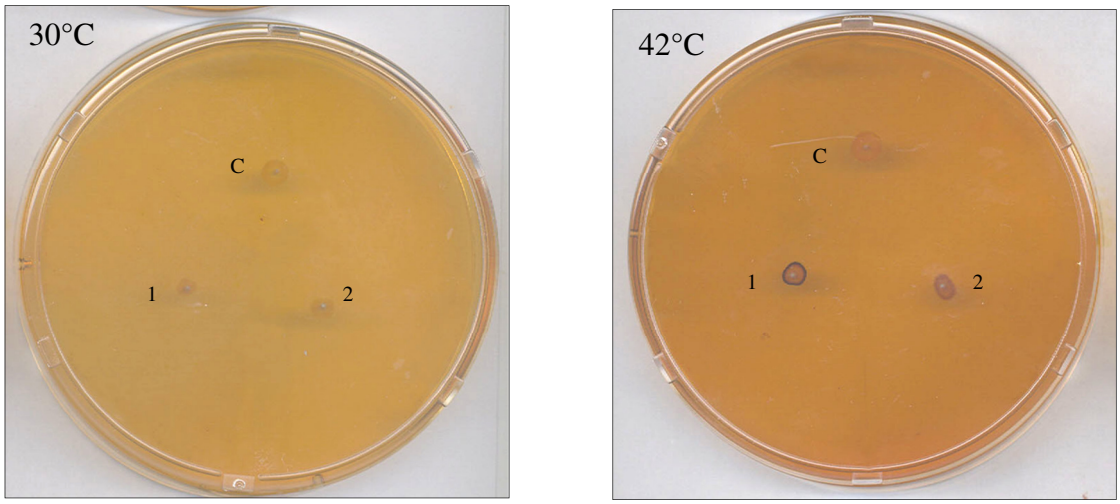


Fig. 2.7: Qualitative plate screen assays showing acid phosphatase enzyme activity. Randomly selected transformants were streaked in duplicate onto LB agar medium. The respective agar plates were stained for acid phosphatase activity after incubation overnight at 30°C and following incubation for a further 3 h at 42°C to induce expression of the *sapS* reporter gene**. Panel A:** Representative results are shown for *E. coli* CU1867 cells harbouring the pNW13*sapS* (1 and 2) or pNW22*sapS* (3 through 5) constructs. As a control, *E. coli* CU1867 harbouring the pNW33N vector was included (C). **Panel B:** Results obtained following transformation of *B. subtilis* 1A46 with pNW33N (C) and with pNW13*sapS* (1) or pNW22*sapS* (2) are shown for comparative purposes. In contrast to the bacterial expression hosts, which lack acid phosphatase activity, the recombinant *E. coli* CU1867 and *B. subtilis* 1A46 strains stained light brown following incubation at 30°C and dark brown following incubation at 42°C. These results therefore indicated that the *sapS* reporter gene was expressed in both bacterial expression hosts, albeit to an apparently lower level in *B. subtilis* 1A46.

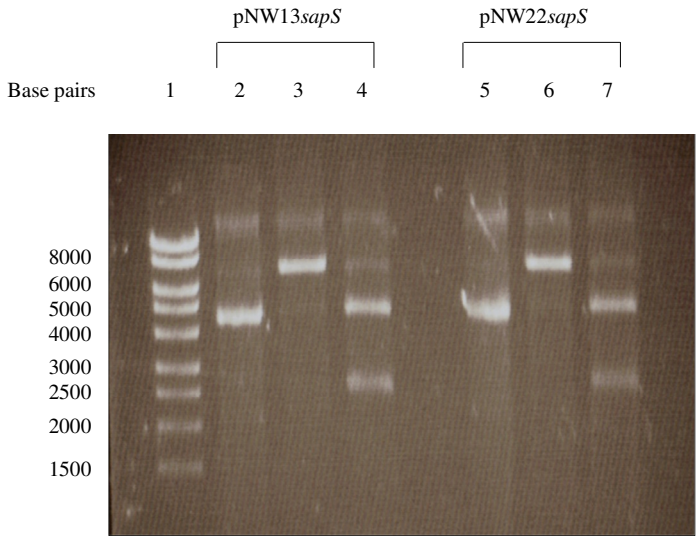


Fig. 2.8: Agarose gel electrophoretic analysis of the recombinant plasmids pNW13*sapS* and pNW22*sapS*. Lane 1, DNA molecular weight marker; lane 2, uncut pNW13*sapS* plasmid DNA; lane 3, pNW13*sapS* linearized by digestion with *Xba*I; lane 4, pNW13*sapS* digested with both *Spe*I and *Xba*I; lane 5, uncut pNW22*sapS* plasmid DNA; lane 6, pNW22*sapS* linearized by digestion with *Xba*I; Lane 7, pNW22*sapS* digested with both *Spe*I and *Xba*I. The sizes of the DNA molecular weight marker, MassRuler™ High Range DNA Ladder (Fermentas), are indicated to the left of the figure.

that both the Pro2 and Pro3 promoters displayed a similar increase in activity at 42°C compared to the activity at 30°C (3.7-fold and 2.7-fold increase, respectively) (Table 2.4). Although the Pro2 promoter was capable of driving a higher level of acid phosphatase activity at 42°C compared to the Pro3 promoter (41 and 9 mU/mg, respectively), the acid phosphatase activity under non-inducing conditions was *ca.* 3.5-fold greater than the activity obtained with the Pro3 promoter (11 and 3 mU/mg, respectively). Cumulatively, these results confirmed those obtained earlier by making use of the *lacZ* reporter gene.

Table 2.4: Basal and induced activities from *sapS* fusions to C1-regulated promoters in *E. coli* CU1867 and *B. subtilis* 1A46

Strain and plasmids	Promoter driving:		Avg. activity in mU/mg (±SD)		Fold induction
	sapS	***c1***	**Basal (30°C)**	**Induced (42°C)**	
E. coli					
pNW13*sapS*	Pro2	ProB	47.91 (3.51)	96.23 (8.87)	2
pNW22*sapS*	Pro3	ProA	16.22 (2.56)	129.04 (58.98)	7.9
B. subtilis					
pNW13*sapS*	Pro2	ProB	11.09 (0.65)	41.33 (5.89)	3.7
pNW22*sapS*	Pro3	ProA	3.20 (0.61)	8.67 (0.40)	2.7

2.4 DISCUSSION

Heterologous gene expression in bacterial hosts such as *E. coli* and *B. subtilis* are typically performed in two phases. In the first phase, the growth phase, the culture is grown until a sufficient cell density, whereas in the second phase the expression system is induced and the desired protein is overproduced. This therefore necessitates the use of tightly regulated promoters to allow for separation of the growth phase from the induction phase. Although numerous such inducible expression systems are available, only a limited number are used widely. These mostly comprise of IPTG-inducible systems in *E. coli* (Baneyx, 1999; Samuelson, 2011), and IPTG- and xylose-inducible ones in *B. subtilis* (Schumann, 2007). It is, however, of interest to search for alternative promoter systems that are efficient (low basal expression and high induction ratio), as well as easy and inexpensive to induce. Consequently, in this part of this study, two temperature-inducible promoter systems were evaluated for their ability to provide regulated protein expression in *E. coli* and *B. subtilis.*

The temperature-inducible promoter systems evaluated in this study had been developed previously for use in pathogenic Gram-positive bacterial species (Schofield *et al*., 2003). Thus, to enable evaluation of the respective promoter systems in both *E. coli* and *B. subtilis* they were cloned into the *E. coli-Bacillus* shuttle vector pNW33N (De Rossi *et al*., 1991). Two different reporter constructs were generated in which transcription of the reporter gene was driven by the Pro2 promoter and the *c1* repressor gene by the ProB promoter (pNW13) or, alternatively, the reporter gene was driven by the Pro3 promoter and the *c1* gene by the ProA promoter (pNW22). The Pro2 and Pro3 promoters differed from each other by the addition of TG nucleotides, whereas the ProA and ProB promoters differed with regards to the spacing between the -35 and -10 sequences to allow for synthesis of differing amounts of the C1 repressor protein (Schofield *et al*., 2003). To assess the strength and inducibility of the respective promoter systems, the Pro2 and Pro3 promoters were transcriptionally fused to either *lac*Z or *sapS* reporter genes that encode for β-galactosidase and acid phosphatase, respectively. The enzyme activities were measured at permissive (30°C) and non-permissive (42°C) temperatures. It should be emphasized that the enzymatic activities obtained with *lac*Z and *sapS* cannot be directly compared due to their different enzymatic characteristics. Nevertheless, the use of two different reporter genes served to validate the results that were obtained since similar trends for both of the reporter genes were observed in *E. coli* and *B. subtilis* (Tables 2.3 and 2.4).

The results obtained in this study indicated that basal expression exhibited by the Pro2 promoter was notably higher than that exhibited by the Pro3 promoter in both *E. coli* and *B. subtilis*. This therefore indicated that the Pro2 promoter was less efficiently repressed in the presence of C1 at the permissive temperature of 30°C. Note should be taken that as a result of the optimization of the synthetic promoter elements, Pro2 and Pro3 carried a number of mismatches in the consensus C1 operator sequences (Schofield *et al*., 2003). Therefore, it is plausible that the difference in basal expression is a reflection of the number of mismatches in the operator sites of the respective C1-regulated promoters and thus their ability to efficiently repress transcription. However, functional C1 binding sites containing mismatches to the consensus sequence have been identified throughout the P1 prophage (Schaefer and Hays, 1991). Considering that the P1 prophage served as the source of the C1-encoding gene used in the construction of the promoter systems under evaluation, it can thus be expected that the operator sites would be effective despite the presence of a number of mismatches. A more likely explanation for the results may be related to the amount of C1 repressor protein that is

being synthesized in the host cells. It has previously been reported that the amount of repressor produced is crucial to the effectiveness of regulated promoter systems; small amounts of repressor can result in partial repression, while too much repressor results in the inability to achieve derepression (Stark, 1987; Schofield *et al*., 2001). In this regard, it is interesting to note that the difference in basal levels of expression from the respective C1-regulated promoters appear to be dependent on the promoter used for C1 expression. In both *E. coli* and *B. subtilis*, basal activity was much higher when ProB, instead of ProA, was used to drive C1 expression, thus suggesting that inadequate amounts of C1 were produced by this promoter to effectively repress transcription. This is not unlikely, since variation in the spacer sequences have been shown to alter promoter strengths up to 400-fold (Jensen and Henner, 1998).

