Development and evaluation of a reporter system for prokaryotic cells based on a secreted acid phosphatase from *Staphylococcus aureus* strain 154

by

ERIKA MARGARETE DU PLESSIS

Submitted in partial fulfillment of the requirements of the degree
Philosophiae Doctor
in the Faculty of Natural and Agricultural Sciences
University of Pretoria
Pretoria

February 2008

This thesis is dedicated to my son

In memory of my grandparents

ACKNOWLEDGEMENTS

I wish to express my sincere thanks and appreciation to:

Prof. J Theron for his unfailing support during the course of my studies. Your enthusiasm, guidance and hard work contributed a great deal to the completion of this thesis.

Dr. Eldie Berger, my friend and colleague, for invaluable scientific guidance and always being there for me.

Dr. Maureen Louw for critical review of the manuscript.

To Michael Crampton for his help and patience with drawing graphs and editing figures.

Lastly, my heartfelt thanks to my family for believing in me and their support through the years.

DECLARATION

I declare that the thesis, which I hereby submit for the degree, Philosophiae Doctor (Microbiology) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at another University.

Signed: …….. Date: ……..

Development and evaluation of a reporter system for prokaryotic cells based on a secreted acid phosphatase from *Staphylococcus aureus* strain 154

by

ERIKA MARGARETE DU PLESSIS

Supervisor: Prof. J. Theron
Department of Microbiology and Plant Pathology
University of Pretoria

Co-supervisor: Dr. M.E. Louw
Biosciences
Council for Scientific and Industrial Research

for the degree PhD

Reporter gene technology has facilitated greatly the analysis of gene expression and the study of individual promoters and their regulation. Although various reporter gene systems are available, none of them are universally applicable and consequently, studies aimed at screening of new reporters are continuing. Toward this end, an acid phosphatase, designated SapS, was identified and characterized from the culture supernatant of a *Staphylococcus aureus* strain isolated from vegetables. Biochemical characterization of the 30-kDa monomeric enzyme indicated that it displayed optimum activity at 40°C and pH 5, using *p*-nitrophenyl phosphate (*p*NPP) as substrate. The enzymatic activity was enhanced by Mg^{2+}, but was inhibited by EDTA and molybdate. Based on its properties and amino acid sequence analyses, SapS was classified as a new member of the bacterial class C family of non-specific acid phosphatases.

The *S. aur eus* SapS enzyme was subsequently evaluated as a reporter for host strain evaluation and cell surface display. *Bacillus hal odurans* of which the major cell wall protease gene (*wprA*) was inactivated was used as expression host, and the cell wall-binding domain of the *cwlC* gene from *B. halodurans* was used as an anchoring motif for cell surface display. The results from *in vitro* enzyme activity assays indicated that extracellular production of the SapS reporter enzyme was improved 3.5-fold in the mutant compared to wild-type *B. hal odurans* strain. Zymographic detection of SapS activity showed that the

SapS-CwlC fusion protein was localized in the *B. halodurans* cell wall fraction, thus demonstrating the potential of SapS as a reporter for cell surface display of heterologous proteins. The versatility of the SapS enzyme as a reporter for gene expression and protein secretion in both Gram-positive and Gram-negative bacteria was also investigated. Transcriptional and translational fusions of the *sapS* gene with selected heterologous promoters and signal sequences were constructed, and expressed in *Escherichia coli*, *B. subtilis* and *B. halodurans*. The strongest promoter for heterologous protein production in each of the host strains was identified, *i.e.* the *E. coli lacZ* promoter in *E. coli*, the *B. halodurans* alkaline protease promoter in *B. subtilis*, and the *B. halodurans* σ^D promoter in *B. halodurans*.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

%	percentage
× *g*	centrifugal force
°C	degrees Celsius
α	alpha
β	beta
µg	microgram
µl	microlitre
µM	micromolar
4-MUP	4-methylumbelliferylphosphate
Å	angstrom
AMP	adenosine monophosphate
Amp^r	ampicillin resistance
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	base pair
C	carboxy
CAT	chloramphenicol transacetyltransferase
Chl^r	chloramphenicol resistance
dNTP	deoxyribonucleoside-5'-triphosphate
DTT	dithiothreitol
e.g.	for example
EDTA	ethylenediaminetetra-acetic acid
Fig.	figure
GFP	green fluorescent protein
GUS	β-glucuronidase
h	hour
HAD	haloacid dehalogenase
i.e.	that is
IPTG	isopropyl β-D-thiogalactoside
kb	kilobase pairs
kDa	kilodalton
L	litre
LB medium	Luria-Bertani medium
M	molar
MG	methyl green

mg	milligram
min	minute
ml	millilitre
mM	millimolar
mU	milliunits
N	amino
NBT	nitro blue tetrazolium
no.	number
NSAP	non-specific acid phosphatase
NTPs	nucleotide triphosphates
o	*ortho*
OD	optical density
ORF	open reading frame
p	*para*
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PDP	phenolphthalein diphosphate
P_i	inorganic orthophosphate
*p*NP	*p*-nitrophenol
*p*NPP	*p*-nitrophenyl phosphate
PVDF	Immobilon-P polyvinylidene difluoride
rpm	revolutions per minute
s	second
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
U	units
UMP	uridine monophosphate
v.	version
v/v	volume per volume
vs.	versus
w/v	weight per volume

LIST OF FIGURES

LIST OF TABLES

RESEARCH COMMUNICATIONS

Papers published:

1. **du Plessis, E.M.,** Theron, J., Joubert, L., Lotter, T. and Watson, T.G. (2002). Characterization of a phosphatase secreted by *Staphylococcus aureus* strain 154, a new member of the bacterial class C family of non-specific phosphatases. Syst. Appl. Microbiol. 25: 21-30.

2. **du P lessis, E .M.,** Theron, J., Berger, E. and Louw, M.E. (2007). Development of the *Staphylococcus au reus* class C non-specific acid phosphatase (*SapS*) as a reporter for gene expression and protein secretion in Gram-negative and Gram-positive bacteria. Appl. Environ. Microbiol. 73: 7232-7239.

Conference contributions:

National conferences:

1. **du Plessis E.M.,** Louw, M.E., Berger, E. and Theron, J. Evaluation of *Staphylococcus aureus* phosphatase (*sapS*) gene as a reporter for gene expression and secretion in Gram-negative and Gram-positive bacteria. The 14th Biennial Congress of the South African Society for Microbiology, April 2006, Pretoria, South Africa.

2. **du Plessis, E.M.** Characterization of the *Staphylococcus aureus* strain 154 acid phosphatase and its development as a reporter system. CSIR Biosciences Science Day, 26 June 2007, Pretoria, South Africa.

International conferences:

1. **du Plessis, E.M.**, Berger, E. and Louw, M.E. (2002). Development of *Staphylococcus aureus* acid phosphatase (*sapS*) gene as a reporter for expression/secretion in Gram-positive bacteria. Seventh Symposium on Lactic Acid Bacteria, September 2002, Egmond aan Zee, The Netherlands.

CHAPTER ONE

LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

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. Moreover, the construction of translational fusions have also allowed for monitoring the fate of a protein of interest, *e.g.*, degradation, subcellular localization, secretion into the extracellular milieu or translocation into other cells (Gober *et al*., 1995; De Kievit *et al*., 2001; Urbain, 2001; Møller *et al*., 2005; Bumann and Valdivia, 2007). The results from these analyses have found application in both basic and applied sciences. In molecular and cellular biology, reporter gene technology has contributed to improved understanding of the cellular events associated with signal transduction and gene expression, and has also provided spatial information about a particular gene product (Welsh and Kay, 1997; Tsien and Miyawaki, 1998; Veening *et al*., 2004; Koga *et al*., 2006). In biotechnology, reporter gene technology has been used for the identification of novel promoters that can be used to establish improved expression systems for high-level production of recombinant proteins (Cagnon *et al*., 1991; Herbst *et al*., 1994; Serrano-Heras *et al.*, 2005; Miksch *et al.*, 2006).

A reporter gene can be defined as a gene with a readily measurable phenotype that can be distinguished easily over a background of endogenous proteins (Wood, 1995; Naylor, 1999). Generally, such reporters are selected on the basis of the sensitivity, dynamic range, convenience and reliability of their assay (Bronstein *et al.*, 1994; Wood, 1995; Schenborn and Groskreutz, 1999). Various reporter genes have been used for molecular genetic analyses and their encoded products all share the ability to glow, to fluoresce or to be assayed colorimetrically (Naylor, 1999; Hautefort and Hinton, 2000). However, none of the reporter systems used is universally applicable, since each reporter has its own advantages and disadvantages that may limit its use in some cells of model organisms and in certain types of studies. Therefore, studies aimed at modifying the widely used reporter systems (Hautefort *et*

al., 2003; Choe *et al*., 2005), extending the methods used for measuring reporter activity (Thibodeau *et al*., 2004; Hampf and Gossen, 2006) and screening of new reporters (Post and Luebeke, 2005; Schmidt *et al*., 2005) are continuing.

Similar to alkaline phosphatase, which has been used successfully as a reporter in both genetic analyses and in enzyme immunoassays (Reuber *et al*., 1991; Manoil, 2000), bacterial non-specific acid phosphatases (NSAPs) may also be exploitable as reporters in molecular biology. Bacterial non-specific acid phosphohydrolases (NSAPs) are secreted enzymes, produced as soluble proteins associated with the periplasmic space or as membrane-bound lipoproteins. They generally dephosphorylate a broad array of substrates and have optimal catalytic activity at an acidic to neutral pH (Rossolini *et al*., 1998). Many bacterial acid phosphatases function as scavengers of organic phosphoesters (Beacham, 1979), while some of them participate in an assortment of essential biological functions, including the regulation of metabolism, energy conversion and signal transduction (Stock *et al*., 1995; Klumpp and Krieglstein, 2002). Indeed, some NSAP-encoding genes have already been exploited as targets for insertional inactivation in cloning vectors that allow direct identification of recombinants. Using these vectors, recombinants were easily identified on the basis of their acid phosphatase-negative phenotype, while clones containing non-recombinant vector exhibited a phosphatase-positive phenotype (Burioni *et al*., 1995; Thaller *et al*., 1998a).

In this review of the literature, information pertinent to reporter molecules and bacterial non-specific acid phosphatases (NSAPs) will be discussed, as they are both closely related to the aims of this study.

1.2 PROKARYOTIC GENETIC REPORTER SYSTEMS

The choice of a reporter system is determined by a number of criteria. An important consideration in this regard, is the availability of simple and sensitive methods for the quantitative and qualitative determination of reporter protein activity against the background of other cell components (Wood, 1995; Naylor, 1999). Other important properties include the ability to follow dynamic changes in gene expression (in this case, important characteristics are half-life and/or duration of protein maturation), the size of reporter gene and its product (for convenience of cloning and analysis of reporter protein expression), the absence of activities similar to that of the reporter protein in the cells under study, as well as

the ability of the reporter protein to tolerate other proteins fused at its N- and C-termini without a loss of activity (Jefferson *et al.*, 1986; Jefferson *et al.*, 1987; Joyeux *et al.*, 1997; Le Loir *et al.*, 1998; Langella *et al.*, 1999; Naylor, 1999). A variety of reporter gene systems is available and has been used extensively for analysis of different aspects of gene expression. The advantages and limitations of selected reporter genes are summarized in Table 1.1, while commonly used reporter genes are discussed in greater detail in the sections below.

1.2.1 Genes encoding 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. These include, amongst other, the *xylE* gene of *Pseudomonas put ida* that encodes catechol 2,3-dioxygenase (Curcic *et al*., 1994), the *phoA* gene of *Escherichia coli* that encodes alkaline phosphatase (Reuber *et al*., 1991), the *licB* gene of *Clostridium the rmocellum* that encodes a thermostable lichenase (Piruzian *et al* ., 2002), the *amyL* gene of *Bacillus subtilis* that encodes an α-amylase (Ugorcakova *et al*., 2000), and the *melA* gene of *Saccaharopolyspora e rythrea* (Post and Luebeke, 2005) and of *Lactobacillus plantarum* (Hörmann *et al*., 2006) that encodes an α-galactosidase. However, the *E. c oli lacZ* and *gusA* genes, encoding β-galactosidase and β-glucuronidase, respectively, are the most popular reporter genes of this type and thus warrant further discussion.

- **β-galactosidase**

The *lacZ* gene, encoding β-galactosidase, from *E. coli* was one of the first genes to be used as a reporter gene (Casabadan and Cohen, 1980; Drahos *et al.*, 1986), and is still one of the most widely used reporter genes (Poyart and Trieu-Cuot, 1997; Talukder *et al* ., 2005). Its popularity has been due to the ease by which *lacZ* gene activity can be assayed quantitatively using a variety of 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, the enzyme activity assay can be performed in cuvettes or in microtiter plates, and the samples can be read on an enzyme-linked immunosorbent (ELISA) plate reader, spectrophotometer, luminometer or fluorometer, depending on the product of the reaction (Jain and Magrath, 1991; Miller, 1992; Schenborn and Groskreutz, 1999).

Table 1.1: Advantages and limitations of selected genetic reporters

Reporter protein	Advantages	Limitations	References
β-galactosidase	Easy to assay Variety of assay formats for use with cell extracts	Many cell types have high endogenous β-galactosidase activity Lower sensitivity in non-chemiluminescent assays	Schenborn and Groskreutz (1999)
β-glucuronidase (GUS)	Various assay formats available for different applications GUS protein is stable Chemiluminscent assay is very sensitive	Assays with best sensitivity require fluorometer or luminometer (scintillation counter can substitute)	Schenborn and Groskreutz (1999) Kim *et al.* (2006)
Chloramphenicol acetyltransferase (CAT)	Various assay formats available Stable protein	Assays are time-consuming and laborious Relatively low sensitivity	Schenborn and Groskreutz (1999)
Luciferase	Fast and easy assays available High sensitivity Large linear range	Short half-life of protein Requires a luminometer for high sensitivity assays	Wood (1995)
Green fluorescent protein (GFP)	No substrates required Stable reporter protein. No apparent toxic effects of GFP expression in bacteria or eukaryotes	Signal intensity may be too weak for some applications	Southward and Surette (2002)
Nuclease	Export-specific reporter gene for isolation and evaluation of secretion signals	Not widely utilized	Le Loir *et al.* (1994)

Chemiluminescent assays, however, display the largest dynamic range and can be developed with 1,2-dioxetane substrates (Jain and Magrath, 1991). It has been reported that the chemiluminescent assay format is similar in sensitivity to the bioluminescent luciferase assays (Schenborn and Groskreutz, 1999). 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* include the stability of the enzyme that prevents the study of temporal gene expression, and the denaturation of the enzyme at pH values below neutrality (Burne and Chen, 2000).