Under inducing conditions at the non-permissive temperature (42°C), the enzyme activity from the C1-regulated Pro2 and Pro3 promoters increased. In *E. coli*, the Pro2 and Pro3 promoters produced similar enzyme activities at this temperature. However, striking differences in the induction ratios were observed, depending on whether ProA or ProB was used to drive C1 expression. Using the Pro3 promoter in combination with the ProA promoter to drive C1 expression (pNW22) resulted in controlled expression in *E. coli*, while using Pro2 in combination with ProB (pNW13) resulted in limited induction (2-fold) due to the high basal expression. In contrast to *E. coli*, expression from the Pro2 promoter was markedly higher compared to Pro3 expression under inducing conditions in *B. subtilis*. Since the activity of both reporter enzymes was higher when the Pro2 promoter was used, it can be concluded that Pro2 is a stronger promoter than Pro3 in *B. subtilis*. Although the highest induction factor was observed when the Pro2 promoter was used in combination with the ProB promoter, it did not differ markedly from that observed using Pro3 in combination with ProA (Tables 2.3 and 2.4).

Based on the above, it is interesting to note that the addition of a TG dinucleotide in the Pro3 promoter did not increase the strength of the promoter in either *E. coli* or *B. subtilis*. Previous analyses of *B. subtilis* promoters demonstrated that *ca.* 45% of the promoters contain TG dinucleotides upstream of the -10 region (Moran *et al*., 1987; Helmann, 1995). Likewise, analysis of *E. coli* promoters identified a TG motif in *ca.* 20% of the promoters (Keilty and Rosenberg, 1987; Burr *et al*., 2000; Mitchell *et al*., 2003). These promoters, referred to as extended -10 promoters, lack identifiable -35 regions and it is thought that the TG motif is

required to compensate for the lack of a -35 region (Chan *et al*., 1990; Kumar *et al*., 1993; Mitchell *et al*., 2003). Since the thermally regulated Pro2 and Pro3 promoters evaluated in this study contain consensus -35 and -10 hexamers, it may therefore not be surprising that the addition of TG dinucleotides in Pro3 had no influence on the promoter strength in *E. coli*. In *B. subtilis*, it has been reported that the TG dinucleotides play an important function in some promoters (*i.e.* those with poor matches to the consensus -10 and -35 hexamers) by providing additional stabilization of the transcription initiation complex (Voskuil and Chambliss, 1998). However, it was subsequently reported that the TG dinucleotides are of minimal benefit in promoters with consensus -35 and -10 sequences and may indeed be detrimental to promoter function, since it is possible that the TG dinucleotides may overstabilize the open complex and hinder promoter clearance (Voskuil and Chambliss, 2002). It is therefore tempting to suggest that such a mechanism may account for the weaker activity of the Pro3 promoter (as compared to Pro2) observed in *B. subtilis*.

Taken together, the results demonstrate that the thermally regulated Pro2 and Pro3 promoter systems may be used for the controlled expression of a cloned gene of interest in *E. coli* and *B. subtilis*. Regulated expression in *E. coli* was obtained with the pNW13 construct, whereas both the constructs resulted in regulated expression in *B. subtilis* (Tables 2.3 and 2.4). At this stage, comparisons of these expression systems with alternative controlled expression systems can be done only in an indirect manner, since many technical aspects of the previously described studies were significantly different from those used in this study. Nevertheless, comparisons were made to other plasmid-based temperature-inducible expression systems that used *lacZ* as reporter gene. In this study, the induction/repression ratio obtained for *E. coli* was 138 and it is *ca.* 10-fold lower than those reported for temperature-inducible systems based on the use of a phage P1 promoter (Schofield *et al*., 2002) or phage lambda P_L and P_R promoters (Remaut *et al*., 1981; Gupta *et al*., 2004). In contrast, the induction/repression ratios obtained in *B. subtilis* (12 and 8 for pNW13*lacZ* and pNW22*lacZ*, respectively) are comparable to an 8-fold induction in *lacZ* gene expression reported by Li *et al*. (2007). With specific reference to *B. subtilis*, the thermally regulated expression systems evaluated in this study may display some advantages over the more widely used IPTG-inducible P_{spac} (Yansura and Henner, 1984) and xylose-inducible P_{xylA} (Kim *et al*., 1996) promoter systems. Since the latter two systems depend on chromosomal integration of the promoter and the gene of interest, plasmid-based gene expression may offer advantages in terms of increased copy number and/or gene dosage. As the level of expression is generally proportional to the

number of transcribed gene copies in the host cell (Summers, 1998), it can therefore be expected that by increasing the plasmid copy number, there will be a concomitant increase in the amount of protein that is being synthesized. Moreover, since the systems evaluated in this study are not derived from sugar-fermenting capacities of *B. subtilis*, it therefore lacks the glucose repression control observed for the P_{xylA} system (Bhavsar *et al*., 2001). Thus, it is not necessary to alter the medium composition prior to the induction of gene expression which is required to obtain tight control in the above system. Finally, since expression is achieved by temperature induction, no inducer molecule is required and is especially relevant in view of the toxic activity of the IPTG inducer at high concentrations (Kosinski *et al*., 1992).

In conclusion, the results of this study indicate that the thermally regulated promoters Pro2 and Pro3 are suitable for the controlled expression of heterologous genes in especially *B. subtilis*. Although the promoters displayed different levels of basal activity during exponential growth, they were both induced upon an increase in growth temperature and thus provide a simple and inexpensive means of induction for target protein production. Consequently, these promoter systems were selected for expression of an enzyme with potential industrial application, the details of which are provided in the following Chapter.

CHAPTER THREE

PRODUCTION OF A RECOMBINANT PURINE NUCLEOSIDE PHOSPHORYLASE IN *B. subtilis* USING THERMALLY REGULATED PROMOTER SYSTEMS

3.1 INTRODUCTION

Purine nucleoside phosphorylase (PNP; EC 2.4.2.1) is a key enzyme of purine metabolism in the salvage pathway of organisms, and catalyzes the reversible phosphorolysis of 2'-deoxypurine ribonucleosides to the free purine base and 2'-deoxyribose-1-phosphate (Bzowska *et al*., 2000; Lewkowicz and Iribarren, 2006). Purine nucleoside phosphorylases (PNPases) are widely distributed in mammalian and bacterial cells. The mammalian PNPases are trimeric complexes with a monomer molecular mass of *ca.* 31 kDa, and usually only take guanosine and inosine as substrates (Parks and Agarwal, 1972; Stoeckler *et al*., 1978). In contrast, most of the prokaryotic PNPases are hexameric complexes with a monomer molecular mass of *ca.* 26 kDa. Their catalytic activity is relatively non-specific compared with that of the mammalian analogues. In addition to inosine and guanosine, they can also cleave adenosine (Jensen and Nygaard, 1975; Ling *et al*., 1994; Koellner *et al*., 2002).

In addition to its biological role, PNPases are also key enzymes for the synthesis of nucleosides and their derivatives, which are commonly used as antiviral (Shirase *et al*., 1989; Ling *et al*., 1990) and anticancer reagents (Utagawa *et al*., 1985; Elizabeth *et al*., 2000; Zhang *et al*., 2005). Specifically, 2'-deoxynucleosides are important raw materials for the preparation of antisense drugs where demand is increasing due to the recent success with their clinical application (Komatsu *et al*., 2001; Dienstag *et al*., 2009). The use of PNPases as biological catalysts offer considerable advantages over chemical synthesis, *e.g.* stereo- and regioselectivity, greater reaction rates, and a reduction of preparation and purification steps (Rocchetti *et al*., 2004). However, their industrial application has been limited by the thermostability of the enzymes (Luo *et al*., 2011).

Research on thermophilic *Bacillus* spp., as promising sources of highly stable enzymes, is an active research subject (Davis, 1998). Thermophiles represent an obvious source of thermostable enzymes, it being reasonable to assume that such character will confer on their proteins a high thermal stability (Adams and Kelly, 1998). Enzymes isolated from these microorganisms are not only thermostable and active at high temperature, but are also often resistant to and active in the presence of organic solvents and detergents (Jaenicke *et al*., 1996). PNPases have been identified in thermophilic bacteria and the enzymes were reported to exhibit a much greater thermostability compared to that produced by *E. coli*. The thermophile *Geobacillus stearothermophilus* (formerly *Bacillus stearothermophilus*) encodes

two thermostable purine nucleoside phosphorylases that have both been characterized (Hori *et al*., 1989a, 1989b; Hamamoto *et al*., 1997a, 1997b). However, moderate thermophilic *Bacillus* spp. may also be good sources for new thermostable enzymes. In this regard, the characterization of a PNPase, designated BHPNP1, of the moderately thermophilic *B. halodurans* Alk36 was recently reported (Visser *et al*., 2010). The enzyme had an optimal pH and temperature of 7 and 70°C, respectively, and had a half-life at 60°C of 20.8 h. Although the substrate specificity of the BHPNP1 enzyme was not determined, the enzyme was capable of phosphorolysis of guanosine and inosine.