As an alternative to the *lacZ* gene from *E. coli*, several publications have reported the use of *lacZ* genes, encoding thermostable β-galactosidases, as reporters for gene expression studies. These have included the thermostable β-galactosidases from *Thermus* sp. A4 (Park and Kilbane, 2004) and from *Thermoanaerobacterium thermosulfurigenes* (Feustel *et al*., 2004). Notably, the thermostable β-galactosidase of *T. thermosulfurigenes* has been used to analyze the transcriptional activity of promoters of genes required for acidogenesis and it has also been suggested to be a valuable reporter for various *Clostridium* spp. (Feustel *et al*., 2004). This is due to its codon usage being similar to that of clostridial species and it also has a low G+C content (31.5%) that is typical of that found in clostridial species (Feustel *et al*., 2004). Moreover, the *celB* gene from the hyperthermophilic *Pyrococcus furiosus* encodes a thermostable β-glucosidase with a high β-galactosidases activity, which can be determined at temperatures up to 100°C (Voorhoorst *et al*., 1995). Since the endogenous β-galactosidase enzyme in most bacteria can be destroyed at high temperature (≥50°C), the advantage of using thermostable β-galactosidase enzymes is that the reporter enzyme activity can be detected at elevated temperatures at which the endogenous enzymes are destroyed, and its use is therefore not limited to strains with a mutated *lacZ* gene (Sessitsch *et al.*, 1996).

- **β-glucuronidase**

The *E. coli gusA* (*uidA*) gene, encoding β-glucuronidase (GUS), has been used widely as a reporter gene in plant molecular biology, since GUS activity has not been detected in most species of plants (Jefferson *et al*., 1986; Jefferson *et al*., 1987). However, there have been several reports regarding the use of *gusA* as a reporter gene in lactic acid bacteria (Platteeuw *et al*., 1994), *Clostridium acetobutylicum* (Girbal *et al*., 2003) and bacteria of agricultural

importance such as *Rhizobium*, *Agrobacterium* and *Pseudomonas* spp. (Reuber *et al*., 1991; Wilson *et al.*, 1992; Sun *et al.*, 2001).

Like the β-galactosidase reporter, one of the principal advantages of GUS is the wide range of assays available for the enzyme (Jefferson and Wilson, 1991). Several different colorimetric assays have been developed using a variety of β-glucuronides as substrates. Substrates such as X-glcA (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) or Magenta-glcA (5-bromo-6-chloro-3-indolyl-β-D-glucuronide) release an indigo or magenta coloured precipitate that allows for easy visualization of the enzymatic activity. In addition, fluorescent and chemiluminescent assays have been developed utilizing substrates such as 4-MUG (4-methylumbelliferylglucuronide) and 1,2-dioxetane aryl glucuronide substrates (Bronstein *et al* ., 1994), respectively. These assays allow for quantification of enzyme activity by fluorometry and luminometry. The sensitivity of the assays obtained with the above substrates vary greatly, with the chemiluminescent assays being 100-fold more sensitive than the fluorescent assays, and the latter can be 100- to 1000-fold more sensitive that most colorimetric assays (Schenborn and Groskreutz 1999; Kim *et al.*, 2006). However, disadvantages noted for this reporter system have included the inhibition of GUS activity by some heavy metals ions (*e.g.*, Cu^{2+} and Zn^{2+}) and its long half-life period (50 h), which precludes studies regarding temporal changes in gene expression (Mascaronhas and Hamilton, 1992; Quaedclieg *et al.*, 1998; Kim *et al.*, 2006).

1.2.2 Antibiotic resistance genes

A number of antibiotic resistance genes have been used as reporter genes, including the gentamycin resistance gene (*aacC1*) (Labes *et al*., 1997), as well as the ampicillin resistance genes *ampC* (Forsman and Jaurin, 1987) and *blaM* (Sibakov *et al*., 1991). The bacterial *cat* genes, encoding chloramphenicol transacetyltransferase (CAT), which confers chloramphenicol resistance upon their host, have been particularly useful. This has been due to the fact that expression of these reporter genes can not only be selected for in growth medium, but also quantified via enzyme assays for CAT activity (Shaw, 1975; Schenborn and Groskreutz, 1999). Consequently, *cat* genes have been employed to monitor qualitative and quantitative gene expression in a wide variety of Gram-negative and Gram-positive bacteria (Osbourne *et al*., 1987; Cao *et al*., 2001; Jeong *et al*., 2006; Kaur *et al*., 2007). CAT seems fairly stable in cells growing at pH values between 4.0 and 7.5 and it has also 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, as well as ELISA reactions with anti-CAT antibodies and anti-CAT substrates. However, assays for CAT activity are time-consuming, more expensive than those for β-galactosidase activity and it displays relatively low sensitivity and a narrow linear range (two orders of a magnitude) (Naylor, 1999).

1.2.3 Bioluminescence genes

Luciferase activity (bioluminescence) is frequently used as a reporter system in both prokaryotic and eukaryotic cells (De Wet *et al* ., 1985; Gould and Subramani, 1988; Palomares *et al*., 1989; Greener *et al*., 1992; Stewart and Williams, 1992). Luciferase is an oxidase that catalyzes the oxidation of reduced flavin ($FMNH_2$) to form intermediate peroxide. The intermediate product then reacts with a long-chain aldehyde to give blue-green luminescence, emitting at 490 nm, as well as oxidized flavin plus the corresponding long-chain fatty acids as products (Wilson and Hastings, 1998). There are several attractive features to using luciferase as a reporter for gene expression, *e.g.*, luciferase has a short half-life and thus ensures that photon production reflects real-time gene expression, low levels of light can be measured and linearly quantified over several orders of magnitude, and since luciferase enzymes are not widespread in bacteria, endogenous background bioluminescence does not present a significant problem (Hautefort and Hinton, 2000).

Although luciferase genes have been cloned from various organisms, including copepods and dinoflagellates (Bae and Hastings, 1994; Li and Hastings, 1998), the most commonly used luciferases for reporter gene assays are the bacterial luciferases (*Vibrio fisheri*; *lux*), the firefly luciferase (*Photinus pyralis*; *luc*) and the Renilla luciferase (from the bioluminescent sea pantsy, *Renilla reniformis*) (De Wet *et al.*, 1987; Meighen, 1991; Lorenz *et al.*, 1994). As a reporter, the *Renilla* luciferase does not appear to offer any particular advantages over firefly luciferase and its assay chemistry is more limited. However, it has become popular as a companion reporter for experiments where two different reporters are needed (McNabb *et al*., 2005; Hampf and Gossen, 2006). The luciferase gene (*gluc*) from the marine copepod *Gaussia pr inces* has also been evaluated and compared to the bacterial *luxAB* reporter, following their expression in *Mycobacterium s megmatis* (Wiles *et al*., 2005). In contrast to *luxAB*, the *gluc* luciferase retained its luminescence output in the stationary phase of growth

and exhibited enhanced stability at low pH, high temperature and in the presenc of hydrogen peroxide. It was subsequently proposed that the copepod luciferase reporter can be used as an alternative to bacterial luciferase, particularly for monitoring responses to environmental stress stimuli (Wiles *et al*., 2005).

Although bioluminescence represents a powerful tool for genetic analysis, it may be problematic to use, since the reaction requires the addition of an exogenous substrate for the light reaction to occur. An aldehyde substrate, usually *n*-decanal, must be supplied to the cells during the assay procedure if using the *luxAB* genes as reporter. With the exception of *Steptomyces* cell types, the substrate penetrates most cells readily, but it can be toxic at relatively low doses (Craney *et al* ., 2007). To circumvent the addition of the aldehyde substrate, investigators have cloned all five genes of the *lux* operon (*luxCDABE*), which allows a completely independent light-generating system that requires no additional substrate (Applegate *et al.*, 1998; Hay *et al.*, 2000; Craney *et al*., 2007). However, cells containing the entire *lux* operon exhibit reduced viability compared to cells containing only *luxAB* (De Weger *et al* ., 1991; Amin-Hanjani *et al* ., 1993). Often the assays also use extracts from samples, rather than whole cells, and therefore destructive sampling is required (Møller *et al.*, 1995).

Bioluminescent reporter genes can be directly monitored by visual or microscopic observations (Masson *et al*., 1993; Flemming *et al.*, 1994), by measuring the light input in a luminometer (Rattray *et al* ., 1990) or a scintillation counter in chemiluminescence mode (Belas *et al* ., 1986). In addition to being able to obtain luciferase assay results within minutes, the sensitivity of the luciferase assay is in the subattomole range and approximately 30- to 1000-fold greater compared to the CAT assay (Pazzagli *et al.*, 1992). The light output is indicative of a metabolically active population of cells, since the luciferase enzymes are dependent on cellular activity reserves or reducing equivalents for bioluminescence. However, after long-term incubation, microbial cells often become starved or stressed and the light production from luciferase enzymes declines as a response to the change in cellular energy status (Duncan *et al.*, 1994). Therefore, *in situ* bioluminescence may not be a reliable indicator and limits its utility.

1.2.4 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; Cubitt *et al*., 1995). 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 (Stewart and Williams, 1992; Chalfie *et al*., 1994; De Weger *et al*., 1994; Inouye and Tsuji, 1994). In addition, GFP is non-toxic, 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 commonly 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 (Tombolini *et al*., 1997). Whereas stability of the protein is advantageous for the environmental monitoring of GFP-tagged cells, it can be problematic in studies regarding temporal changes in gene expression, since, once the reporter protein is synthesized, it may persist. In this regard, the utility of GFP as a reporter has been optimized through the isolation and engineering of variants with different folding rates and different *in vivo* stabilities (Heim *et al*., 1994; Cormack *et al*., 1996; Keiler *et al*., 1996; Andersen *et al*., 1998). Additionally, various groups have obtained GFP mutants exhibiting diverse spectral properties that may allow simultaneous analysis of gene expression from a number of different promoters (Delgrave *et al.*, 1995; Ehrig *et al.*, 1995; Mena *et al*., 2006).

Depending on the nature of the study, GFP fluorescence can be monitored by exposing bacterial colonies to UV light (Tresse *et al.*, 1998), by fluorometric detection (Burlage *et al*., 1996), epifluorescence microscopy (Chalfie *et al*., 1994; De Kievit *et al*., 2001), confocal laser scanning microscopy (Eberl *et al*., 1997) or flow cytometry (Tombolini *et al*., 1997; Mena *et al*., 2006). Numerous *gfp*-based reporter systems have been developed for use in especially environmental applications to study GFP-tagged bacteria (Chalfie *et al*., 1994; Eberl *et al*., 1997; Southward and Surette, 2002), as well as for studying gene expression in Gram-positive (Chen *et al*., 2003; Chary *et al*., 2005; Serrano-Heras *et al*., 2005) and Gram-negative bacteria (De Kievit *et al.*, 2001; Miksch *et al.*, 2006).

1.2.5 Export-specific reporter genes

Different export-specific reporters have been described that allows for the identification and characterization of the exported proteins, as well as for screening and characterization of signal peptides. The *E. coli* PhoA alkaline phosphatase, encoded by *phoA* (Hoffman and Wright, 1985; Manoil and Beckwith, 1985; Lim *et al*., 1995; Manoil, 2000; Gibson and Caparon, 2002), and TEM β-lactamase, encoded by *blaM* (Broom-Smith *et al*., 1990; Sibakov *et al.*, 1991), has each been used successfully as export reporters in different Gram-negative bacteria. However, they are not well suited for use in Gram-positive bacteria. Not only did expression of the *E. coli* reporter genes decrease, probably as a consequence of differences in codon usage and G+C content, but also fusions to these reporters displayed little activity in Gram-positive bacteria, which has been ascribed to improper folding of the fusion proteins (Smith *et al.*, 1987; Pearce *et al.*, 1993; Poquet *et al.*, 1998).

To overcome the above limitations, different Gram-positive export reporters have been described, including α-amylase, encoded by the *amyL* genes of *Bacillus amyloliquefaciens* (Palva *et al.*, 1982) and *B. licheniformis* (Hols *et al.*, 1992), and a tyrosinase, encoded by the *melC* operon, of *Streptomyces glaucescens* (Adham *et al*., 2003). However, the *Staphylococcus aureus* secreted nuclease (Nuc) that is encoded by *nuc*, has remained the most popular export-specific reporter for use in Gram-positive bacteria. The *S. aureus* secreted nuclease has been genetically and biochemically well characterized, and is a small, stable monomeric protein, devoid of cysteines residues (Shortle *et al*., 1983). Nuc is also efficiently secreted by various Gram-positive bacteria as an active 168-amino-acid polypeptide, which may undergo subsequent proteolytic cleavage of the N-terminal 19- to 21-amino-acid propeptide to give rise to another active form, called NucA (Kovacevic *et al*., 1985; Lieble *et al*., 1992; Miller *et al*., 1997). Since the staphylococcal nuclease is inactive intracellularly, it has been particularly effective in the screening and characterization of signal peptides (Poquet *et al.*, 1998; Ravn *et al.*, 2000). Moreover, translational fusions to the N-terminus and/or the C-terminus of the mature nuclease protein have been reported to be enzymatically active (Piard *et al*., 1997; Le Loir *et al*., 1998; Langella *et al*., 1999). The enzymatic activity can be readily detected by spectrophotometric assays (Kovacevic *et al*., 1985), zymography (Lieble *et al.*, 1992) and plate assays, which are non-toxic to the colonies and thus eliminates the need for replica plating (Le Loir *et al.*, 1994; Poquet *et al.*, 1998).

1.3 BACTERIAL NON-SPECIFIC ACID PHOSPHOHYDROLASES

Bacterial non-specific acid phosphohydrolases or phosphatases (NSAPs; EC 3.1.3.2) are secreted enzymes that function as soluble periplasmic proteins or as membrane-bound lipoproteins (Rossolini *et al*., 1998). They are a diverse group of monomeric or oligomeric proteins with monomer molecular weight in the range of 25-30 kDa. Bacterial NSAPs do not exhibit marked substrate specificity and can dephosphorylate dispartate phosphomonoesters (*e.g.*, nucleotides, sugar phosphates and phytic acid), by hydrolysis of phosphoester or phosphoanhydride bonds (Vincent *et al*., 1992) at acidic to neutral pH (Beacham, 1979).

Although the physiological function of bacterial NSAPs is largely unclear, it is generally believed that they function in the periplasmic space as organophosphate-degrading enzymes. The hydrolysis of organophosphates in the periplasm allows inorganic orthophosphate (P_i) and organic by-products, released during the catalysis, to be transported across the cytoplasmic membrane, thus providing the bacterial cell with essential nutrients (Oliver, 1996). Moreover, some secreted phosphatases such as the respiratory burst-inhibiting acid phosphatase of *Francisella tularensis* (Reilly *et al*., 1996) and the tyrosine phosphatases of *Yersinia* spp. (Bliska *et al*., 1991) and *Salmonella enterica* serovar Typhimurium (Kaniga *et al.*, 1996) have been implicated in bacterial virulence and pathogenecity. Other phosphatases, localized to the cytosol, may be involved in dephosphorylating reactions occurring in signal transduction and in metabolic pathways (Stock *et al*., 1995; Klumpp and Krieglstein, 2002; Kirstein and Turgay, 2005). In addition to their multiple roles in cell physiology and pathogenecity, interest in bacterial acid phosphatases also extends to the possibility of exploiting these enzymes as reporter genes for analysis of gene expression and regulation (Payne and Jackson, 1991; Makino *et al* ., 1994), paradigms for molecular evolution (Thaller *et al*., 1998b), markers for bacterial taxonomy and identification (Pompei *et al*., 1990; Pompei *et al*., 1993), and in bioremediation of heavy metals from wastewater via acid phosphatase-mediated metal biomineralization processes (Macaskie, 1990; Basnakova *et al*., 1998).

Despite some bacterial NSAPs having been identified and described more than 30 years ago (Dvorak *et al*., 1967; Malveaux and Clemente, 1967; Kier *et al*., 1977; Weppelman *et al*., 1977), additional insights regarding their distribution (Satta *et al.*, 1988; Pompei *et al.*, 1993; Groisman *et al*., 1992; Thaller *et al*., 1995a), properties (Thaller *et al*., 1995a; Thaller *et al*.,

1997b; Godlewska *et al.*, 2002; Chen *et al.*, 1999) and structure (Makde *et al.*, 2003b; Felts *et al.*, 2006; Calderone *et al*., 2006) have only more recently been obtained. Consequently, in the following sections current knowledge on these enzymes relating to methods for studying bacterial NSAPs and descriptions of known bacterial NSAPs will be presented and compared.

1.3.1 Qualitative an d q uantitative as says f or s tudying b acterial n on-specific ac id phosphatase enzyme activity

Numerous screening methods have been developed for the isolation of acid phosphatase-producing bacteria (Pompei *et al*., 1990; Thaller *et al*., 1992; van Ommen Kloeke *et al*., 1999), the selection of acid phosphatase-encoding genes (Boquet *et al*., 1987; Pond *et al*. 1989; Riccio *et al*., 1997; Passariello *et al*., 2003), and the discrimination of microbial taxa (Satta *et al*., 1988, Pompei *et al*., 1993; Thaller *et al*., 1995a). Screening methods based on acid phosphatase activity have also been applied to detect bacteria transformed with recombinant plasmids as an alternative to the conventional *lacZ* system. In these studies, vectors harbouring different acid phosphatases have been designed as tools for cloning-dependent insertional inactivation (Burioni *et al*., 1995; Thaller *et al*., 1998a). The screening methods reported in the above studies comprised qualitative plate screen assays, zymogram techniques and quantitative *in vitro* enzyme assays.

1.3.1.1 Plate screen assays

Methods for assaying acid phosphatase activity of bacterial colonies rely on phosphorylated substrates that are either added to the growth medium or provided by flooding colonies with the catalytic solution. Although this approach has the advantage that many different bacterial isolates can be screened in a single step, it does not yield much information regarding the properties of the enzyme. However, gross differences in acid phosphatase production can be detected (Satta *et al.*, 1988; Pompei *et al.*, 1990; Pompei *et al.*, 1993). The substrates used in plate screen assays typically yield a coloured or fluorescent product after orthophosphate (P_i) release. Bacterial colonies stain yellow, blue, deep green, or black when P_i is released from *p*-nitrophenyl phosphate (*p*NPP) (Boquet *et al.*, 1987), 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Chaffin and Rubens, 1998), phenolphthalein diphosphate/methyl green (PDP-MG) (Riccio *et al*., 1997; Thaller *et al*., 1998a), or α-naphtyl phosphate/Fast Garnett GBC salt (Golovan *et al*., 2000), respectively. Acid phosphatase activity can also be detected by monitoring the fluorescence that follows the hydrolysis of 2-(5'-chloro-2'-

phosphoryloxyphenyl)-4-[3H]-quinazolinone (van Ommen Kloeke *et al .*, 1999) or 4-methylumbelliferylphosphate derivatives (4-MUP) (Gee *et al.*, 1999).

Of the different assays, the PDP-MG plate screen assay has been used the most frequently. It has been reported that optimal concentrations of MG and PDP vary significantly with different groups of microorganisms. Whereas the differences in the MG concentration required are likely to be dependent upon the varying permeability of different microorganisms to the dye, the differences in the PDP concentration may depend on quantitative differences in the acid phosphatase activity expressed by different microorganisms (Satta *et al .*, 1979; Pompei *et al .*, 1990). However, the PDP-MG acid phosphatase assay is neither affected by the type of base medium nor the pH of the medium (Satta *et al .*, 1984), and its sensitivity can be improved by substituting PDP with phenolphthalein monophosphate (PMP) or 6-benzoyl-naphtyl phosphate (6-BNP) (Pompei *et al.*, 1990).

1.3.1.2 Zymogram assays

Acid phosphatase activities in crude bacterial preparations can also be analyzed by zymogram techniques. In this case, crude cellular extracts are first subjected to electrophoretic separation, and acid phosphatase activities are detected *in situ* by means of chromogenic reactions. Different detection systems, based upon dephosphorylation of the substrates to yield coloured products, have been described. Acid phosphatases can be detected by the presence of either green-stained activity bands when PDP-MG is used as substrate (Thaller *et al*., 1995a; Thaller *et a l.*, 1997a), as blue-stained activity bands when BCIP is used as substrate (Thaller *et al .*, 1995a; Malke, 1998), or as black-stained activity bands when α-naphtyl phosphate/Fast Garnet GBC salt is used as substrate (Gabriel, 1971). Alternatively, reactions based on detection of the released P_i by means of acidified ammonium molybdate, which yields a blue precipitate, have also been described (Thaller *et al.*, 1994).

In contrast to performing electrophoresis under non-denaturing conditions, an advantage of using denaturing SDS-PAGE, followed by treatment that allows renaturation of the enzyme in the gel matrix, is that proteins are separated on the basis of the size of the polypeptide component of the enzyme. Thus, the migration distance at which the band activity is detected depends on the molecular weight of the polypeptide component of the enzyme. Zymogram assays are suitable for analyzing several bacterial isolates simultaneously and since different

substrates can be used for acid phosphatase activity development, substrate profiles of the enzymes can be determined. Moreover, enzyme inhibitors can be added to the equilibration buffer at different concentrations to determine their effect on enzyme activity (Rossolini *et al.*, 1994; Thaller *et al.*, 1995a; Riccio *et al.*, 1997).

1.3.1.3 *In vitro* enzyme assays with crude preparations and purified enzymes

As an alternative to zymogram assays, acid phosphatase activities can also be analyzed using *in vitro* assays with crude cellular extracts or purified enzymes. Although the measurement of acid phosphatase activity of whole cells or of crude cell extracts using chromogenic substrates is suitable for analyzing large numbers of bacterial isolates (Bhargava *et al.*, 1995; Thaller *et al.*, 1995a; Riccio *et al.*, 1997), screening can, however, be complicated by the fact that the bacterial cell may contain multiple phosphatases with overlapping substrate profiles (Rossolini *et al*., 1998). Rather than using crude enzyme extracts, the individual bacterial acid phosphatases can be purified, followed by biophysical and biochemical characterization of the purified protein. This approach has been used for the analysis of some bacterial NSAPs (Uerkvitz and Beck, 1981; Chen *et a l.*, 1999). Since the enzyme purification procedures can be complex and have to be adjusted for each new enzyme, it is not suitable for simultaneous screening of several bacterial isolates.

For *in vitro* bacterial acid phosphatase activity assays, *p*-nitrophenyl phosphate (*p*NPP) is typically used as substrate (Thaller *et al.*, 1994; Uchiya *et al*., 1996; Malke, 1998; Golovan *et al.*, 2000; Passarriello *et al*., 2006). The *p*-nitrophenol formed after ester hydrolysis of the substrate can be detected by addition of NaOH to the reaction mixture, which not only terminates the assay but the hydroxide ions react with the *p*-nitrophenol to remove the phenolic proton, thereby resulting in the formation of *p*-nitrophenolate. The latter, which is a yellow coloured compound absorbing at 405 nm, can be detected spectrophotometrically. Since not much else in the reaction mixture absorbs light at this wavelength, it represents a simple assay whereby the amount of *p*-nitrophenol formed in the enzyme-catalyzed reaction can be determined quantitatively (Golovan *et al.*, 2000).

1.3.2 Classification of bacterial non-specific acid phosphatases

Classification of acid phosphatases was initially based on their biochemical and biophysical properties such as their pH optima (neutral, acid or alkaline), substrate profiles (specific vs. non-specific) and molecular sizes (high molecular weight vs. low molecular weight). However, subsequent to the availability of amino acid sequence data for different bacterial acid phosphatases the enzymes have been classified into three classes, *i.e.* class A, B and C, respectively. Whereas the amino acid sequences of class A acid phosphatases are completely unrelated to those of class B and class C acid phosphatases, class B and class C acid phosphatases show amino acid sequence similarity (identity 12-22%) (Thaller *et al*., 1998b; Dissing and Uerkvitz, 2006). The unique properties of the different classes of acid phosphatases are summarized in Table 1.2, and will be discussed in greater detail in the following sections.

Table 1.2: Unique properties of class A, B and C bacterial non-specific acid phosphatases

	Class A	**Class B**	**Class C**
Secreted as:	Phosphohydrolase	Phosphohydrolase	Lipoprotein
Molecular weight	25-27 kDa	25 kDa	24-30 kDa
Subclasses	A1, A2 and A3	None	None
Substrate profile	Active towards various phosphomonoesters, but not diesters	Active towards various organic phosphomonoesters, but not diesters	Narrow spectrum of substrates
	Subclass A3 apparently more specific towards nucleotide triphosphates (NTPs)	In some instances, the pH optimum for activity is substrate dependant	
Inhibition properties	Activity inhibited by low SDS concentrations	Activity inhibited by EDTA, inorganic orthophosphate (P_i) and nucleosides	Activity inhibited by EDTA
	Activity not inhibited by EDTA	Activity not inhibited by low SDS concentrations	Activity enhanced by cations

1.4 CLASS A BACTERIAL NON-SPECIFIC ACID PHOSPHATASES

The class A acid phosphatases are a group of secreted phosphatases, which contain a polypeptide component with a molecular weight of 25-27 kDa, and can be identified based on a conserved sequence motif (Table 1.3). Class A acid phosphatases can be detected by zymography after renaturing SDS-PAGE using BCIP as the chromogenic substrate; however, low concentrations of SDS inactivate these enzymes (Table 1.2). Moreover, a distinct feature of class A enzymes is that enzyme activity is not inhibited by EDTA. The class A acid phosphatases has been divided into three subclasses, *i.e.* A1, A2 and A3. Whereas subclass A1 enzymes are resistant to fluoride and contain a slightly smaller polypeptide component (25 kDa), subclass A2 enzymes are inhibited by fluoride and contain a slightly larger polypeptide component (27 kDa). Subclass A3 enzymes consist of monomeric enzymes, of which the activity can be inhibited by divalent cations, and they display a clear preference for nucleotide triphosphates as substrates (Thaller *et al*., 1995a; Thaller *et al*., 1998b). Seven different class A acid phosphatase-encoding genes have been cloned, sequenced and their products have been characterized to varying extents.

Table 1.3: Conserved sequence motif identified in bacterial class A non-specific acid phosphatases (adapted from Thaller *et al.*, 1998b)

	Protein (bacterium)	**Conserved motif**	**Reference**
Class A (subclass A1)	PhoC (*Morganella morganii*)	GSYPSGH-[TA]	Thaller *et al*. (1994)
Class A (subclass A1)	PhoN (*Providencia stuartii*)	GSYPSGH-[TA]	Rossolini *et al*. (1998)
Class A (subclass A1)	PhoN (*Shigella flexneri*)	GSYPSGH-[TA]	Uchiya *et al*. (1996)
Class A (subclass A2)	PhoN (S*almonella enterica* serovar Typhimurium)	GSYPSGH-[TA]	Kasahara *et al*. (1991)
Class A (subclass A3)	Apy (*Shigella flexneri*)	GSYPSGH-[TA]	Bhargava *et al*. (1995)
Class A (subclass A3)	PhoC (*Zymomonas mobilis*)	GSYPSGH-[TA]	Pond *et al*. (1989)
Class A (subclass A3)	PiACP (*Prevotella intermedia*)	GSYPSGH-[TA]	Chen *et al*. (1999)

1.4.1 PhoN acid phosphatase of *Salmonella enterica* serovar Typhimurium

The PhoN acid phosphatase enzyme of *Salmonella enterica* serovar Typhimurium was the first class A NSAP enzyme to be purified and characterized (Uerkvitz and Beck, 1981). PhoN is a homodimeric protein containing two 27-kDa subunits. It is active against various different substrates, including 3'- and 5'-nucleoside monophosphates, nucleoside di- and triphosphates, hexose and pentose phosphates, aryl-phosphates (*p*-nitrophenyl phosphate [*p*NPP] and phenolphthalein diphosphate [PDP]), α-naphthyl phosphate and pyrophosphate. The pH optimum is approximately 5.5 when 5'-adenosine monophosphate (AMP) is used as substrate. PhoN activity is inhibited by fluoride and mercury ions, but is unaffected by EDTA, tartrate and divalent cations such as Mg^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+}, Ba^{2+}, Ni^{2+} and Zn^{2+}. Inorganic orthophosphate (P_i) partially inhibits enzyme activity at concentrations in excess of 0.1 M, although the inhibitory effect is more evident with 5'-nucleotides than with *p*NPP as substrate (Uerkvitz and Beck, 1981; Kasahara *et al.*, 1991). It has subsequently been reported that the *phoN* gene, encoding the acid phosphatase, is found in different *Salmonella* serovars, suggesting that the gene may have been acquired through horizontal transfer of genetic material prior to diversification of the salmonellae (Groisman *et al*., 1992).