In the previous Chapter, it was shown that derivatives of the *E. coli-Bacillus* shuttle vector pNW33N, which harbour different thermally regulated promoter systems, represented useful expression vectors for controlled expression in *B. subtilis*. Notably, *B. subtilis* is generally considered to have great industrial potential for production of enzymes of industrial interest, such as proteases (Ho and Lim, 2003), α-amylases (Huang *et al*., 2004) and lipases (Ho and Lim, 2003). Consequently, the aim of this part of the study was to evaluate the utility of the thermally regulated promoter systems for efficient and controlled overexpression of an industrially important enzyme in *B. subtilis*. For this purpose, the previously characterized purine nucleoside phosphorylase enzyme (BHPNP1) of *B. halodurans* Alk36 was selected. Moreover, due to the potential industrial applications of the PNPase, the substrate specificity of the heterologously expressed enzyme was also determined.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains, plasmids and culturing conditions

E. coli DH10B (F$^-$ *mrc*A Δ(*mrr-hsdRMS-mcrBC)* Δ*lac*X74 *end*A1 *rec*A1 *deo*R Δ(*ara-leu*) 7697 *ara*D139 *gal*U *gal* K*nup* G*rps*L λ$^-$), obtained from Invitrogen, was used as an intermediary cloning host. Expression studies were performed with *B. subtilis* 1A46 (*recE*4 *thr*-5 *trp*C2), which was obtained from the *Bacillus* Genetic Stock Centre (Columbus, Ohio, USA). *E. coli* and *B. subtilis* strains were cultured in Luria-Bertani broth (LB: 0.5% [w/v] yeast extract, 1% [w/v] tryptone, 1% [w/v] NaCl; pH 7) at 37°C with shaking (175 rpm). When appropriate, *E. coli* growth medium was supplemented with ampicillin (100 µg/ml) or chloramphenicol (20 µg/ml), and *B. subtilis* growth medium was supplemented with chloramphenicol (5 µg/ml). All antibiotics were obtained from Roche Diagnostics.

The plasmid constructs pDAS113 and pDAS122 (Chapter 2; Table 2.1) were kindly provided by Prof. D.A. Schofield (Department of Microbiology and Immunology, Medical University of South Carolina, USA). Plasmids pBluescript II SK (+) and pNW33N, an *E. coli-Bacillus-Geobacillus* shuttle vector, were obtained from Stratagene and the *Bacillus* Genetic Stock Centre, respectively.

3.2.2 DNA amplification

3.2.2.1 Primers

To obtain the nucleoside phosphorylase gene (BH1531) of *B. halodurans* Alk36, primers were designed from the *B. halodurans* C-125 genome sequence (GenBank accession no. NC-002570; Takami *et al*., 2000) owing to the high level of sequence identity between these two *B. halodurans* strains (Visser *et al*., 2010). The forward primer was designed to anneal 33 bp upstream from the start codon (ATG), thus excluding the promoter sequence. To facilitate cloning of the amplicon, restriction endonuclease recognition sequences were incorporated at the 5' terminus of the respective primers. The primers, indicated in Table 3.1, were obtained from Inqaba Biotechnical Industries.

Table 3.1: Primers used for amplification of the *B. halodurans* Alk36 nucleoside phosphorylase gene

Primers[a]	Sequence	Relevant properties
B1 forward	5'-CGGGTCGACGAACTTTAAGGGGGAACT-3'	*Sal*I site incorporated
B1 reverse	5'-CGGGCATGCCGTAGTCCTCTCCTTTGT-3'	*Sph*I site incorporated

[a] Relevant restriction endonuclease sites are underlined

3.2.2.2 Polymerase chain reaction (PCR)

Genomic DNA was isolated from *B. halodurans* Alk36 according to the method of Lovett and Keggins (1979), as described previously (Chapter 2, Section 2.2), and used as template in the PCR reaction. The PCR reaction mixture (100 µl) contained 20 ng of template DNA, 1 × PCR buffer (75 mM Tris-HCl [pH 8.8], 16 mM $(NH_4)_2SO_2$, 0.1% [v/v] Tween-20), 2 mM $MgCl_2$, 200 µM of each dNTP, 0.5 µM of each of the forward and reverse primers and 1 U of Biotaq DNA polymerase (Bioline, Inc.). The tubes were placed in a Progene thermocycler

(Techne). The thermocycling profile consisted of an initial denaturation step at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 58°C for 1 min and elongation at 72°C for 1.5 min. After the last cycle, the reactions were kept at 72°C for 4 min to complete synthesis of all DNA strands. For control purposes, a reaction mixture identical to that above was prepared, except that template DNA was omitted. An aliquot of the reaction mixtures were subsequently analyzed by agarose gel electrophoresis in the presence of an appropriate DNA molecular weight marker.

3.2.3 Plasmid constructs

All molecular cloning techniques employed in the construction of the recombinant plasmids indicated below were performed in accordance with the procedures described in Chapter 2 (Section 2.2.4 to Section 2.2.11). The plasmid constructs were first established in *E. coli* DH10B and then transferred to *B. subtilis* 1A46. Transformation of bacteria was performed by electroporation for *E. coli* (Tung and Chow, 1995) and by protoplasting for *B. subtilis* (Chang and Cohen, 1979). All plasmid constructs were verified by restriction endonuclease digestion. The cloning strategies are indicated diagrammatically in Fig. 3.1.

- **pSKB1**

Primers B1 forward and B1 reverse were used with genomic DNA of *B. halodurans* Alk36 as template DNA to PCR amplify the promoterless nucleoside phosphorylase gene (BH1531). The amplicon was subsequently cloned into T-tailed pBluescript SK II (+) vector DNA to generate pSKB1.

- **pNW13B1**

Plasmid pDAS113 was digested with both *Sal*I and *Sph*I to excise the *lacZ* reporter gene. Digestion of pSKB1 with both *Sal*I and *Sph*I yielded two nucleoside phosphorylase gene-specific DNA fragments of *ca.* 670 and 200 bp, due to an internal *Sph*I cleavage site. These two DNA fragments were subsequently used in a three-way ligation, together with digested pDAS113, to generate pDS13B1. The transcription cassette was then recovered from pDS13B1 by digestion with both *Nae*I and *Xba*I, and cloned into the *E. coli-Bacillus* shuttle vector pNW33N that had been digested with both *Sma*I and *Xba*I to generate pNW13B1.

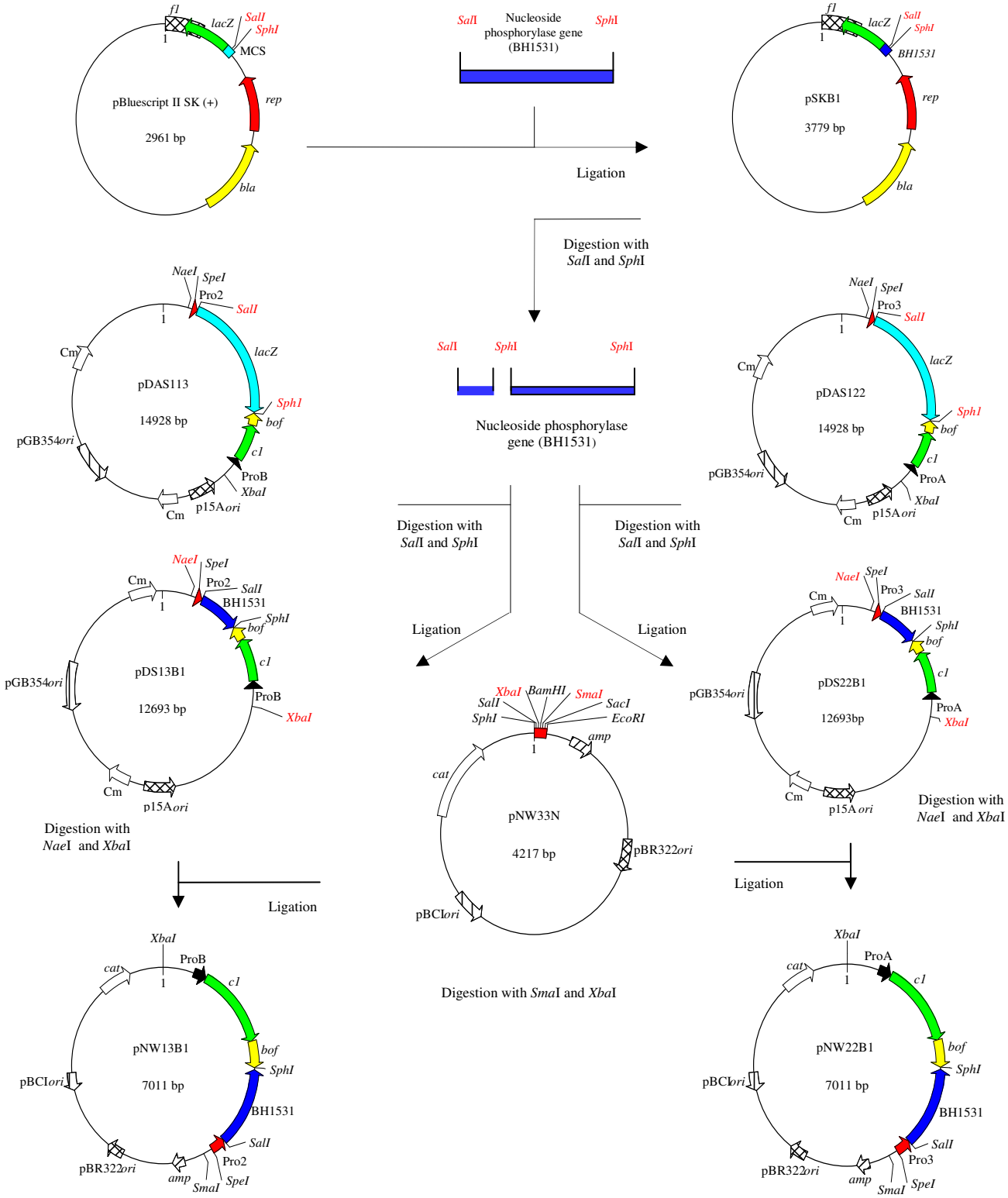


Fig. 3.1: Diagrammatic representation of the cloning strategies used to construct plasmids pNW13B1 and pNW22B1.

- **pNW22B1**

Using a cloning strategy similar to that described above, the promoterless nucleoside phosphorylase gene was cloned into the *Sal*I and *Sph*I sites of plasmid pDAS122 to generate pDS22B1. The transcription cassette was subsequently recovered from pDS22B1 and cloned as a *Nae*I-*Xba*I restriction DNA fragment into the *Sma*I and *Xba*I sites of pNW33N to generate pNW22B1.

3.2.4 Nucleoside phosphorylase enzyme assays

3.2.4.1 Preparation of crude enzyme extracts

A single colony of the recombinant *B. subtilis* 1A46 strains was inoculated into 50 ml of LB broth and cultured overnight at 30°C. An aliquot (4 ml) of the overnight cultures was inoculated into 420 ml of LB broth supplemented with the appropriate antibiotic, and the cultures were incubated at 30°C until an OD_{600} of 0.1 was reached. Each culture was then divided into two equal volumes (200 ml each) and incubated at 30°C and 42°C, respectively. The cells from 160 ml of each culture was collected by centrifugation at 12 000 rpm for 15 min. The cell pellets were suspended in 5 ml of sonication buffer (50 mM Tris; pH 7.5) and sonicated on ice at 10-min intervals for 30 min with a Bandelin Sonoplus Ultrasonic Homogenizer (Model HD 2070). The cell lysates were clarified by centrifugation at 12 000 rpm for 10 min and the supernatants, considered the intracellular fractions, were used in subsequent enzyme assays. The protein concentration of the samples was determined spectrophotometrically according to the method of Bradford (1976), using the Microtiter Plate Protein Assay kit (BioRad) and bovine serum albumin (BSA) as standard.

3.2.4.2 Quantitative enzyme assays

Nucleoside phosphorylase activity was determined spectrophotometrically in UV-compatible microtiter plates (Nunc). The microtiter plate assay is based on a modified method of Erion *et al*. (1997), using inosine as the substrate. In this assay, xanthine oxidase (XO) acts as a dehydrogenase allowing reduction of a tetrazolium salt to a formazan product that can be measured in the visible spectrum.

$$\text{Inosine} \xrightarrow{\text{PNP}} \text{Hypoxanthine} + \text{Ribose-1-phosphate} + 2\ \text{Tetrazolium salt} \xrightarrow{\text{XO}} \text{Uric acid} + 2\ \text{Formazan}$$

Ten microliters (10 µl) of the enzyme preparation was added to 190 µl of 50 mM sodium phosphate buffer (pH 8), containing 0.5 mM inosine and 0.2 U/ml xanthine oxidase (Sigma-Aldrich). The rate of change in absorbance at 293 nm due to the release of uric acid was measured in a Powerwave HT spectrophotometer (BioTek Instruments). One unit of nucleoside phosphorylase is defined as the amount of enzyme needed to release 1 µmol of uric acid from inosine in the presence of xanthine oxidase in 1 min (Visser *et al*., 2010). The enzyme assays were performed in triplicate and the results are indicated as means ± standard deviation.

3.2.5 Modulation of nucleoside phosphorylase expression in shake-flask cultures

To determine the optimal induction temperature and induction time period, the recombinant strain *B. subtilis*(pNW13B1) was cultured, as described above, except that the overnight culture was inoculated into 1.2 L of LB broth to an OD_{600} of 0.06. The culture was then incubated at 30°C until an OD_{600} of 0.1 was reached. To determine the optimal induction temperature, the culture was divided into equal volumes (200 ml each) and incubated at 30°C, 34°C, 38°C, 42°C, 46°C or 50°C. Upon reaching an OD_{600} of 0.6, the cells were harvested and crude enzyme extracts were prepared. To determine the optimal induction time, cultures were incubated at 42°C or 46°C, and samples were taken at 30-min intervals. All enzyme assays were performed, as described above. The assays were performed in triplicate and the results are indicated as means ± standard deviation.

3.2.6 Substrate specificity of the nucleoside phosphorylase

Thin layer chromatography (TLC) analysis was used to determine the substrate specificity of the *B. halodurans* Alk36 nucleoside phosphorylase enzyme. The analyses were performed on crude enzyme extracts prepared from the recombinant strain *B. subtilis*(pNW13B1). The substrate specificity of the enzyme was tested against adenosine, inosine, 2'-deoxyinosine, uridine and thymidine. For this purpose, 950 µl of a 2 mM solution of each substrate was incubated at 60°C. After the temperature was reached, 10 µl of enzyme preparation was added to each of the substrates and incubation was continued for 30 min with gentle shaking at 60°C. Following incubation, the samples were spotted on a 20 cm × 10 cm silica F254 aluminium plate (Merck). The mobile phase prepared for separation consisted of different mixtures of chloroform and methanol. When the nucleoside phosphorylase enzyme was tested against the substrates thymidine and uridine, the mobile phase prepared for separation

consisted of a mixture 75:25 chloroform and methanol. For the substrates inosine and 2'-deoxyinosine, the mobile phase consisted of a mixture of 85:15, and for adenosine the mixture was 9:1. The TLC spots were visualized under UV light at 266 nm.

3.3 RESULTS

3.3.1 Construction of the expression vectors pNW13B1 and pNW22B1

In the previous Chapter, results were obtained that indicated that the thermally regulated Pro2 and Pro3 promoters (Schofield *et al*., 2003) could be used in *B. subtilis* for the controlled overexpression of two different reporter genes. Consequently, in this part of the study, both these promoters were evaluated for their ability to overproduce an enzyme of industrial importance. For this purpose, the purine nucleoside phosphorylase enzyme (BHPNP1) of the moderately thermophilic bacterium *B. halodurans* Alk36 was selected.

The purine nucleoside phosphorylase gene (BH1531) of *B. halodurans* Alk36, excluding the native promoter sequence, was obtained by PCR amplification using genomic DNA as template, together with primers B1 forward and B1 reverse, which contain *Sal*I and *Sph*I restriction endonuclease sites, respectively. Following PCR, the 879-bp amplicon was cloned into T-tailed pBluescript SK II (+) vector DNA to generate pSKB1 and digestion of the derived recombinant plasmid with both *Sal*I and *Sph*I yielded three DNA fragments of *ca.* 2.9 kb, 670 bp and 200 bp. Since the purine nucleoside phosphorylase gene contains a unique *Sph*I site that is located 206 bp from the 5'-terminal end, the gene was thus excised as two DNA fragments of *ca.* 670 and 200 bp, respectively (Fig. 3.2, lane 2). The integrity of the cloned insert DNA was verified by nucleotide sequencing prior to it being used in further DNA cloning steps. The complete nucleotide sequence and deduced amino acid sequence of the cloned nucleoside phosphorylase gene is provided in the Appendix to this dissertation.

To enable cloning of the purine nucleoside phosphorylase gene under control of the thermally regulated Pro2 and Pro3 promoters, the *lacZ* reporter gene was excised from plasmids pDAS113 and pDAS122 by digestion with both *Sal*I and *Sph*I. The vector DNA fragments, together with the 670-bp and 200-bp restriction fragments of the purine nucleoside phosphorylase gene were used in a three-way ligation, yielding plasmids pDS13B1 and pDS22B1 in which the *lac*Z gene was replaced with the purine nucleoside phosphorylase coding sequence. The promoter-purine nucleoside phosphorylase transcriptional fusions were

subsequently recovered by digestion with both *Nae*I and *Xba*I, and cloned into pNW33N vector DNA that had been prepared by digestion with *Sma*I and *Xba*I. Following transformation of *B. subtilis* 1A46 protoplasts, plasmid DNA was extracted from a number of randomly selected chloramphenicol-resistant transformants and analyzed by agarose gel electrophoresis. Plasmid DNA migrating slower than the parental pNW33N vector DNA was selected and characterized by restriction endonuclease digestion.

Digestion of the recombinant plasmid DNA with both *Spe*I and *Xba*I, which flank the insert DNA in the multiple cloning site (MCS) of pNW33N, yielded two DNA fragments that corresponded in size to the pNW33N vector DNA (4.217 kb) and the cloned insert DNA (2.794 kb), whereas digestion of the recombinant plasmid DNA with *Xba*I yielded an expected DNA band corresponding to 7.011 kb (Fig. 3.3). Based on these results, it was concluded that plasmids pNW13B1 and pNW22B1 contained the purine nucleoside phosphorylase gene under control of the Pro2 and Pro3 promoters, respectively. The recombinant strains *B. subtilis*(pNW13B1) and *B. subtilis*(pNW22B1) were used in further assays.

3.3.2 Analysis of purine nucleoside phosphorylase activity in *B. subtilis*

To compare the thermally regulated Pro2 and Pro3 promoter systems for controlled overexpression of the purine nucleoside phosphorylase enzyme, *B. subtilis* 1A46 harbouring pNW13B1 or pNW22B1 were cultured in selective medium at 30°C and 42°C. The cells were harvested and the purine nucleoside phosphorylase activity of intracellular fractions was subsequently assayed using inosine as substrate.

The results, presented in Table 3.2 below, indicated that with the Pro2 promoter basal purine nucleoside phosphorylase activity could be detected at 30°C (0.7 U/mg), but the enzyme activity increased 5.4-fold at 42°C (3.8 U/mg). When the purine nucleoside phosphorylase activity was driven by the Pro3 promoter, the basal enzyme activity was reduced to 0.1 U/mg and increased 32-fold at 42°C (3.2 U/mg).