1.4.2 PhoN and Apy acid phosphatases of *Shigella flexneri*

Two class A enzymes, but belonging to different subclasses, have been identified in *Shigella flexneri*. Both enzymes, *i.e.* PhoN and Apy, are encoded by genes carried on the large virulence-associated plasmid harboured by clinical isolates of *S. flexneri* (Bhargava *et al*., 1995; Uchiya *et al*., 1996). The *Shigella* PhoN acid phosphatase enzyme is a 27-kDa monomer and exhibits a broad substrate profile, including nucleotides, *p*NPP, glucose-6-phosphate and β-glycerophosphate. The pH and temperature optima are at 6.6 and 37ºC, respectively. The enzymatic activity is inhibited by diisopropylfluorophosphates, *N*-bromosuccinimide and dithiothreitol (DTT), but not by EDTA, fluoride, tartrate and benzamide (Uchiya *et al*., 1996). The deduced amino acid sequence of the PhoN protein displays higher amino acid sequence homology to PhoN of *P. stuartii* (83.2%) and PhoC of *M. morganii* (80.6%), compared to PhoN of *S. enterica* serovar Typhimurium (47.8%) and PhoC of *Z. mobilis* (34.8 %). Consequently, the PhoN enzyme of *S. flexneri* has been classified under subclass A1 of the class A NSAPs (Rossolini *et al*., 1998).

The *S. flexneri* Apy enzyme displays some unique properties compared to other class A enzymes. The native Apy enzyme is a 25-kDa monomer. The enzyme exhibits preferential activity on nucleoside triphosphates (NTPs), which are hydrolysed sequentially to the corresponding diphosphates and monophosphates, through release of P_i. It is also active on pyrophosphate and, to a lesser extent, on *p*NPP, but not on 5'-adenosine monophosphate (AMP). The optimum pH for activity is between pH 7 and pH 7.5 (Bhargava *et al*., 1995). Similar to other class A enzymes, the Apy enzyme activity is not inhibited by EDTA, while it is inhibited by fluoride, *o*-vanadate, sodium azide and various divalent cations, including Ba^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+} and Cu^{2+} (Bhargava *et al*., 1995). In contrast to the *S. flexneri* PhoN enzyme, which is produced by only some shigellae and thus apparently not involved in the virulence phenotype of these bacteria (Uchiya *et al*., 1996), the *apy* gene is carried by virulent *Shigella* spp. (Bhargava *et al*., 1995). This observation, together with the localization of the enzyme in the periplasmic space, the specific activity of the enzyme on NTPs and the dramatic decrease of the NTPs pool in eukaryotic cells invaded by shigellae, suggests that Apy could be involved in the virulence phenotype of these pathogens (Mantis *et al.*, 1996). Based on the degree of amino acid sequence divergence of the Apy protein compared to the other known class A NSAPs, together with its peculiar functional features, the Apy protein has thus been classified as the prototype enzyme of subclass 3 under the class A enzymes (Rossolini *et al*., 1998).

1.4.3 PiACP acid phosphatase of *Pretovella intermedia*

A novel acid phosphatase with phosphotyrosyl phosphatase (PTPase) activity, designated PiACP, has been purified from *Pretovella intermedia* (Chen *et al*., 1999). The PiACP protein is a monomer with a molecular weight of approximately 30 kDa. Characterization of the biochemical properties of the PiACP enzyme indicated that is highly active against *p*NPP, glucose-6-phosphate, adenosine triphosphate (ATP) and *O*-phospho-DL-tyrosine, while low activity was observed when *O*-phospho-DL-serine, *O*-phospho-DL-threonine and 3'- and 5'-adenosine monophosphate (AMP) were used as substrates. The optimum pH and temperature for PiACP activity is 4.9 and 46°C, respectively, using *p*NPP as substrate. Similar to the Apy enzyme from *S. flexneri*, the PiACP enzyme activity is inhibited by fluoride, molybdate, *o*-vanadate and the divalent cations Cu^{2+} and Zn^{2+}. However, enzyme activity is unaffected by EDTA, DTT, tartrate and okadaic acid (Chen *et al* ., 1999). The deduced amino acid sequence of the PiACP enzyme displays similarity (25 to 64% amino acid sequence identity)

to the other members of class A bacterial acid phosphatases. Despite the absence of a highly conserved motif typical of the active domain of PTPase, it was, however, reported that based on mutagenesis of recombinant PiACP, the histidine residue located in the conserved motif identified in class A phosphatases (Table 1.3) is essential for activity. Based on its inhibitor resistance and sensitivity profile, it has been proposed that PiACP be classified under subclass A3 of the class A NSAPs (Chen *et al*., 1999).

1.4.4 Other class A acid phosphatases

The PhoC enzyme of *Zymomonas mobilis* is the major P_i-irrepressible acid phosphatase produced by this species and was the first class A enzyme of which the sequence had been determined (Pond *et al* ., 1989). The enzyme has, however, not yet been purified or characterized. The characterization and sequence of PhoC, the principal P_i-irrepressible acid phosphatase of *Morganella morganii* has been reported by Thaller *et al*. (1994). PhoC is a homotetrameric protein containing four 25-kDa subunits. The enzyme exhibits activity against a broad range of substrates, including 3'- and 5'-nucleoside monophosphates, glucose-6-phosphate, β-glycerophosphate and aryl-phosphates (*p*NPP and PDP). The pH optimum is 6, using *p*NPP as substrate. The enzyme activity is only slightly inhibited by a P_i concentration of 0.1. M, but is not inhibited by EDTA, tartrate or fluoride (Thaller *et al*., 1994). The PhoN enzyme of *Providencia stuartii* has not been purified or characterized in detail, but based on its similarity to PhoC of *M. m organii* (84% amino acid sequence identity), it has been proposed that it might display similar properties to those of *M. morganii* (Rossolini *et al*., 1998).

1.5 CLASS B BACTERIAL NON-SPECIFIC ACID PHOSPHATASES

The class B acid phosphatases include a group of secreted bacterial phosphatases, which in their native form are 100-kDa homotetrameric proteins comprising four polypeptide subunits (25 kDa), and share two conserved sequence domains (Table 1.4). Based on amino acid sequence, the class B NSAPs is completely unrelated to class A enzymes. Class B enzymes can furthermore be distinguished from class A enzymes due to their inability to dephosphorylate the chromogenic substrate BCIP in zymographic analyses. Moreover, unlike class A enzymes, the class B enzymes retain their activity in the presence of low SDS concentrations and are inhibited by EDTA (Table 1.2). Four different class B acid

phosphatase-encoding genes have been cloned and sequenced, and their products have been characterized to varying extents.

Table 1.4: Conserved sequence motifs (domain A and domain B) identified in class B non-specific acid phosphatases (adapted from Thaller *et al.*, 1998b)

Protein (bacterium)	*Domain A		Domain B	Reference
AphA (*Escherichia coli*)	67- FDIDDTVLFSSP-	110	-YGDSDNDI-40	Thaller *et al.* (1997a)
AphA (S*almonella enterica* serovar Typhimurium)	66- FDIDDTVLFSSP-	110	-YGDSDNDI-40	Rossolini *et al.* (1998)
NapA (*Morganella morganii*)	66- FDIDDTVLFSSP-	110	-YGDADADI-40	Thaller *et al.* (1995b)
NapA (*Haemophilus influenzae*)	66-FDIDDTVLFSSP-	110	-YGDSDDDV-40	Rossolini *et al.* (1998)

*Numbers at the beginning of domain A indicate the number of amino acid residues from the N-terminus of the native protein; numbers in the middle of domain A and B indicate the number of residues between the two domains; numbers at the end of domain B indicate the number of residues from the C-terminus of the protein

1.5.1 AphA acid phosphatase of *Salmonella enterica* serovar Typhimurium

The first class B acid phosphatase to be characterized was purified from *Salmonella enterica* serovar Typhimurium strain LT2 and designated AphA (Uerkvitz and Beck, 1981). The gene encoding the AphA enzyme has been cloned from *S. enterica* serovar Typhi and restriction analysis of the *aphA* genes from serovars Typhi and Typhimurium has indicated that the two genes are highly conserved at the nucleotide sequence level (Rossolini *et al* ., 1998). Characterization of the AphA enzyme of *S. enterica* serovar Typhimurium indicated that it is active on various organic phosphomonoesters, including 3'- and 5'-uridine monophosphate (UMP), *p*NPP and α-naphtol (Uerkvitz *et al* ., 1988). The pH optimum for phosphatase activity is in the acidic range and appears to be substrate dependent, being lower (5-5.5) with 3'-UMP or *p*NPP and higher (pH 6.5) with 5'-UMP. The AphA activity is inhibited by EDTA, by high P_i concentrations (50% reduction in activity is observed in the presence of 0.1 M of P_i) and by nucleosides (28-79% reduction in activity, depending on the nucleoside type, in the presence of a 0.1 mM concentration) (Uerkvitz and Beck, 1981; Uerkvitz *et al*.,

1988). The AphA enzyme has also been reported to function as a phosphotransferase in the presence of organic compounds carrying a free hydroxyl group that serve as phosphate acceptors, together with a hydrolysable phosphoester that can function as a phosphate donor. The phosphotransferase activity was demonstrated using *p*NPP as a phosphate donor and either alkylalcohols (methanol, ethanol, ethylene glycol or glycerol) at high concentrations (0.2 to 2 M) or nucleosides at low concentrations (0.1 mM) as phosphate acceptors. The products resulting from transphosphorylation of nucleosides were mostly 3'-nucleotides, with only minor amounts of 5'- and 2'-nucleotides being obtained (Uerkvitz, 1988; Dissing and Uerkvitz, 2006).

1.5.2 NapA acid phosphatase of *Morganella morganii*

The *Morganella morganii* NapA acid phosphatase enzyme was the first class B NSAP to be cloned and sequenced (Thaller *et al*., 1995b). In contrast to the major P_i-irrepressible class A PhoC enzyme of this species, the NapA enzyme was initially identified as a minor P_i-irrepressible NSAP. The NapA enzyme is active on various organic phosphomonoesters, including 3'- and 5'-nucleoside monophosphates, aryl-phosphates (*p*NPP and PDP), β-glycerophosphate and sugar phosphates (glucose-6-phosphate and ribose-5'-phosphate). With *p*NPP as substrate, the pH optimum of phosphatase activity is 6 (Thaller *et al*., 1995b). The enzyme activity is inhibited by EDTA, Ca^{2+}, high P_i concentrations (a partial inhibitory effect becomes apparent at P_i concentrations in excess of 20 mM) and by nucleosides (31-63% reduction in activity, depending on the nucleoside type, in the presence of a 0.1 mM concentration). The phosphatase activity of NapA is unaffected by fluoride and tartrate, but is stimulated by low concentrations (1 mM) of Mg^{2+}, Co^{2+} and Zn^{2+}. Similar to the *Salmonella* AphA enzyme, the *M. m organii* NapA enzyme is also able to function as a phosphotransferase using *p*NPP as a phosphate donor and either alkylalcohols or nucleosides as phosphate acceptors (Thaller *et al*., 1995b).

1.5.3 AphA acid phosphatase of *Escherichia coli*

The presence of a periplasmic acid phosphatase in *E. coli* with features typical of class B NSAPs was initially detected by zymogram assays (Rossolini *et al*., 1994; Thaller *et al*., 1995a). Subsequent cloning and overexpression in *E. coli* of the acid phosphatase-encoding gene, designated *aphA*, allowed for characterization of the encoded AphA enzyme (Thaller *et al*., 1997a). Its biophysical and functional properties are similar to those of the *S. enterica*

serovar Typhimurium AphA enzyme and of the *M. morganii* NapA enzyme. The *E. coli* AphA enzyme is active against a broad array of organic phosphomonoesters, including 3'- and 5'-nucleoside monophosphates, aryl-phosphates (*p*NPP, PDP, phenyl phosphate and *O*-phospho-L-tyrosine), non-aromatic phosphoamino acids (*O*-phospho-L-serine and *O*-phospho-L-threonine), β-glycerophosphate, ribose-5'-phosphate and phytic acid. The highest activity is obtained when aryl-phosphates and nucleotides were used as substrates. No activity is detectable against adenosine triphosphate (ATP), glucose-1-phosphate, glucose-6-phosphate or diesters. Similar to the *Salmonella* AphA enzyme, the pH optimum for the phosphatase activity of the *E. coli* AphA enzyme is higher (6-6.5) for 5'-nucleoside monophosphates and lower (5.5-6) for *p*NPP. The enzyme activity is not affected by fluoride and stimulated by low concentrations of Mg^{2+}. The *E. coli* AphA enzyme is inhibited by EDTA, Ca^{2+}, by P_i (a slight decrease in enzyme activity is evident at a P_i concentration of 5 mM and increases concomitantly with the P_i concentration) and by nucleosides (67-80% reduction in activity, depending on the nucleoside type, in the presence of a 0.1 mM concentration). Similar to the other class B NSAPs, the *E. coli* AphA enzyme is also able to function as a phosphotransferase using *p*NPP as a phosphate donor and either alkylalcohols at high concentrations or nucleosides at low concentrations as phosphate acceptors (Thaller *et al.*, 1997a). More recently, Passarriello *et al*. (2006) purified and characterized the AphA acid phosphatase of *E. coli* strain MG 1655. In addition to the above results, the enzyme was reported to be inactive on cyclic nucleotides and nucleotide di- and triphosphates, and that the enzyme activity is inhibited by EGTA, 1,10-phenanthroline and dipicolinic acid. It was suggested, based on its high activity towards 3'- and 5'-mononucleotides, that the AphA enzyme be considered as a broad spectrum nucleotidase (Passarriello *et al.*, 2006).