Table 3.2: Basal and induced activities from nucleoside phosphorylase gene fusions to C1-regulated promoters in *B. subtilis* 1A46

Construct	Promoter driving:		Avg activity in Units/mg (±SD)	
	BH1531	***c1***	**Basal (30°C)**	**Induced (42°C)**
pNW13B1	Pro2	ProB	0.73 (0.09)	3.79 (0.78)
pNW22B1	Pro3	ProA	0.13 (0.02)	3.19 (0.53)

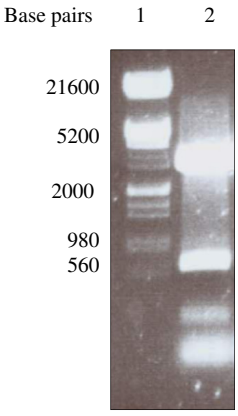


Fig. 3.2: Agarose gel electrophoretic analysis of the recombinant plasmid pSKB1. Lane 1, DNA molecular weight marker; lane 2, recombinant plasmid pSKB1 digested with both *Sal*I and *Sph*I. The sizes of the DNA molecular weight marker, phage λ DNA digested with *Eco*RI and *Hind*III, are indicated to the left of the figure.

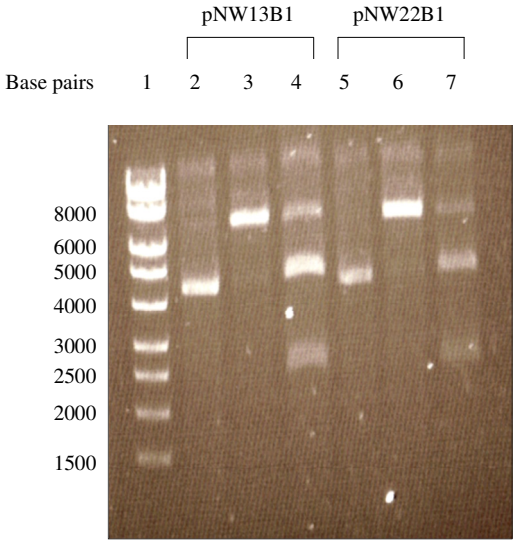


Fig. 3.3: Agarose gel electrophoretic analysis of the recombinant plasmids pNW13B1 and pNW22B1. Lane 1, DNA molecular weight marker; lane 2, uncut pNW13B1 plasmid DNA; lane 3, pNW13B1 linearized by digestion with *Xba*I; lane 4, pNW13B1 digested with both *Spe*I and *Xba*I; lane 5, uncut pNW22B1 plasmid DNA; lane 6, pNW22B1 linearized by digestion with *Xba*I; lane 7, pNW22B1 digested with both *Spe*I and *Xba*I. The sizes of the DNA molecular weight marker, MassRuler™ High Range DNA Ladder (Fermentas), are indicated to the left of the figure.

Based on the higher enzyme activity expressed by the Pro2 promoter under inducing conditions, the pNW13B1 expression vector was selected for further use. While direct visualization of a recombinant protein by immunoblot analysis would provide a more direct measure of protein production levels, measurement of the nucleoside phosphorylase activities does, however, yield an indication of the promoter strength and thus its capacity for enzyme production. Consequently, it was reasoned that enzyme production with the Pro2 promoter would be higher than with the Pro3 promoter since Pro2 was capable of driving a higher level of enzyme activity than Pro3 at 42°C. Moreover, the higher level of basal enzyme activity from Pro2 may contribute further to increased production levels of the purine nucleoside phosphorylase enzyme.

3.3.3 Optimization of purine nucleoside phosphorylase production in *B. subtilis*

Although the above results indicated an increased level of purine nucleoside phosphorylase activity after temperature induction, relatively low levels of enzyme activity were observed. Consequently, in an attempt to improve the expression level of the recombinant enzyme in *B. subtilis* the effects of different induction temperatures and the duration of thermal induction were investigated.

To determine the optimal temperature at which synthesis of the recombinant enzyme was induced in *B. subtilis* 1A46 cells harbouring plasmid pNW13B1, the purine nucleoside phosphorylase activity of intracellular extracts prepared from cultures incubated at different temperatures (30-50°C) was measured. The results (Fig. 3.4) indicated that the level of enzyme activity increased slightly at temperatures from 30°C to 38°C (0.05 U/mg at 30°C and 0.39 U/mg at 38°C). Although the enzyme activity was increased by growing the cells at 42°C (4.28 U/mg), maximal enzyme activity was detected at 46°C (11.64 U/mg). Incubation of the cells at 50°C led to a reduction in the purine nucleoside phosphorylase activity (5.54 U/mg) and was *ca.* 50% less than the activity detected at 46°C.

To determine the optimal duration of thermal induction, the culture was grown at 30°C and then incubated at either 42°C or 46°C. The intracellular extracts of culture samples collected at 30-min intervals after thermal induction were used to quantify purine nucleoside phosphorylase activity. Analysis of the time course of temperature induction at 42°C (Fig. 3.5a) indicated a low level of enzyme activity after 2 h (0.93 U/mg), which increased

gradually to 4.38 U/mg after 3 h and a maximum purine nucleoside phosphorylase activity of 5.17 U/mg was measured after 4 h. In contrast, not only were higher levels of purine nucleoside phosphorylase activity measured for the culture incubated at 46°C, but a faster rate of induction could be observed (Fig. 3.5b). After 30 min of incubation the enzyme activity was 0.55 U/mg and increased sharply to 10.53 U/mg after 2 h, whereafter the enzyme activity increased gradually before reaching a maximum of 13.47 U/mg after 4 h.

From the above results, it can therefore be concluded that for maximal induction of recombinant purine nucleoside phosphorylase expression in *B. subtilis*, the culture has to be induced at 46°C for 4 h. However, higher induced enzyme activity may be obtained by a further increase in the duration of thermal induction.

3.3.4 Determination of the substrate specificity of the purine nucleoside phosphorylase

The substrate specificity of the *B. halodurans* purine nucleoside phosphorylase was analyzed by testing several purine and pyrimidine nucleosides as substrates of the enzyme. Excluded from the analysis were guanosine, which has previously been shown to be a substrate of the enzyme (Visser *et al*., 2010), and cytidine, which is not cleaved by any known nucleoside phosphorylase (Pugmire and Ealick, 2002). To investigate, intracellular enzyme extracts were mixed with the different substrates, incubated at 60°C and then subjected to thin layer chromatography (TLC) since it allows for the clear separation of nucleosides and purine or pyrimidine bases.

The results indicated that the recombinant enzyme was unable to hydrolyze thymidine and uridine, as was evidenced by the absence of corresponding free pyrimidine bases (thymine or uracil) on the chromatogram (Fig. 3.6a). The enzyme was capable of hydrolyzing selected purine nucleosides. Incubation of the enzyme with inosine and 2'-deoxyinosine resulted in the phosphorolysis of the respective substrates to free hypoxanthine (Fig. 3.6b). These substrates were not completely converted to free purine bases, indicating that further optimization of the reaction conditions used in this study may be required. In contrast, incubation with adenosine did not lead to the appearance of adenine on the chromatogram (Fig. 3.6c). These results therefore provide supporting evidence that the *B. halodurans* BHPNP1 is indeed a purine nucleoside phosphorylase, albeit that the enzyme exhibits a narrow substrate specificity.

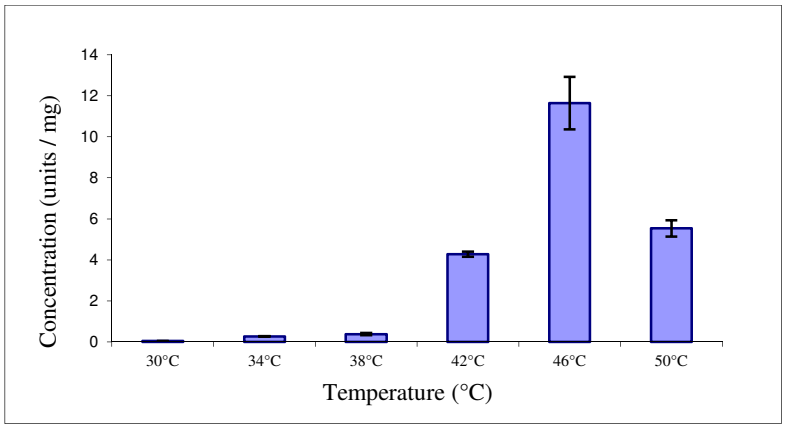
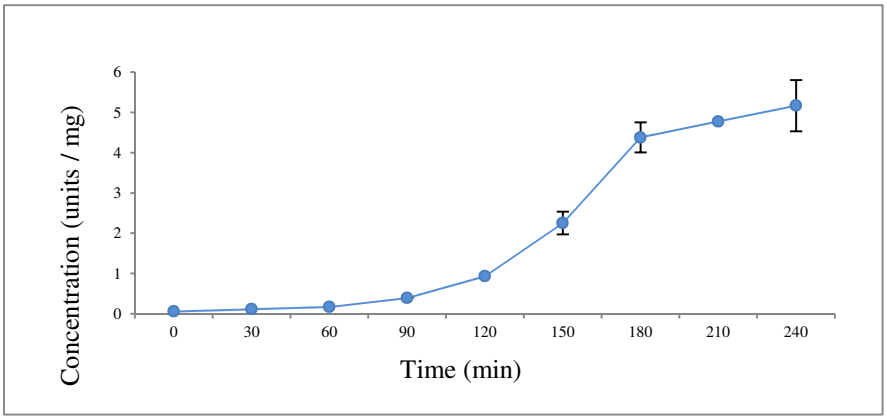


Fig. 3.4: Effect of different induction temperatures on BHPNP1 production in *B. subtilis* 1A46. An overnight culture of *B. subtilis*(pNW13B1) was inoculated into LB broth and grown at 30°C to an OD_{600} of 0.1. The culture was divided in equal volumes and incubated at the indicated temperatures until an OD_{600} of 0.6 was reached. The cells were harvested and the intracellular fractions were assayed for nucleoside phosphorylase activity. Values (± SD) are means of triplicate cultures assayed in triplicate.

(a)



(b)

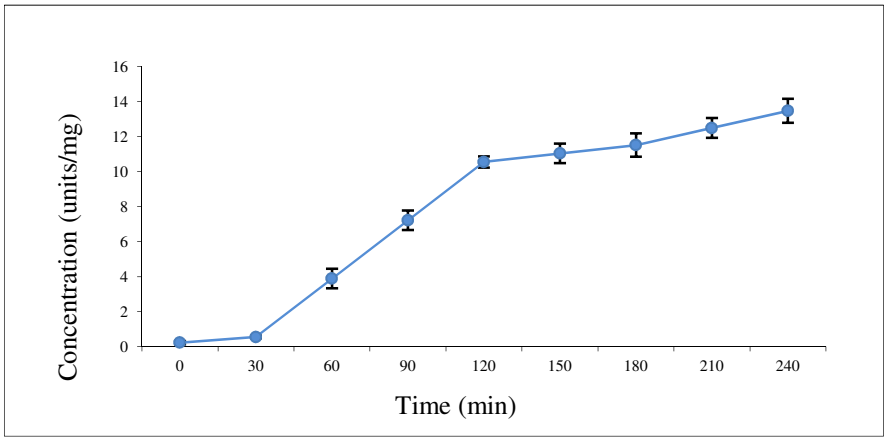
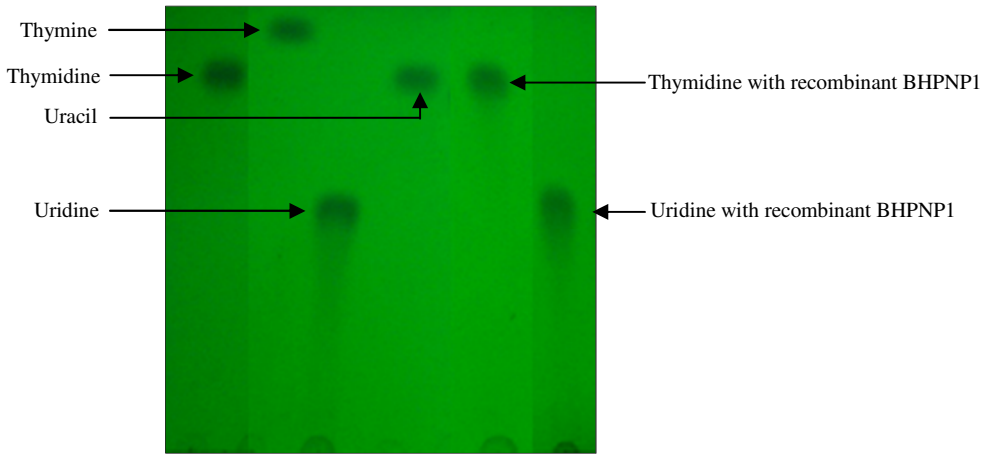
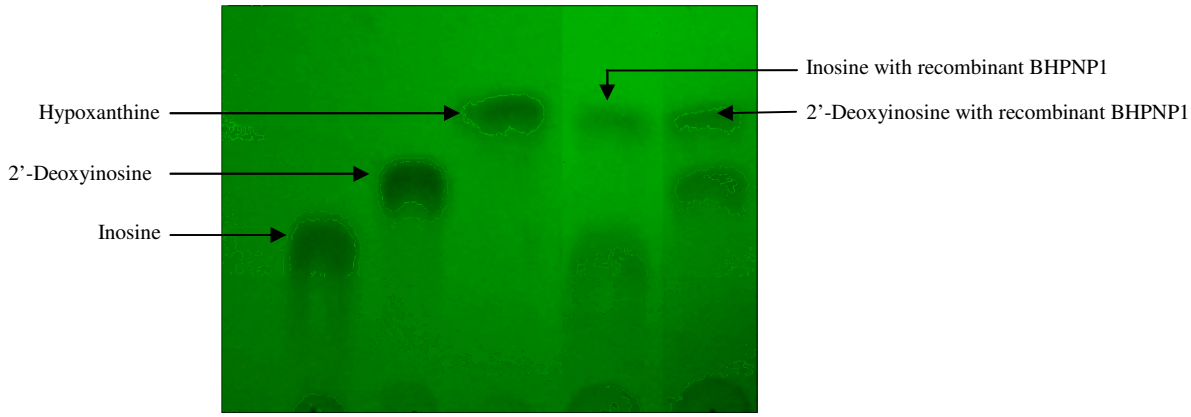


Fig. 3.5: Time course analysis of BHPNP1 production in *B. subtilis* 1A46 following temperature induction. An overnight culture of *B. subtilis*(pNW13B1) was inoculated into LB broth and grown at 30°C to an OD_{600} of 0.1. Equal volumes of the culture were then incubated at 42°C (a) or 46°C (b), and samples were collected at 30-min intervals for nucleoside phosphorylase activity assays. Values (± SD) are means of triplicate cultures assayed in triplicate.

(a)



(b)



(c)

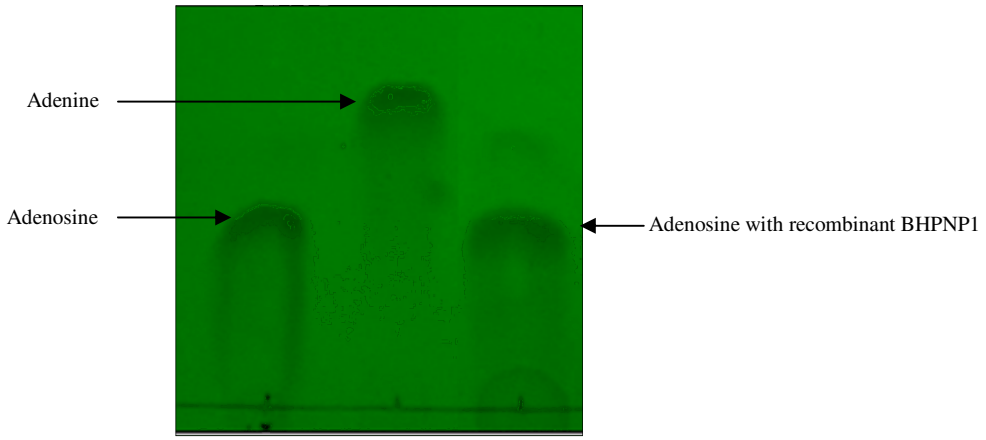


Fig. 3.6: Substrate specificity of the recombinant BHPNP1 nucleoside phosphorylase. Thin layer chromatography (TLC) was used to test intracellular enzyme extracts prepared from induced cultures of *B. subtilis*(pNW13B1) against thymidine and uridine (a), inosine and 2'-deoxyinosine (b), and adenosine (c). The mobile phase consisted of different mixtures of chloroform and methanol, as indicated under Materials and Methods (Section 3.2.6), and the TLC plates were visualized under UV light.

3.4 DISCUSSION

Since purine nucleoside phosphorylases (PNPases) are potentially important for a variety of industrial applications, there is considerable interest to produce these enzymes at a large scale (Hori *et al*., 1991; Hamamoto *et al*., 1996; Li *et al*., 2008; Visser *et al*., 2010; Luo *et al*., 2011). The first step needed to isolate a PNPase for industrial applications is usually overexpression of the corresponding gene of interest. Often this step is considered to be trivial, because proteins can be easily overexpressed in *E. coli* using commercially available systems (Terpe, 2006; Samuelson, 2011). Although *E. coli* has remained one of the most attractive hosts for the synthesis of heterologous proteins, recent years has witnessed an increase in interest in the use of Gram-positive bacteria such as *B. subtilis* as expression hosts (Schumann and Ferreira, 2004; Schumann, 2007). In this part of the study, the controlled overexpression of a PNPase by means of thermally regulated promoter systems in *B. subtilis* was specifically investigated.

Purine nucleoside phosphorylases (PNPases) are used in the enzymatic synthesis of nucleosides and nucleoside analogues (Lewkowicz and Iribarren, 2006). Of these, nucleoside analogues have been used extensively in anticancer and antiviral therapies as monomers (Elizabeth *et al*., 2000; Van Rompay *et al*., 2003; De Clercq and Field, 2006) and as building blocks for oligonucleotide therapeutics (Dienstag *et al*., 2009). Although PNPases derived from mesophillic bacteria have mostly been used in the synthesis reactions (Mao *et al*., 1997; Esipov *et al*., 2002; Trelles *et al*., 2004; Luo *et al*., 2011), thermostable enzymes may, however, provide several advantages. Not only can the reactions be carried out at a high temperature for extended periods of time, but the reaction rate is higher at elevated temperatures. Moreover, the solubility of the reactants is increased at a higher temperature which, in turn, allows synthesis reactions with higher concentrations of reactants (Hori *et al*., 1989c; Cacciapuoti *et al*., 1994; Rocchietti *et al*., 2004).

In this study, the moderate thermophile *B. halodurans* was selected as a source of thermostable PNPases. Genomic analysis has indicated that *B. halodurans* encodes two putative PNPases (BH1531 and BH1532), in addition to a putative pyrimidine nucleotide phosphorylase (Visser *et al*., 2010). Why *B. halodurans* has two PNPases is not clear, but it is conceivable that the two PNPases may have different functions in the salvage pathway in this bacterium. Interestingly, it has been reported that the thermophiles *Geobacillus*

stearothermophilus JTS859 (Hori *et al*., 1989a, 1989b) and *G. stearothermophilus* TH6-2 (Hamamoto *et al*., 1996) both produce two kinds of thermostable PNPases. Characterization of these PNPases, designated as Pu-NPaseI and Pu-NPaseII, indicated that the respective PNPases have different substrate specificities. For both these strains, the Pu-NPaseI enzymes were shown to catalyze the phosphorolysis of inosine and guanosine, but not adenosine. The latter is the primary substrate of Pu-NPaseII (Hori *et al*., 1989a, 1989b; Hamamoto *et al*., 1997a, 1997b). It is thus tempting to speculate that the putative PNPases of *B. halodurans* may likewise have different substrate specificities.