1.5.4 Other class B acid phosphatases

Rossolini *et al*. (1998) reported the presence of a gene, encoding a hypothetical protein similar to other class B NSAPs, in the genome of *Haemophilus influenzae*. The gene was named *napA* and its acid phosphatase activity was confirmed by zymogram analysis, following its cloning and overexpression in *E. coli*. Although the NapA enzyme has not been characterized further, its amino acid sequence displayed 46% identity to the cognate class B enzymes of the above-mentioned *Enterobacteriaceae*.

1.6 CLASS C BACTERIAL NON-SPECIFIC ACID PHOSPHATASES

Of the three classes of bacterial NSAPs, class C enzymes have been identified the most recent (Thaller *et al.*, 1998b). The four acid phosphatase enzymes classified as class C NSAPs are characterized by a polypeptide component with a molecular weight of 24-30 kDa, narrow spectrum of substrates, as well as EDTA-mediated inhibition and cation-mediated enhancement of enzymatic activity. In addition, they all share two conserved sequence domains (Table 1.5). Unlike the class A and B NSAPs, which are secreted across the cytoplasmic membrane, the class C NSAPs have a signal peptide typical of bacterial lipoproteins and are membrane-associated. Class B, class C and some plant acid phosphatases (vegetative storage proteins) show amino acid sequence similarity (identity 12-22%) and all of them contain four aspartate residues (D) in the two most conserved domains (Thaller *et al*., 1998b). The four invariant aspartate residues have been proposed to be activity-linked, as the acid phosphatase activity was abolished by mutagenesis of the aspartate residues (Reilly *et al.*, 2001; Godlewska *et al.*, 2002).

Table 1.5: Conserved sequence motifs (domain A and domain B) identified in class C non-specific acid phosphatases (adapted from Thaller *et al.*, 1998b)

Protein (bacterium)	*Domain A		Domain B	Reference
LppC (*Streptococcus equisimilis)*	96-VLDIDETVLDNSPY-	102	-FGDNLVDF-65	Gase *et al*. (1997)
e(P4) (*Haemophilus influenzae*)	81-VADLDETMLDNSPY-	103	-VGDNLDDF-68	Green *et al*. (1991)
OlpA (*Chryseobacterium meningosepticum*)	72-VLDLDETVLDNSPY-	101	-FGDNLSDF-72	Thaller *et al*. (1997b)
HppA (*Helicobacter pylori*)	55-ILDLDETVLNTFDY-	101	-VGDTLHDF-52	Tomb *et al*. (1997) Godlewska *et al*. (2002)

*Numbers at the beginning of domain A indicate the number of amino acid residues from the N-terminus of the native protein; numbers in the middle of domain A and B indicate the number of residues between the two domains; numbers at the end of domain B indicate the number of residues from the C-terminus of the protein

1.6.1 OlpA acid phosphatase of *Chryseobacterium meningosepticum*

The OlpA protein of *C. meningosepticum* was the first class C NSAP to be identified, and was discovered as a zymograhically detectable NSAP activity while screening non-enterobacterial bacteria for the presence of NSAPs (Thaller *et al*., 1997b). More recently, the *olpA* gene has been cloned and expressed in *E. c oli*, and its biochemical properties investigated (Passariello *et al*., 2003). The native *C. meningosepticum* OlpA protein has a molecular weight of approximately 30 kDa. Enzyme activity studies showed that the enzyme is able to hydrolyze *p*NPP, as well as 5'- and 3'-nucleotide monophosphates, with a strong preference for 5'-nucleotides and 3'-adenosine monophosphate (AMP). The enzyme is also able to hydrolyze ribose-5'-phosphate, glucose-6-phosphate and β-glycerol phosphate, although with a lower efficiency, and is inactive against nucleotide di- and triphosphates, diesters and phytate. The pH optimum of OlpA with *p*NPP as substrate is 6. Concerning divalent cations, the OlpA activity is stimulated by Mg^{2+} and Cu^{2+}, while it is moderately inhibited by Zn^{2+}. The activity of OlpA is inhibited by EDTA, molybdate, fluoride, tartrate, *o*-vanadate and P_i (Passariello *et al*., 2003). Based on the results obtained, Passariello *et al*. (2003) suggested that OlpA may behave as a broad-spectrum nucleotidase with an overall preference for 5'-nucleotides rather than it being a true non-specific acid phosphatase.

1.6.2 *e*(P4) and HppA acid phosphatases of *Haemophilus influenzae*

The *e*(P4) outer membrane lipoprotein, encoded by the *hel* gene (Reilly *et al*., 1999; Reilly and Smith, 1999) and the HppA cell envelope protein, encoded by the *hppA* gene (Godlewska *et al*., 2002; Reilly and Calcutt, 2004), of *H. influenzae* are the only other members of the class C enzymes that have been purified and characterized in some detail. The 28-kDa *e*(P4) enzyme shows a narrow substrate specificity with an apparent preference for aryl-phosphates (*p*NPP and 4-MUP) and phenylphosphate. Using *p*NPP as substrate, the enzyme exhibits optimal activity at pH 5 in the presence of Cu^{2+}. The *e*(P4) enzyme is inhibited by EDTA, *o*-vanadate and molybdate, but not by P_i, tartrate or fluoride (Reilly *et al*., 1999). A secreted soluble derivative of the *e*(P4) lipoprotein, lacking the lipid anchor and expressed in *E. coli*, was found to retain identical functional activity while being easier to purify (Reilly and Smith, 1999). Concerning a physiological role, the *e*(P4) lipoprotein of *H. influenzae* has been reported to be essential for uptake of hemin as a source of porphyrin, which is an essential growth factor for *H. i nfluenzae* when grown aerobically (Riedl and Mekalanos, 1996; Morton *et al.*, 2007). However, the relationship between this function, which is carried

out by hemin-binding motifs within the protein (Riedl and Mekalanos, 1996), and the non-specific acid phosphatase activity of the protein has yet to be elucidated.

Godlewska *et al*. (2002) reported that the *hppA* gene of *H. pylori* encodes a class C acid phosphatase precursor and demonstrated the necessity of two aspartic acid residues in the DDDD motif (D_{73} and D_{192}) for activity of the *H. pylori* HppA enzyme. The *hppA* gene of *H. pylori* has been cloned and expressed *E. coli*, allowing for its biochemical characterization (Reilly and Calcutt, 2004). The recombinant HppA protein exhibits a molecular weight of 24 kDa. The HppA enzyme has narrow substrate specificity with highest activity for aryl-phosphates (*p*NPP and 4-MUP) and significant activity for 5' nucleoside monophosphates, but not against phenyl or tyrosine phosphate. The pH optimum for enzyme activity is dependent on the substrate, and was 4.6 and 5.2 for purine and pyrimidine 5' monophosphates, respectively. The enzyme activity for hydrolysis of *p*NPP, as well as other substrates, is enhanced in the presence of divalent cations, including Cu^{2+}, Ni^{2+}, Co^{2+} and Mg^{2+}. Although tartrate, DTT and P_i have little effect on phosphatase activity, molybdate, *o*-vanadate and EDTA have significant inhibitory effects on enzyme activity. Unlike class B NSAPs, the HppA enzyme was reported to lack transphosphorylation activity (Reilly and Calcutt, 2004).

1.6.3 Other class C acid phosphatases

Thaller *et al*. (1998b) reported that the *C. meningosepticum* OlpA protein displayed amino acid sequence similarity to the LppC cytoplasmic membrane protein of *Streptococcus equisimilis*. Subsequently, Malke (1998), in a brief report, confirmed that the *S. equisimilis* LppC protein (32 kDa) displayed acid phosphatase activity, as was evidenced by LppC enzyme activity being optimal at pH 5 using *p*NPP as substrate, and by the enzyme activity being unaffected by EDTA, tartrate and TritonX-100.

1.7 STRUCTURAL ANALYSIS OF BACTERIAL NON-SPECIFIC ACID PHOSPHATASES

Recently, there have been several reports regarding the successful crystallization of class A, B and C bacterial non-specific acid phosphatases (NSAPs) with a view to elucidating the structure-function relationship of the enzymes, and to gain an understanding of their catalytic mechanisms. In only a few cases, however, have the crystal structures been solved.

1.7.1 PhoN and AphA acid phosphatases of *Salmonella enterica* serovar Typhimurium

The *S. enterica* serovar Typhimurium PhoN and AphA proteins, members of the class A and class B NSAPs, respectively, have been expressed in *E. coli*, purified, crystallized and subjected to limited X-ray diffraction studies (Makde *et al*., 2003a; 2003b). Whereas two forms of PhoN crystals were harvested, comprising either of four monomers or two monomers (Makde *et al.*, 2003a), the AphA crystals comprised two tetramers (Makde *et al*., 2003b). The crystals composed of dimeric PhoN and tetrameric AphA were shown to be biologically active as addition of *p*NPP turned the crystals yellow, presumably due to the formation *p*-nitrophenolate. However, the authors noted that the low-resolution (2.5-2.9 Å) partial structure models obtained for the respective acid phosphatases required further bioinformatic and fold-prediction analyses in order to elucidate their experimental structures. Notably, in the case of the AphA protein, it was reported that storage of partially purified AphA at 4°C for one month resulted in an inactive truncated AphA protein, lacking 16 N-terminal residues. The inactive protein was observed to be a monomer, suggesting a role for the N-terminal residues in constituting the active tetramer of the AphA protein (Makde *et al*., 2003b).

1.7.2 AphA acid phosphatase of *Escherichia coli*

The class B non-specific acid phosphatase AphA of *E. coli* has also been expressed in *E. coli*, purified and crystallized (Forleo *et al.*, 2003). The crystal structure of the AphA enzyme was subsequently solved at 1.7 Å resolution, the results of which indicated that the enzyme is comprised of four identical 25-kDa subunits assembled to form a flat molecule with two catalytic sites on each face (Calderone *et al*., 2004). The subunits of the homotetramer interacted via intermolecular β-sheet formation, involving an N-terminal extension (Calderone *et al*., 2004). Each catalytic site contained an Mg^{2+} ion that is coordinated by three of four conserved aspartate residues, *i.e.* the carboxylate groups of Asp_{44} and Asp_{167}, and the backbone carbonyl oxygen atom of Asp_{46}. Moreover, AphA exhibited a haloacid dehalogenase (HAD) fold and the active site crevice is comprised of hydrophobic pocket, made by residues Phe_{56}, Leu_{71}, Trp_{77} and Tyr_{193}. It was suggested that the hydrophobic pocket could accommodate aromatic ring structures of nucleotides and that the respective amino acid residues could function in positioning the nucleotide substrates in the catalytic site (Calderone *et al.*, 2004).

Towards understanding the catalytic mechanism of AphA, a series of crystallographic studies have more recently been undertaken (Calderone *et al*., 2006). The crystal structures of the AphA enzyme in complex with the hydrolysis products of nucleotide monophosphate substrates (AMP and dCMP, which are the preferred substrates by the enzyme), and a proposed intermediate analogue (osmate, which is a phosphate analogue) were reported. Based on the set of X-ray structures obtained, an associative catalytic mechanism for phosphoester hydrolysis and phosphoryl transfer reactions was proposed. The pathway appears to proceed via a two-step reaction, where the enzyme acts as intermediate acceptor of the phosphoryl group by forming a covalent adduct with it, and following formation of the phosphoenzyme, the product phosphate is subsequently hydrolysed (Calderone *et al*., 2006). Support for the formation of a phospho-aspartyl intermediate in the AphA catalytic cycle was provided by the structure of the osmate adduct of AphA, in which an osmate species was covalently linked to Asp_{44} bridging it to Mg^{2+}. These results also indicated that Asp_{44} therefore acts as a nucleophile towards the substrate, which is activated by the coordination bond to Mg^{2+}. The results furthermore indicated that the catalysis of phosphomonoesters by AphA entails the cyclic movement of a loop (loop 6), which forms one of the walls of the active site cleft. The loop undergoes movement that allows the substrate aromatic moiety to bind into the hydrophobic pocket between Phe_{56} and Tyr_{193}, after which it then locks the substrate in the active site and facilitates the phosphoryl transfer reaction (Calderone *et al*., 2006).

1.7.3 *e*(P4) acid phosphatase of *Haemophilus influenzae*

Crystallization of a recombinant *H. influenzae e*(P4) protein, a member of the class C NSAPs, has been reported (Ou *et al*., 2006), and the crystal structure of the ligand-free recombinant protein and a tungstate-inhibited form of the protein was subsequently solved at 1.7 Å resolution (Felts *et al*., 2007). The *e*(P4) acid phosphatase was shown to have a two-domain architecture, consisting of a core α/β domain and a smaller α domain. Whereas the core domain, which contained the residues responsible for catalysis and binding the active site Mg^{2+} ion, had a structure reminiscent of the haloacid dehalogenase (HAD) superfamily, the α domain appeared to be unique and may play roles in substrate binding and dimerization. The latter was proposed to be important for catalysis since intersubunit contacts appeared to stabilize the active site. The active site is located in a cleft between the two domains and is solvent accessible. Similar to the *E. coli* AphA enzyme, the conserved DDDD motif also

plays a prominent role in metal binding. The ligands of the Mg^{2+} ion are the carboxyl groups of the first and third Asp residues of the DDDD motif (Asp_{64} and Asp_{181}), the backbone carbonyl of the second Asp of the DDDD motif (Asp_{66}), and two water molecules. Moreover, based on the tungstate-bound *e*(P4) structure, in which tungstate mimics the phosphate moiety of phosphomonoester substrates, it was suggested that that Asp_{64} is the nucleophile that attacks the substrate phosphate atom. Despite both *E. coli* AphA and *H. influenzae e*(P4) acid phosphatases featuring the DDDD motif and a HAD superfamily core domain, Felts *et al*. (2007) noted that the α domain serves to distinguish *e*(P4) from other HAD superfamily enzymes, including class B NSAPs. Since the protein-protein interaction surface observed in the *e*(P4) dimer is not seen in AphA, it was suggested that the mode of self-association may represent a major structural difference between class B and C NSAPs.

1.8 AIMS OF THIS INVESTIGATION

Reporter gene technology is indispensable for monitoring gene expression and characterization of promoter strength and regulation. Not only has this technology been used extensively in molecular genetic analyses to monitor the cellular events associated with signal transduction and gene expression (Veening *et al.*, 2004; Koga *et al*., 2006), but also as a means to identify promoters that can be exploited for the development of improved expression vectors (Serrano-Heras *et al*., 2005; Miksch *et al*., 2006). The principal advantages of reporter assays are their high sensitivity, reliability, simpler manipulation procedures (*e.g.*, reduced purification or cell lysis) and adaptability to large-scale measurements (Wood, 1995; Naylor, 1999). Despite the availability of various reporter gene systems, as highlighted in this review, new reporter genes are nevertheless introduced every year. This is due to the fact that none of the used reporter systems is universally applicable since all of them, alongside with advantages, have some shortcomings that limit their application for a number of model organisms, cells and in some studies (Janatova *et al*., 2003; Chary *et al*., 2005; Kim *et al.*, 2006). In this regard, bacterial non-specific acid phosphatases (NSAPs) may potentially be exploited as novel reporter proteins.

Bacterial non-specific acid phosphatases (NSAPs) are found as soluble enzymes associated with the periplasmic space or as constituents of bacterial membranes (Rossolini *et al.*, 1998), and participate in an assortment of biological functions, including the regulation of

metabolism, energy conversion and signal transduction (Stock *et al*., 1995; Klumpp and Kriegelstein, 2002). Although the existence of these enzymes has been known for a relatively long time, they have only fairly recently been characterized with regards to their enzymology and structure (Thaller *et al*., 1995a; Chen *et al*., 1999; Reilly and Calcutt, 2004; Felts *et al*., 2006; Calderone *et al*., 2006). Notably, bacterial non-specific acid phosphatase-encoding genes have been utilized in the construction of cloning-dependent insertional inactivation vectors, thus allowing rapid and inexpensive screening of recombinants (Burioni *et al*., 1995; Thaller *et al*., 1998a). Reports have also indicated that, in addition to plate screen assays, acid phosphatase enzymatic activity can also be detected by zymography (Thaller *et al*., 1995a; Malke, 1998), and quantitatively by *in vitro* enzyme assays with both the purified enzyme and whole-cell enzyme extracts (Uerkvitz and Beck, 1981; Bhargava *et al*., 1995; Chen *et al*., 1999; Passarrielo *et al*., 2006). Thus, the availability of qualitative and quantitative enzyme assay methods, in addition to their moderate size (25-30 kDa), provided further impetus for the development of bacterial NSAP enzymes as molecular reporters.

Towards the development and evaluation of an acid phosphatase-based prokaryotic reporter system, the aims of this investigation were the following:

- To isolate and characterize an acid phosphatase secreted by *Staphylococcus aureus*.

- To evaluate the *S. aureus* acid phosphatase as a reporter gene for host strain evaluation and cell surface display, using *B. halodurans* as expression host.

- To evaluate the *S. aureus* acid phosphatase as a reporter for heterologous gene expression and protein secretion in Gram-negative and Gram-positive bacteria.

1.9 REFERENCES

Adham, S.A., Rodriguez, S., Ramos, A., Santamaria, R.I. and Gil, J.A. (2003). Improved vectors for transcriptional/translational signal screening in corynebacteria using the *melC* operon from *Streptomyces glaucescens* as reporter. Arch. Microbiol. **180**: 53-59.

Amin-Hanjani, S., Meikle, A., Glover, L.A., Proser, J.I. and Killham, K. (1993). Plasmid and chromosomally encoded luminescence marker systems for detection of *P. fluorescens* in soil. Mol. Ecol. **2**: 47-54.

Andersen, J.B., Sternberg, C., Poulsen, L.K., Bjørn, S.P., Givskov, M. and Molin, S. (1998). New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. Appl. Environ. Microbiol. **64**: 2240-2246.

Applegate, B.M., Kehrmeyer, S.R. and Slayer, G.S. (1998). A chromosomally based *tod-luxCDABE* whole cell reporter for benzene, toluene, ethylbenzene, and xylene (BTEX) sensing. Appl. Environ. Microbiol. **64:** 2730-2735.

Bae, Y.M. and Hastings, J.W. (1994). Cloning, sequencing and expression of dinoflagellate luciferase DNA from a marine alga, *Gonyaulax polyedra*. Biochim. Biophys. Acta **1219**: 449-456.

Basnakova, B.G., Stephens, E.R., Thaller, M.C., Rossolini, G.M. and Macaskie, L.E. (1998). The use of *Escherichia coli* bearing a *phoN* gene for the removal of uranium and nickel from aqueous flows. Appl. Microbiol. Biotechnol. **50** 266-272.

Beacham, I.R. (1979). Periplasmic enzymes in Gram-negative bacteria. Int. J. Biochem. **10**, 877-883.

Belas, R., Simon, M. and Silverman, M. (1986). Regulation of lateral flagella gene transcription in *Vibrio parahaemolyticus*. J. Bacteriol. **167**: 210-218.

Bhargava, T., Datta, S., Ramchandran, V., Ramakrishnan, R., Roy, R.K., Sankaran, K. and Subrahmanyam, Y.V.B.K. (1995). Virulent *Shigella* codes for a soluble apyrase: Identification, characterization and cloning of the gene. Curr. Sci. **68**: 293-300.

Bliska, J.B., Guan, K., Dixon, J.E. and Falkow, S. (1991). Tyrosine phosphate hydrolysis of host proteins by an essential *Yersinia* virulence determinant. Proc. Natl. Acad. Sci. USA **88**: 1187-1191.

Boquet, P.L., Manoil, C. and Beckwith, J. (1987). Use of *TnphoA* to detect genes for exported proteins in *Escherichia coli*: Identification of the plasmid-encoded gene for a periplasmic acid phosphatase. J. Bacteriol. **169**: 1663-1669.

Bronstein, I., Fortin, J., Stanley, P.E., Stewart, G.S.A.B. and Kricka, L.J. (1994). Chemiluminescent and bioluminescent reporter gene assays. Anal. Biochem. **219**: 169-181.

Broom-Smith, J.K ., T adayyon, M . an d Z hang, Y . (1990). β-lactamase as a probe of membrane protein assembly and protein export. Mol. Microbiol. **4**: 1637-1644.

Bumann, D. an d V aldivia, R.H. (2007). Identification of host-induced pathogen genes by differential fluorescence indiction reporter sysytems. Nature Protoc. **2**: 770-777.

Burioni, R ., P laisant, P ., R iccio, M .L., R ossolini, G .M. an d S atta, G . (1995). A new plasmid-based cloning vector for direct detection of recombinant clones, based on inactivation of a bacterial acid phosphatase-encoding gene. New Microbiol. **18**:201-206.

Burlage, R.S., Y ang, Z.K. an d Mehlhorn, T . (1996). A transposon for green fluorescence protein transcriptional fusions: Applications for bacterial transport experiments. Gene **173**: 53-58.

Burne, R.A. and Chen, Y-Y. M. (2000). Analysis of gene expression in biofilm bacteria, pp. 91-102. In: Handbook of bacterial adhesion: principles, methods and applications (Y.H. An and R.J. Friedman, eds.), Totowa, New Jersey, Humana Press Inc.

Cagnon, C ., V alverde, V . an d M asson, J.L . (1991). A new family of sugar-inducible expression vectors for *E. coli*. Protein Eng. **4**: 843-847.

Calderone, V., Forleo, C., Benvenuti, M., Thaller, M.C., Rossolini, G.M. and Mangani, S. (2004). The first structure of a bacterial class B acid phosphatase reveals further structural heterogeneity among phosphatases of the haloacid dehalogenase fold. J. Mol. Biol. **335**: 761-773.

Calderone, V., Forleo, C., Benvenuti, M., Thaller, M.C., Rossolini, G.M. and Mangani, S. (2006). A structure-based proposal for the catalytic mechanism of the bacterial acid phosphatase AphA belonging to the DDD superfamily of phosphohydrolases. J. Mol. Biol. **355**:708-721.

Cao, Q ., Q u, Z ., Wan , Y ., Z hang, H . an d S hen, D . (2001). Cloning, molecular characterization, and application of rice epiphytic *Bacillus pimulus* promoter fragments. Curr. Microbiol. **43:** 244-248.

Casabadan, M.J. and Cohen, S.N. (1980). Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J. Mol. Biol. **138**: 179-207.

Chaffin, D.O. and Rubens, C.E. (1998). Blue/White screening of recombinant plasmids in Gram-positive bacteria by interruption of alkaline phosphatase gene (*phoZ*) expression. Gene **219**: 91-99.
``

Chalfie, M ., T u, Y ., Euskirchen, G ., War d, W.W., P rasher, D .C.C. (1994). Green fluorescent protein as a marker of gene expression. Science **263**: 802-805.

Chary, V.K., Busui, M., Renye, J.A. and Piggot, P.J. (2005). Vectors that facilitate the replacement of transcriptional *lacZ* fusions in *Streptococcus mutans* and *Bacillus subtilis* with fusions to *gfp* or *gusA*. FEMS Microbiol. Lett. **247**: 171-176.

Chen, X., Ansai, T., Awano, S., Iida, T., Barik, S. and Takehara, T. (1999). Isolation, cloning, and expression of an acid phosphatase containing phosphotyrosyl phosphatase activity from *Prevotella intermedia*. J. Bacteriol. **181**: 7107-7114.

Chen, Y., Yan, J., Yang, M., Wang, J. and Shen, D. (2003). Expression of green fluorescent protein in *Bacillus brevis* under the control of a novel constitutive promoter F1 and insertion mutagenesis of F1 in *Escherichia coli* DH5α. FEMS Microbiol. Lett. **229**: 111-117.

Choe, J.H., Guo, H. and van den Engh, G. (2005). A dual-fluorescence reporter system for high-throughput clone characterization and selection by cell sorting. Nucl. Acids Res. **33**: e49.

Cody, C.W., Prasher, D.C., Westler, W.M., Prendergast, F.G. and Ward, W.W. (1993). Chemical structure of the hexapeptide chromophore of the *Aequorea* green fluorescent protein. Biochem. **32**: 1212-1218.

Cormack, B.P., Valdivia, R.H. and Falkow, S. (1996). FACS-optimized mutants of the green fluorescent protein (GFP). Gene **173**: 33-38.

Craney, A., Hohenauer, T., Xu, Y., Navani, N.K., Li, Y. and Nodwell, J. (2007). A synthetic *luxCDABE* gene cluster optimized for expression in high-GC rich bacteria. Nucl. Acid Res. **35**: e46.

Cubitt, A.B., Heim, R., Adams, S.R., Boyd, A.E., Gross, L.A. and Tsien, R.Y. (1995). Understanding, improving and using green fluorescent proteins. Trends Biochem. Sci. **20**: 448-455.

Curcic, R., Dhandayuthapani, S. and Deretic, V. (1994). Gene expression in mycobacteria: Transcriptional fusion based on *xylE* and analysis of promoter region of the response regulator of *Mycobacterium tuberculosis*. Mol. Microbiol. **13**: 1057-1064.

De Kievit, T.R., Gillis, R., Marx, S., Brown, C. and Iglewski, B.H. (2001). Quorum-sensing genes in *Pseudomonas aeruginosa* biofilms: Their role and expression patterns. Appl. Environ. Microbiol. **67**: 1865-1873.

De Weger, L.A., Dekkers, L.C., Van der Bij, A.J. and Lungtenberg, B.J.J. (1994). Use of phosphate-reporter bacteria to study phosphate limitation in the rhizosphere and in bulk soil. Mol. Plant-Microbe Interact. **7**: 32-38.

De We ger, L .A., D unbar, P ., M ahaffee, W. F., L ugtenberg, B .J.J. an d S ayler, G .S. (1991). Use of bioluminescence markers to detect *Pseudomonas* spp. in the rhizosphere. Appl. Environ. Microbiol. **57**: 3641-3644.

De Wet, J.R., Wood, K.V., De Luca, M., Helinski, D.R. and Subramani, S. (1987). Firefly luciferase gene: Structure and expression in mammalian cells. Mol. Cell. Biol. **7**: 725-737.

De Wet, J.R., Wood, K.V., Helinski, D.R. and De Luca, M. (1985). Cloning of the firefly luciferase cDNA and expression of active luciferase in *E. coli*. Proc. Natl. Acad. Sci. USA **82**: 7870-7873.

Delgrave, S ., H awtin, R.E., S ilva, C .M., Y ang, M .M. an d Y ouvan, D.C. (1995). Red-shifted excitation mutants of the green fluorescent protein. BioTechn. **13**: 151-154.

Dissing, K . an d U erkvitz, W. (2006). Class B non-specific acid phosphatase from *Salmonella typhimurium* LT2 phosphotransferase activity, stability and thiol group reactivity. Enz. Microbial. Technol. **38**: 683-688.

Drahos, D ., H emming, B.C. an d M cPherson, S . (1986). Tracking recombinant organisms in the environment: β-galactosidase as a selectable non-antibiotic marker for fluorescent pseudomonads. BioTechn. **4**: 439-444.

Duncan, S ., G lover, L .A., K illham, K. an d P rosser, J.I . (1994). Luminescence-based detection of activity of starved and viable but nonculturable bacteria. Appl. Environ. Microbiol. **60**: 1308-1316.

Dvorak, H .F., B rockman, R .W. an d H eppel, L.A. (1967). Purification and properties of two acid phosphatase fractions isolated from osmotic shock fluid of *Escherichia coli*. Biochem. **6**: 1743-1751.

Eberl, L ., S chulze, R ., A mmendola, A ., G eisenberger, O ., E arhart, R., S teinberg, C ., Molin, S. and Amann, R. (1997). Use of green fluorescent protein as a marker for ecological studies of activated sludge communities. FEMS Microbiol. Lett. **149**: 77-83.