The cloning, purification and partial biochemical characterization of a thermostable PNPase encoded by BH1531 of *B. halodurans* Alk36 was recently reported (Visser *et al*., 2010). Consequently, this PNPase, which was designated BHPNP1, was selected for overexpression in *B. subtilis* in this study. In the previous Chapter, two thermally regulated promoter systems were evaluated and it was shown that they are capable of controlled expression of two different reporter genes. To further evaluate the usefulness of these promoter systems for the synthesis of an industrially important enzyme, the BHPNP1-encoding open reading frame (ORF) was cloned downstream of the temperature-sensitive C1-regulated Pro2 and Pro3 promoters in pNW13 and pNW22, respectively. The expression experiments in *B. subtilis* showed that the gene was expressed in a controlled manner by both vector constructs. The enzyme activity expressed from the Pro2 promoter was higher compared to that expressed from the Pro3 promoter under inducing conditions. These results are in agreement with those obtained earlier (Chapter 2), indicating that the Pro2 promoter is a stronger promoter than Pro3 in *B. subtilis.* Subsequently, the recombinant pNW13B1 construct was selected and the enzyme activity was measured at different induction temperatures. The enzyme activity was 4.23 U/mg, 11.64 U/mg or 5.54 U/mg in cultures that were heat-induced at 42°C, 46°C or 50°C, respectively. These results therefore indicated that induction was achieved at temperatures of 42°C or higher, which is in agreement with previous reports indicating instability of the C1 repressor protein at 42°C and above (Heinrich *et al*., 1989; Schofield *et al*., 2003). The C1 repressor protein has been shown to be more thermally stable when tightly bound to DNA than it is in its unbound form (Heinrich *et al*., 1989). Therefore, the optimum induction temperature of 46°C may thus reflect the need for a higher temperature to efficiently dissociate C1 from the DNA in order to derepress the promoter and allow transcription to proceed. Since *B. subtilis* is a mesophile, the decrease in enzyme activity at 50°C is most likely due to cell death at this elevated temperature. In addition, the enzyme

activity was also measured at different times post-induction of cultures that had been induced at either 42°C or 46°C. The results indicated that not only did the C1-regulated Pro2 promoter have a faster rate of induction at 46°C than at 42°C, but the level of enzyme activity was 2.6-fold higher than that measured after 4 h of induction at 42°C. Indeed, the maximum enzyme activity measured at 42°C was obtained after *ca.* 75 min of incubation in cultures induced at 46°C (Fig. 3.5). It is important to note that the above results also indicate that it is possible to modulate the Pro2 promoter system. The ability to obtain different levels of expression by partial induction of the promoter is an important feature of a controlled expression system, as it may allow for the synthesis of especially toxic proteins and may minimize deleterious effects that protein overexpression may have on cell growth and viability (Glick, 1995; Schumann, 2007).

The substrate specificity of the recombinant BHPNP1 enzyme was evaluated based on its relative activity towards different purine and pyrimidine nucleosides. For this purpose, thin layer chromatography (TLC) was used to monitor the disappearance of the substrates and the appearance of the products. In accordance with its classification as a purine nucleoside phosphorylase, the recombinant enzyme was unable to catalyze the phosphorolysis of pyrimidine nucleosides. However, the substrate specificity of BHPNP1 resembled that of Pu-NPaseI of thermophilic *G. stearothermophilus* strains, since it was able to catalyze phosphorolysis of guanosine (Visser *et al*., 2010) and inosine but adenosine was not utilized as a substrate. The ability of BHPNP1 to utilize guanosine and inosine, together with its reported thermal stability (60°C for 20.8 h) (Visser *et al*., 2010), suggests that it may be a very useful biological catalyst for the synthesis of modified nucleosides. Most notably, the recombinant enzyme could find application in the synthesis of 5'-methyluridine (5'-MU) which is a non-natural nucleoside that can be used as intermediate in the synthesis of the nucleoside analogues zidovudine (AZT) and stavudine, both of which are used for the treatment of HIV/AIDS (Ishii *et al*., 1989). 5'-MU is synthesized by a PNPase through the transglycosylation of D-ribose-1-phosphate, using guanosine or inosine as a donor and thymine as receptor (Hori *et al*., 1989c). Since a high temperature (60-70°C) is required to solubilize these substrates, the BHPNP1 enzyme may thus be well suited for the development of a preparative or industrial process whereby large amounts of 5'-MU can be synthesized.

In conclusion, the results obtained during the course of study indicated that the thermally regulated Pro2 promoter system can be used for the controlled expression of BHPNP1, a

purine nucleoside phosphorylase of *B. halodurans* Alk36. Based on its thermostability and substrate specificity profile, the recombinant BHPNP1 enzyme may find application in the synthesis of nucleoside analogues. In this regard, it is envisaged that cell lysates induced to overexpress the enzyme or purified BHPNP1 may be used in such applications.

CHAPTER FOUR

CONCLUDING REMARKS

The aims of this study were essentially (i) to evaluate two thermally regulated promoters in *Escherichia coli* and *Bacillus subtilis*, and (ii) to evaluate their utility for the overexpression of an enzyme of industrial importance in *B. subtilis*. The details of the results obtained in the course of achieving these objectives have been discussed in the individual Chapters. Here, the information that has evolved during this study will be summarized briefly and suggestions regarding future research will be made.

In the first part of the study, transcription fusions of the thermally regulated Pro2 and Pro3 promoters to different reporter genes (*lacZ* and *sapS*) were used to demonstrate controlled expression of the respective reporter genes in *E. coli* and *B. subtilis*. Quantitative enzyme activity measurements indicated that under inducing conditions both the Pro2 and Pro3 promoters are capable of driving similar high levels of enzyme activity in *E. coli*, whereas Pro2 is the stronger of the two promoters in *B. subtilis.* However, in both expression hosts, high basal expression was measured when ProB, rather than ProA, was used to drive expression of the temperature-sensitive C1 repressor protein. In this regard, it can be envisaged that higher repression/induction ratios could be obtained by minimizing basal expression levels. Future studies could therefore be directed to construct a promoter system comprising of a combination of the Pro2 and ProA promoters. The strength, as well as the basal expression and induced expression of the newly constructed thermally regulated promoter can then be evaluated according to the procedures described in this dissertation.

It is interesting to note that although previous studies have reported on the use of different temperature-inducible expression systems in *B. subtilis* (Liu *et al*., 2004; Serrano-Heras *et al*., 2005; Li *et al*., 2007), none of these have discussed the stress effects caused by the up-shift in temperature. In contrast to other expression systems, heat induction is likely to trigger a heat shock response, which includes a rapid and selective synthesis of heat shock proteins soon after the temperature increases (Georgopoulos and Welch, 1993; Price, 2002). Such an increase in protein synthesis may generate an unstable cellular environment that causes a critical metabolite burden which, in turn, impacts negatively the growth rate and the quantity of protein produced (Glick, 1995; Schumann, 2000). In *E. coli*, investigations regarding stress effects caused by recombinant protein production and the up-shift in temperature have indicated that, in addition to the heat shock response, a stringent stress response can also be elicited (Gill *et al*., 2000; Hoffman and Rinas, 2004). During the stringent stress response, the synthesis of tRNA and rRNA, as well as the transcription of genes from the transcriptional-

translational machinery are downregulated and thus the translation process is interrupted. This limits protein synthesis and the cell growth capacities during recombinant protein production (Dong *et al*., 1995; Cashel *et al*., 1996; Sanden *et al*., 2003). With regard to *B. subtilis*, transcriptomic and proteomic approaches may be used in future to analyze the nature and effects of cellular responses elicited by the simultaneous stress effects of recombinant protein production and the up-shift in temperature. Knowledge gained by these studies could be useful in order to develop improved strategies for high-yield protein production in this expression host.

In the second part of the study, the controlled expression of a thermostable purine nucleoside phosphorylase (PNP) of *B. halodurans*, designated BHPNP1, was demonstrated in *B. subtilis* and the substrate specificity of the recombinant enzyme was determined. Optimization of the induction temperature indicated that increased levels of recombinant BHPNP1 could be obtained at a temperature of 46°C and incubation for at least 4 h. Morover, the BHPNP1 enzyme catalyzes the phosphorolysis of guanosine and inosine but not adenosine. These properties make the BHPNP1 enzyme potentially useful in the production of 5'-methyluridine (5'-MU), an intermediate compound used for the synthesis of the antiviral nucleoside analogues zidovudine (AZT) and stavudine. Thus, future studies could aim to use the recombinant enzyme in the synthesis of 5'-MU by either using *B. subtilis* cell lysates in which BHPNP1 activity has been induced or by making use of the purified enzyme. Alternatively, the use of immobilized purified enzyme or intact cells can be investigated. The use of immobilized enzymes on solid supports has several advantages. Not only does it enable the reuse of the enzyme, but it may also increase the stability of the enzyme under a wide range of environmental conditions (Hori *et al*., 1989c, 1991; Rocchietti *et al*., 2004; Bavaro, 2008; Xie *et al*., 2011). In comparison, the immobilization of whole microbial cells could eliminate the costly process of enzyme extraction and purification (Yokozeki *et al*., 1982; Trelles *et al*., 2004; Luo *et al*., 2011). However, this approach may depend on whether the enzyme is located intracellularly or extracellularly, cell permeability, as well as the molecular weight and structure of the substrates and products. Furthermore, due to the barrier of the cell membrane, the activity of intracellular enzymes may be lower than that of purified enzymes (Numanoglu and Sungur, 2004; Luo *et al*., 2011). Despite these concerns, this latter approach may still be feasible since it has been reported that chemical and/or physical methods to increase cell permeability also resulted in improving the activity and catalytic efficiency of the enzymes (Numanoglu and Sungur, 2004).