Ehrig, T ., O'Kane, D .J. an d P rendergast, F.G. (1995). Green fluorescent protein mutants with altered fluorescence excitation spectra. FEBS Lett. **367**: 163-166.

Felts, R .L., O u, Z ., Reilly, T.J. an d T anner, J.J. (2007). Structure of recombinant *Haemophilus influenzae e*(P4) acid phosphatase reveals a new member of the haloacid dehalogenase superfamily. Biochem. **46:**11110-11119.

Feustel, L., Nakotte, S. and Dürre, P. (2004). Characterization of two reporter gene systems for *Clostridium acetobutylicum*. Appl. Environ. Microbiol. **70**: 798-803.

Flemming, C.A., Leung, K.T., Lee, H., Trevors, J.T. and Greer, C.W. (1994). Survival of *lux-lac*-marked biosurfactant-producing *Pseudomonas ae ruginosa* UG2L in soil monitored by non-selective plating and PCR. Appl. Environ. Microbiol. **60**: 1606-1613.

Forleo, C., Benvenutti, M., Calderone, V., Schippa, S., Docquier, J.D., Thaller, M.C., Rossolini, G.M. and Mangani, S. (2003). Expression, purification, crystallization and preliminary X-ray characterization of the class B acid phosphatase (AphA) from *Escherichia coli*. Acta Crystallogr. D Biol Crystallogr. **59**: 1058-1060.

Forsman, M. and Jaurin, B. (1987). Chromogenic identification of promoters in *Streptomyces lividans* by using an *ampC* beta-lactamase promoter-probe vector. Mol. Gen. Genet. **210**: 23-32.

Gabriel, O. (1971). Locating enzymes on gels. Methods Enzymol. **17A**: 578-604.

Gase, K., Liu, G., Bruckmann, A., Steiner, K., Ozegowski, J. and Malke, H. (1997). The *lppC* gene of *Streptococcus equisimilis* encodes a lipoprotein that is homologous to the *e*(P4) outer membrane protein from *Haemophilus influenzae*. Med. Microbiol. Immunol. **186**: 63-73.

Gee, K.R., Sun, W.C., Bhalgat, M.K., Upson, R.H., Klaubert, D.H., Latham, K.A. and Haugland, R.P. (1999). Fluorogenic substrates based on fluorinated umbelliferones for continuous assays of phosphatases and beta-galactosidases. Anal. Biochem. **273**: 41-48.

Gibson, C.M. and Caparon, M.G. (2002). Alkaline phosphatase reporter transposon for identification of genes encoding secreted proteins Gram-positive microorganisms. Appl. Environ. Microbiol. **68**: 928-932.

Girbal, L., Mortier-Barriere, I., Raynard, F., Rouianet, C., Croux, C. and Soucaille, P. (2003). Development of a sensitive gene expression system and an inducible promoter system for *Clostridium acetobutylicum*. Appl. Environ. Microbiol. **69**: 4985-4988.

Gober, J.W., Boyd, C.H., Jarvis, M., Mangan, E.K., Rizzo, M.F. and Wingrove, J.A. (1995). Temporal and spatial regulation of flip, an early flagellar gene of Caulobacter crescentus that is required for motility and normal cell division. J. Bacteriol. **177**: 3656-3667.

Godlewska, R., Bujnicki, J.M., Ostrowski, J. and Jagusztyn-Krynicka, E.K. (2002). The *hppA* gene of *Helicobacter pylori* encodes the class C acid phosphatase precursor. FEMS Lett. **525**: 39-42.

Golovan, S., Wang, G., Zhang, J. and Forsberg, C.W. (2000). Characterization and overproduction of the *Escherichia coli appA*-encoded bifunctional enzyme that exhibits both phytase and acid phosphatase activities. Can. J. Microbiol. **46**: 59-71.

Gould, S.J. and Subramani, S. (1988). Firefly luciferase as a tool in molecular and cell biology. Anal. Biochem. **175**: 5-13.

Green, B.A., Farley, J.E., Quinn-Dey, T., Deich, R.A. and Zlotnick, G.W. (1991). The *e*(P4) outer membrane protein of *Haemophilus influenzae*: Biologic activity of anti-*e* serum and cloning and sequencing of the structural gene. Infect. Immun. **59**: 3191-3198.

Greener, A., Lehman, S.M. and Helinski, D.R. (1992). Promoters of the broad host range plasmid RK2: Analysis of transcription (initiation) in five species of Gram-negative bacteria. Genetics **130**: 27-36.

Groisman, E.A., Saier, M.H. and Ochman, H. (1992). Horizontal transfer of a phosphatase gene as evidence for mosaic structure of the *Salmonella* genome. EMBO J. **11**: 1309-1316.

Hampf, M. and Gossen, M. (2006). A protocol for combined *Photinus* and *Renilla* luciferase quantification compatible with protein assays. Anal. Biochem. **356**: 94-99.

Hautefort, I., Proenca, M.J. and Hinton, J.C.D. (2003). Single-copy green fluorescent protein gene fusions allow accurate measurement of *Salmonella* gene expression *in vitro* and during infection of mammalian cells. Appl. Environ. Microbiol. **69**: 7480-7491.

Hautefort, I.H. and Hinton, J.C.D. (2000). Measurement of bacterial gene expression *in vivo*. Phil. Trans. R. Soc. Lond. B **355**: 601-611.

Hay, A.G., Rice, J. F., Applegate, B.M., Bright, N.G. and Slayer, G.S. (2000). A bioluminescent whole-cell reporter for detection of 2,4-dichlorophenoxyaceticacid and 2,4-dichlorophenol in soil. Appl. Environ. Microbiol. **66**: 4589-4594.

Heim, R., Prasher, D.C. and Tsien, R.Y. (1994). Wavelength mutations and posttranslational autooxidation of green fluorescent protein. Proc. Natl. Acad. Sci. USA **91**: 12501-12504.

Herbst, B.S., Kneip, S. and Bremer, E. (1994). pOSEX: Vectors for osmotically-controlled and finally tuned gene expression *Escherichia coli*. Gene **151**: 137-142.

Hoffman, C.S. and Wright, A. (1985). Fusions of secreted proteins to alkaline phosphatase: An approach for studying protein secretion. Proc. Natl. Acad. Sci. USA **82**: 5107-5111.

Hols, P., Baulard, A., Gantiyn, D., Delplace, B., Hogan, S. and Delcour, J. (1992). Isolation and characterization of genetic expression and secretion signals from *Enterococcus faecalis* through the use of broad-host-range α-amylase probe vectors. Gene **118**: 21-30.

Hörmann, S., Vogel, R.F. and Ehrmann, M. (2006). Construction of a new reporter system to study the NaCl-dependent *dnaK* promoter activity of *Lactobacillus safranciscensis*. Appl. Microbiol. Biotechnol. **70**: 690-697.

Inouye, S. and Tsuji, F.I. (1994). Evidence for redox forms of the *Aequorea* green fluorescent protein. FEBS Lett. **351**: 211-214.

Jain, V.K. and Magrath, I.T. (1991). A chemiluminescent assay for quantification of β-galactosidase in *lacZ*-transfected cells. Anal. Biochem. **199**: 119-124.

Janatova, I., Costaglioli, P., Wesche, J., Masson, J.M. and Meilhoc, E. (2003). Development of a reporter system for the yeast *Schwanniomyces occidentalis*: Influence of DNA composition and codon usage. Yeast **20**: 687-701.

Jefferson, R.A., Burgess, S.M. and Hirsh, D. (1986). Beta-glucuronidase from *E. coli* as a gene fusion marker. Proc. Nat. Acad. Sci. USA. **83**: 8451-8457.

Jefferson, R.A., Kavanagh, T.A. and Beven, M.W. (1987). Gus fusions: Beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. **6**: 3901-3907.

Jeong, D.W., Choi, Y.C., Lee, J.M., Kim, J. H., Lee, J.H., Kim, K.H. and Lee, H.J. (2006). Isolation and characterization of promoters from *Lactococcus lactis* subsp. Cremoris LM0230. Food Microbiol. **23**: 82-89.

Joyeux, A., Balaguer, P., Germain, P., Boussiox, A.M., Pons, M. and Nicholas, J.C. (1997). Engineered cell lines as a tool for monitoring biological activity of hormone analoges. Anal. Biochem. **249**: 119-130.

Kaniga, K., Uralil, J.B. and Galan, J. (1996). A secreted protein tyrosine phosphatase with modular effector domains in the bacterial pathogen *Salmonella typhimurium*. Mol. Microbiol. 21: 663-641.

Kasahara, M., Nakata, A. and Shinagawa, H. (1991). Molecular analysis of the *Salmonella typhimurium phoN* gene, which encodes non-specific acid phosphatase. J. Bacteriol. **173**: 6770-6775.

Kaur, J., Rajamohan, G. and Dikshit, K.L. (2007). Cloning and characterization of promoter-active DNA sequences from *Streptococcus equisimilis*. Curr. Microbiol. **54**: 48-53.

Keiler, K.C., Waller, P.R.H. and Sauer, R.T. (1996). Role of a peptide tagging system in the degradation of proteins synthesized from damaged messenger RNA. Science **271**: 990-993.

Kier, L.D., Weppelman, R. and Ames, B.N. (1977). Resolution and purification of three periplasmic phosphatases of *Salmonella typhimurium*. J. Bacteriol. **130**: 411-419.

Kim, K.W., Franceschi, V.R., Davin, L.B.V. and Lewis, N.G. (2006). Beta-glucuronidase as reporter gene: Advantages and limitations. Methods Mol. Biol. **323**: 263-273.

Kirstein, J. and Turgay, K. (2005). A new tyrosine phosphorylation mechanism involved in signal transduction in *Bacillus subtilis*. J. Mol. Microbiol. Biotechnol. **9**: 182-188.

Klumpp, S. and Krieglstein, J. (2002). Phosphorylation and dephosphorylation of histidine residues in proteins. Eur. J. Biochem. **269**: 1067-1071.

Koga, K ., I kegami, A ., N akasone, K ., M urayama, R ., A kanuma, G ., N atori, Y ., Nanamiya, H. and Kawamura, F. (2006). Construction of *Bacillus subtilis* strains carrying the transcriptional *bgaB* fusion with the promoter region of each *rrn* operon and their differential transcription during spore development. J. Gen. Appl. Microbiol. **52**: 119-124.

Kovacevic, S., Veal, L.E., Hsiung, M. and Miller, J. (1985). Secretion of Staphylococcal nuclease by *B. subtilis*. J. Bacteriol. **162**: 521-528.

Labes, G., Bibb, M. and Wollheben, W. (1997). Isolation and characterization of a strong promoter element from the *Streptomyces ghanae nsis* phage l19 using the gentamycin resistance gene (*aacC1*) of Tn1696 as reporter. Microbiol. **143**: 1503-1512.

Langella, P. and Le Loir,Y. (1999). Heterologous protein secretion in *Lactococcus lactis*: A novel antigen delivery system. Braz. J. Med. Biol. Res. **32**: 191-198.

Le L oir, Y., G russ, A ., E hrlich, S .D. an d L angella, P . (1998). A nine-residue synthetic propeptide enhances secretion efficiency of heterologous proteins in *Lactococcus l actis*. J. Bacteriol. **180**: 1895-1903.

Le L oir, Y .L., Gruss, A., E hrlich, S .D. an d L angella, P. (1994). Direct screening of recombinants in Gram-positive bacteria using the secreted Staphylococcal nuclease as a reporter. J. Bacteriol. **176:** 5135-5139.

Li, L . and H astings, J.W. (1998). The structure and organization of the luciferase gene in the photosynthetic dinoflagellate *Gonyaulax polyedra*. Plant Mol. Biol. **36**: 275-284.

Lieble, W., Sinskey, A.J. and Schleifer, K.H. (1992). Expression, secretion, and processing of Staphylococcal nuclease by *Corynbacterium glutamicum*. J. Bacteriol. **174:**1854-1861.

Lim, E.M., Rauzier, J., Timm, J., Torrca, G., Murray, A., Gicquel, B. and Portnoi, D. (1995). Identification of *Mycobacterium t uberculosis* DNA sequences encoding exported proteins by using *phoA* gene fusions. J. Bacteriol. **177**: 59-65.

Lorenz, W.W., McCann, R .O., L ongiaru, M . and C ormier, M .J. (1991). Isolation and expression of a cDNA encoding *Renilla reniformis* luciferase. Proc. Natl. Acad. Sci. USA **88**: 4438-4442.

Macaskie, L .E. (1990). An immobilized cell bioprocess for the removal of heavy metals from aqueous flows. J. Chem. Technol. Biotechnol. **49**: 357-379.

Makde, R.D., Kumar, V., Gupta, G.D., Jasti, J., Singh, T.P. and Mahajan, S.K. (2003b). Expression, purification, crystallization and preliminary X-ray diffraction studies of

recombinant class b non-specific acid phosphatase of *Salmonella t yphimurium*. Acta Crystallogr.: Bio, Crystallogr, **59**: 1849-1852.

Makde, R .D., K umar, V ., R ao, A .S., Y adava, V .S. an d M ahajan, S .K. (2003a). Purification, crystallization and preliminary X-ray diffraction studies of recombinant class A non-specific acid phosphatase of *Salmonella typhimurium*. Acta Cryst: Bio. Crystallogr. **59**: 515-518.

Makino, K ., A memura, M ., Kim, S -K., N akata, A . an d S hinagawa, H . (1994). Mechanism of transcriptional activation of the phosphate regulon in *Escherichia coli*, pp. 5-12. In: Phosphate in microorganisms (A.M. Torriani-Gorini, E. Yagil, S. Silver, eds.), Washington DC, ASM Press.

Malke, H . (1998). Cytoplasmic membrane lipoprotein LppC of *Streptococcus e quisimilis* functions as an acid phosphatase. Appl. Environ. Microbiol. **64**: 2439-2442.

Malveaux, F.J. and San Clemente, C.L. (1967). Elution of loosely bound acid phosphatase from *Staphylococcus aureus.* Appl. Environ. Microbiol. **15**: 738-743.