Transglycosylation reactions involving the use of purine nucleoside phosphorylase enzymes and guanosine or inosine as substrates requires the use of high temperatures (60-70°C) in order to solubilize suitable concentrations of the substrates and products (Hori *et al*., 1989c, 1991). Considering that the *B. halodurans* BHPNP1 purine nucleoside phosphorylase enzyme has an optimum temperature of 70°C and a half-life of 20.8 h at 60°C (Visser *et al*., 2010), the availability of purified BHPNP1 may provide an opportunity to investigate the structure and function of the enzyme at high temperatures. The elucidation of mechanisms by which this thermophilic enzyme acquires its thermostability will not only yield information on the structure-stability relationship of the enzyme, but may also allow evaluation of strategies to improve its stability under such harsh reaction conditions. In this regard, detailed structural studies can be conducted on the purified BHPNP1 enzyme, and the results can be compared with those previously obtained for its mesophilic counterparts (Mao *et al*., 1997; Appleby *et al*., 1999, 2001). Such comparisons, together with mutational studies, may lead to the identification of key interactions and the role thereof in protein stability.

In summary, the data indicated that the two thermally regulated promoter systems evaluated during the course of this study may be used for the controlled expression of heterologous proteins in both *E. coli* and *B. subtilis*. Although future studies are required to minimize basal expression from the Pro2 promoter system, the utility of the promoter was nevertheless demonstrated through the controlled production of BHPNP1, a purine nucleoside phosphorylase with potential industrial application. A number of future research directions have also been highlighted that may result in increased production levels and improved stability of the enzyme at high temperatures. Information generated by these types of studies may culminate in a strategy for feasible large-scale bioproduction of nucleosides and nucleoside analogues.

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APPENDICES

A1 NUCLEOTIDE AND AMINO ACID SEQUENCE ALIGNMNETS

Nucleotide sequence of the *sapS* reporter gene that was used in this study.

sapS forward primer — Start codon

```
1     GGGTCGACCA TGAGGTGATA AGATGAATAAA ATTTCAAAGT ATATTGCAAT AGCATCATTA
61    TCGGTAGCGG TTACAGTTTC AGCACCACAAA CGACAAATTC TACAGCGTTT GCCAAAAGTT
121   CTGCTGAAGT TCAACAAACG CAACAAGCTTC TATACCAGCA TCACAAAAGG CGAATCTTGG
181   TAATCAAAAT ATTATGGCAG TGGCTTGGTAT CAAAATTCAG CTGAAGCAAA AGCATTATAT
241   TTACAAGGTT ATAACAGTGC AAAGACACAGT TAGATAAAGA GATTAAAAAG AATAAAGGTA
301   AACATAAGTT AGCTATTGCT TTGGATTTAGA TGAAACAGTT TTAGATAATT CTCCATATCA
361   AAGCTATGCA TCAATACATA ATAAACCTTTC CCAGAAGGTT GGCANGAATG GGTACAAGCT
421   GCTAAAGCTA AACCTGTCTT GGCGCAAAAGA ATTCTTGAAA TATGCTGACA AAAAAGGTGT
481   CGATATCTAC TATATTTCTG ATAGAGATAAA GAAAAAGATT TAAAGGCAAC ACAAAAGAAC
541   TTAAAACAAC AAGGTATCCC TCAAGCTAAGA AGAGTCATAT TTTACTAAAA GGTAAAGATG
601   ATAAGAGTAA AGAATCACGC AGACAAATGGT TCAAAAGGAT CATAAACTTG TCATGCTATT
661   TGGAGATATT TTATTAGACT TTACAGATCCA AAAGAAGCTA CAGCTGAATC TCGTGAAGCA
721   TTAATTGAAA AACATAAAGA CGATTTCGGTA AGAAATATAT CATTTTCCCT AACCCAATGT
781   ATGGTAGTTG GGAAGCTACG ATTTACAACAA TAACTATAAA GCAAGTGACA AAGCAAAAGA
841   TAAATTACGT AAAAATGCTA TTAAGCAATTC GATCCTAAAA CAGGCGAAGT TAAATAAGCA
901   TGCCC
```

sapS reverse primer — Stop codon

Amino acid sequence alignment of the SapS acid phosphatase used in this study and that reported previously by Du Plessis *et al.* (2002) (GenBank accession no. AY061973).

Upper line: SapS amino acid sequence, as determined in a previous study
Lower line: Amino acid sequence deduced from the above nucleotide sequence for the *sapS* gene

```
1     MNKISKYIAIASLSVAVTVSAPQTTNSTAFAKSSAEVQQTQQASIPASQKANLGNQNIMA
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1     MNKISKYIAIASLSVAVTVSAPQTTNSTAFAKSSAEVQQTQQASIPASQKANLGNQNIMA

61    VAWYQNSAEAKALYLQGYNSAKTQLDKEIKKNKGKHKLAIALDLDETVLDNSPYQGYASI
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
61    VAWYQNSAEAKALYLQGYNSAKTQLDKEIKKNKGKHKLAIALDLDETVLDNSPYQGYASI

121   HNKPFPEGWHEWVQAAKAKPVYGAKEFLKYADKKGVDIYYISDRDKEKDLKATQKNLKQQ
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
121   HNKPFPEGWHEWVQAAKAKPVYGAKEFLKYADKKGVDIYYISDRDKEKDLKATQKNLKQQ

181   GIPQAKKSHILLKGKDDKSKESRRQMVQKDHKLVMLFGDNLLDFTDPKEATAESREALIE
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
181   GIPQAKKSHILLKGKDDKSKESRRQMVQKDHKLVMLFGDNLLDFTDPKEATAESREALIE

241   KHKDDFGKKYIIFPNPMYGSWEATIYNNNYKASDKAKDKLRKNAIKQFDPKTGEV
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||
241   KHKDDFGKKYIIFPNPMYGSWEATIYNNNYKASDKAKDKLRKNAIKQFDPKTGEV
```

A2 NUCLEOTIDE AND AMINO ACID SEQUENCE ALIGNMNETS

Nucleotide sequence of the *B. halodurans* Alk36 BH1531 gene that was expressed in this study.

B1 forward primer — Start codon

```
1      CGGGTCGACG AACTTTAAGG GGGAACTGCC ATTATGCTTA ACGTAACTCA ATTGCAAGAA
61     GCGACTACAT TTATTCAACA GCAAATAGAA ACAAAACCAA CGATCGGTTT AATTTTAGGT
121    TCTGGTTTAG GGATTTTAGC TGATGAGATC GAACAGCCGG TGAAAGTTCC TTACAGTGAC
181    ATTCCACATT TTCCTGTCTC TACCGTCCAA GGACATGCGG GCCAGCTTGT GATCGGCATG
241    CTTGAAGGAA AGCAAGTGAT TGCGATGCAA GGGCGATTTC ATTTTTATGA AGGCTACAGC
301    CTTGAGGTTG TCACATTCCC TGTCCGCGTC ATGAAAGCTC TAGGTGTAGA ACAAATCATT
361    GTCACAAATG CAGCGGGCGG TGTGAACGAA TCGTTCGAAG CGGGTGACCT AATGATCATT
421    CGCGACCATA TTAACAACAT GGCACAAAAC CCACTAATTG GGCCGAATGA TGAGGCGTTC
481    GGCGTACGTT TTCCGGATAT GTCAAATGCC TACTCTGAGC GCTTACGAAC GTTAGCGAAG
541    GAAAAGGGAA ATACGTTGAA TCTCAAGCTA CAGGAAGGGG TCTATGTTGC GAACACAGGT
601    CCTGTTTATG AAACCCCTGC TGAAGTGCGG ATGATTCGAA AACTTGGCGG AGATGCTGTA
661    GGTATGTCAA CCGTACCAGA AGTCATTGTC GCTCGTCATG CAGGACTTGA GGTTCTTGGA
721    ATTTCATGTA TTTCTAACAT GGCGGCAGGA ATTTTACCAC AGCCTTTATC ACATGATGAA
781    GTCATTGAAA CAACGGAACG AGTGAGGCAA GACTTTCTTA ATCTTGTCAA AGCAATCGTT
841    AAAGACATGT AAACAAAGGA GAGGACTACG GCATGCCCG
```

Stop codon — B1 reverse primer

Amino acid sequence alignment of the BH1531 purine nucleoside phosphorylase expressed in this study and that reported previously for *B. halodurans* C-125 by Takami *et al*. (2000) (GenBank accession no. NC-002570).

Upper line: Purine nucleoside phosphorylase amino acid sequence, as determined in a previous study
Lower line: Amino acid sequence deduced from the above nucleotide sequence for the *B. halodurans* Alk36 BH1531 gene

```
1      MLNVTQLQEATTFIQQQIETKPTIGLILGSGLGILADEIEQPVKVPYSDIPHFPVSTVQG
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
55     MLNVTQLQEATTFIQQQIETKPTIGLILGSGLGILADEIEQPVKVPYSDIPHFPVSTVQG

61     HAGQLVIGMLEGKQVIAMQGRFHFYEGYSLEVVTFPVRVMKALGVEQIIVTNAAGGVNES
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
115    HAGQLVIGMLEGKQVIAMQGRFHFYEGYSLEVVTFPVRVMKALGVEQIIVTNAAGGVNES

121    FEAGDLMIIRDHINNMAQNPLIGPNDEAFGVRFPDMSNAYSERLRTLAKEKGNTLNLKLQ
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
175    FEAGDLMIIRDHINNMAQNPLIGPNDEAFGVRFPDMSNAYSERLRTLAKEKGNTLNLKLQ

181    EGVYVANTGPVYETPAEVRMIRKLGGDAVGMSTVPEVIVARHAGLEVLGISCISNMAAGI
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
235    EGVYVANTGPVYETPAEVRMIRKLGGDAVGMSTVPEVIVARHAGLEVLGISCISNMAAGI

241    LPQPLSHDEVIETTERVRQDFLNLVKAIVKD
       ||||||||||||||||||||||||||||||
295    LPQPLSHDEVIETTERVRQDFLNLVKAIVKD
```