Manoil, C . (2000). Tagging exported proteins using *Escherichia coli* alkaline phosphatase gene fusions. Methods Enzymol. **326**: 35-47.

Manoil, C. and Beckwith, J. (1985). Tn*phoA*: A transposon probe for protein export signals. Proc. Natl. Acad. Sci. USA **82**: 8129-8133.

Mantis, N., Prevost, M.C. and Sansonetti, P. (1996). Analysis of epithelial stress response during infection by *Shigella flexneri*. Infect. Immun. **64**: 2474-2482.

Mascaronhas, J.P. and Hamilton, D.A. (1992). Artifacts in the localization of *gus* activity in anthers of petunia transformed with a CAMV 35S-*gus* construct. Plant J. **2**: 405-408.

Masson, L., Comeau, Y., Brousseau, R., Samson, R. and Greer, C. (1993). Construction and application of chromosomally integrated *lac-lux* gene markers to monitor the fate of a 2,4-dichlorophenoxyacetic acid-degrading bacterium in contaminated soils. Microb. Releases **1**: 209-216.

McNabb, D .S., R eed, R. an d M arciniak, R .A. (2005). Dual luciferase assay system for rapid assessment of gene expression in *Saccharomyces cerevisiae*. Eukar. Cell **4**: 1539-1549.

Meighen, E .A. (1991). Molecular biology of bacterial luminescence. Microbiol. Rev. **55**: 123-142.

Mena, M .A., T reynor, T .P., M ayo, S .L. an d D augherty, P .S. (2006). Blue fluorescent proteins with enhanced brightness and photostability from a structurally targeted library. Nature Biotech. **24**: 1569-1571.

Miksch, G., Bettenworth, F., Friehs, K., Flaschel, E., Saalbach, A. and Nattkemper, T.W. (2006). A rapid reporter system using GFP as a reporter protein for identification and screening of synthetic stationary-phase promoters in *E. coli*. Appl. Microbiol. Biotechnol. **70**: 229-236.

Miller, J.H. (1992). A short course in bacterial genetics, p. 72. Cold Spring Harbor, Cold Spring Harbour Laboratory Press.

Miller, J.R., Kovacevic, S. and Veal, L.E. (1997). Secretion and processing of staphylococcal nuclease by *Bacillus subtilis*. J. Bacteriol. **169**: 3508-3514.

Møller, S., Kristensen, C.S., Poulsen, L.K., Carstensen, J.M. and Molin, S. (1995). Bacterial growth on surfaces: Automated image analysis for quantification of growth rate-related parameters. Appl. Environ. Microbiol. **61**: 741-748.

Morton, D.J., Smith, A., Van Wagoner, M., Seale, T.W., Whitby, P.W. and Stull, T.L. (2007). Lipoprotein *e*(P4) of *Haemophilus influenzae*: Role in heme utilization and pathogenesis. Microb. Infect. **9**: 932-939.

Naylor, L.H. (1999). Reporter gene technology: The future looks bright. Biochem. Pharmacol. **58**: 749-757.

Oliver, D.B. (1996). Periplasm, pp. 88-103. In: *Escherichia coli* and *Salmonella* (F.C. Neidhardt, R. Curtiss, E.C.C. Lin, J.L. Ingraham, K.B. Low, B. Magasanik, W. Reznikoff, M. Riley, M. Schaechter, H.E. Umbarger, eds.), Washington DC, ASM Press.

Osbourne, A.E., Barver, C.E. and Daniels, M.S. (1987). Identification of plant-induced genes of the bacterial pathogen *Xanthomonas campestris* pv. *campestris* using a promoter-probe plasmid. EMBO J. **6**: 23-28.

Ou, Z., Felts, R.L., Reilly, T.J., Nix, J.C. and Tanner, J.J. (2006). Crystallization of recombinant *Haemophilus influenzae e*(P4) acid phosphatase. Acta Cryst. **62**: 464-466.

Palomares, A.J., De Luca, M.A. and Helinski, D.R. (1989). Firefly luciferase as a reporter enzyme for measuring gene expression in vegetative and symbiotic *Rhizobium meliloti* and other Gram-negative bacteria. Gene **81**: 55-64.

Palva, I., Sarvas, M., Lehtovaara, P., Sibakov M. and Kaariainen, L. (1982). Secretion of *Escherichia coli* β-lactamase from *Bacillus subtilis* by the aid of α-amylase signal sequence. Proc. Natl. Acad. Sci. USA. **79**: 5582-5586.

Park, H.S. and Kilbane, J.J. (2004). Gene expression studies of *Thermus thermophilus* promoters P_{dnaK}, P_{arg} and $P_{scs\text{-}mdh}$. Lett. Appl. Microbiol. **38**: 415-224.

Passarriello, C., Schippa, S., Iori, P., Berlutti, F., Thaller, M.C. and Rossolini, G.M. (2006). Biochemical characterization of the class B acid phosphatase (AphA) of *E. coli* MG1655. Biochim. Biophys. Acta **1764**: 13-19.

Passarriello, C., Schippa, S., Leone, R., Mangani, S., Thaller, M.C. and Rossolini, G.M. (2003). The molecular class C acid phosphatase of *Chryseobacterium meningosepticum* (OlpA) is a broad spectrum nucleotidase with preferential activity of 5' nucleotides. Biochim. Biophys. Acta **1648**: 203-209.

Payne, M.S. and Jackson, E.N. (1991). Use of alkaline phosphatase fusions to study protein secretion in *Bacillus subtilis*. J. Bacteriol. **173**: 2278-2282.

Pazzagli, M., Devine, J.H., Peterson, D.O. and Baldwin, T.O. (1992). Use of bacterial and firefly luciferases as reporter genes in DEAE-dextran-mediated transfection of mammalian cells. Anal. Biochem. **204**: 315-323.

Pearce, B.J., Yin, Y.B. and Masure, H.R. (1993). Genetic identification of exported proteins in *Streptococcus pneumoniae*. Mol. Microbiol. **9**: 1037-1050.

Piard, J.C., Jimenez-Diaz, R., Ehrlich, S.D., Fischetti, V.A. and Gruss, A. (1997). The M6 protein of *Streptococcus pyogenes* and its potential as a tool to anchor biologically active molecules at the surface of lactic acid bacteria. Adv. Exp. Med. Biol. **418**: 545-550.

Piruzian, E.S., Glodenkova, I.V., Musiychuk, K.A., Kobets, N.S., Arman, I.P., Bobrysheva, I.V., Chekuta, I.A., and Glazkova, D. (2002). A reporter system for prokaryotic and eukaryotic cells based on the thermostable lichenase from *Clostridium thermocellum*. Mol. Genet. Genomics **266**: 778-786.

Platteeuw, C., Simons, G. and De Vos, W.M. (1994). Use of *Escherichia coli* β-glucuronidase (*gusA*) gene as a reporter gene for analyzing promoters in lactic acid bacteria. Appl. Environ. Microbiol. **60**: 587-593.

Pompei, R., Cornaglia, G.M., Ingianni, A. and Satta, G. (1990). Use of a novel phosphatase test for simplified identification of the tribe *Proteae*. J. Clin. Microbiol. **28**: 214-218.

Pompei, R., Ingianni, A., Foddis, G., Di Pietro, G. and Satta, G. (1993). Patterns of phosphatase activity among enterobacterial species. Int. J. Syst. Bacteriol. **43**: 174-178.

Pond, J.L., Eddy, C.K., Mackensie, K.F., Conway, T., Borecky, D.J. and Ingram, L.O. (1989). Cloning, sequencing and characterization of the principal acid phosphatase, the phoC$^+$ product, from *Zymomonas mobilis.* J. Bacteriol. **171**: 767-774.

Poquet, I., Ehrlich, S.D. and Gruss, A. (1998). An export-specific reporter designed for Gram-positive bacteria: Application to *Lactococcus lactis*. J. Bacteriol. **180**: 1904-1912.

Post, D .A. an d L uebeke, V .E. (2005). Purification, cloning, and properties of α-galactosidase from *Saccharomyces e rythraea* and its use as a reporter system. Appl. Microbiol. Biotechnol. **67**: 91-96.

Poyart, C. and Trieu-Cuot, P. (1997) A broad host range mobilizable shuttle vector for the construction of transcriptional fusions to β-galactosidase in Gram-positive bacteria. FEMS Microbiol. Lett. **156**: 193-198.

Prasher, D .C., E ckenrode, V .K., War d, W. W., P rendergast, F.G. and C ormier, M.J. (1992). Primary structure of *Aequoria victoria* green-flourescent protein. Gene **111**: 229-233.

Quaedclieg, N .E.M., S chlaman, H .R.M., A dmiraal, P .C., Wi jting, S .E., S tougaard, J., Spaink, H .P. (1998). Fusions between GFP and GUS as sensitive and vital bifunctional reporters in plants. Plant. Mol. Biol. **38**: 861-873.

Rattray, E .A., Prosser, J.I ., K illham, K. an d G lover, L .A. (1990). Luminescence-based non-extractive technique for *in situ* detection of *Escherichia coli* in soil. Appl. Environ. Microbiol. **56**: 3368-3374.

Ravn, P., Arnau, J., M adsen, S.M., V rang, A. and Israelsen, H. (2000). The development of Tn*Nuc* and its use for the isolation of novel secretion signals in *Lactococcus lactis*. Gene **242**: 347-356.

Reidl, J. an d M ekalanos, J.J. (1996). Lipoprotein e(P4) is essential for hemin uptake by *Haemophilus influenzae*. J. Exp. Med. **183**:621-629.

Reilly, T.J. and C alcutt, M.J. (2004). The class C acid phosphatase of *Helicobacter pylori* is a 5' nucleotidase. Protein Expr. Purif. **33**: 48-56.

Reilly, T .J. an d S mith, A .L. (1999). Purification and characterization of a recombinant *Haemophilus i nfluenzae* outer membrane phosphomonoesterase *e*(P4). Protein Expr. Purif. **17**: 401-409.

Reilly, T.J., Baron, G.S., Nanp, F. and Kuhlenschmidt, M.S. (1996). Characterization and sequencing of a respiratory burst-inhibiting acid phosphatase from *Francisella t ularensis.* J. Biol. Chem. **271**: 10973-10983.

Reilly, T .J., C hance, D .L. an d S mith, A .L. (1999). Outer membrane lipoprotein *e*(P4) of *Haemophilus influenzae* is a novel phosphomonoesterase. J. Bacteriol. **181**: 6797-6805.

Reilly, T .J., G reen, B .A., Z lotnick, G .W. an d S mith, A .L. (2001). Contribution of the DDDD motif of *H. i nfluenzae e*(P4) to phosphomonoesterase activity and heme transport. FEBS Lett. **494**: 19-23.

Reuber, T.L., Long, S.L. and Walker, G.C. (1991). Regulation of *Rhizobium meliloti* exogenes in free-living cells and in plant a examined using *TnphoA* fusions. J. Bacteriol. **173**: 426-434.

Riccio, M.L., Rossolini, G.M., Lombardi, G., Chiesurin, A. and Satta, G. (1997). Expression cloning of different bacterial phosphatase-encoding genes by histochemical screening of genomic libraries onto indicator medium containing phenolphthalein diphosphate and methyl green. J. Appl. Microbiol. **82**: 177-185.

Rossolini, G.M., Scippa, S., Riccio, M.L., Berlutti, F., Macaskie, L.E. and Thaller, M.C. (1998). Bacterial non-specific acid phosphohydrolases: Physiology, evolution and use as tools in microbial technology. CMLS Cell. Mol. Life Sci. **54**: 833-850.

Rossolini, G.M., Thaller, M.C., Pezzi, R. and Satta, G. (1994). Identification of an *Escherichia coli* periplasmic acid phosphatase containing a 27-kDa polypeptide component. FEMS Microbiol. Lett. **118**: 167-173.

Satta, G. Grazi, G., Varaldo, P.E. and Fontana, R. (1979). Detection of bacterial phosphatase activity by means of an original and simple test. J. Clin. Pathol. **32**: 391-395.

Satta, G., Pompei, R. and Ingianni, A. (1984). The selective staining mechanism of phosphatase producing colonies in the diphosphatephenolphthalein-methyl green method for the detection of bacterial phosphatase activity. Microbiologica **7**: 159-170.

Satta, G., Pompei, R., Grazi, G. and Cornaglia, G. (1988). Phosphatase activity is a constant feature of all isolates of all major species of the family *Enterobacteriaceae*. J. Clin. Microbiol. **26**: 2637-641.

Schenborn, E. and Groskreutz, D. (1999). Reporter gene vectors and assays. Mol. Biotech. **13**: 29-44.

Schmidt, S., Wolf, N., Strey, J., Nahrstedt, H., Meinhardt, F. and Waldeck, J. (2005). Test systems to study transcriptional regulation and promoter activity in *Bacillus megaterium*. Appl. Microbiol. Biotechnol. **68**: 647-655.

Serrano-Heras, G., Salas, M. and Bravo, A. (2005). A new plasmid vector for regulated gene expression in *Bacillus subtilis*. Plasmid **54**: 278-282.

Sessitsch, A., Wilson, K.J., Akkermans, A.D.L. and de Vos, W.M. (1996). Simultaneous detection of different *Rhizobium* strains marked with the *Escherichia coli gusA* and the *Pyrococcus furiosus celB* gene. Appl. Environ. Microbiol. **62**: 4191-4194.

Shaw, W.V. (1975). Chloramphenicol acetyltransferase from chloramphenicol-resistant bacteria. Methods Enzymol. **43**: 737-755.

Shortle, D. (1983). A genetic system for analysis of a staphylococcal nuclease. Gene **22**: 181-189.

Sibakov, M., Koivula, T., von Wright, A. and Palva, I. (1991). Secretion of TEM α-lactamase with signal sequences isolated from the chromosome of *Lactococcus lactis* subsp. *lactis*. Appl. Environ. Microbiol. **57**: 341-348.

Smith, H., Bron, S., Van Ee, J. and Venema, G. (1987). Construction and use of signal sequence selection vectors in *Escherichia coli* and *Bacillus subtilis*. J. Bacteriol. **169**: 3321-3328.

Southward, C.M., and Surette, M.G. (2002). The dynamic microbe: Green fluorescent protein brings bacteria to light. Mol. Microbiol. **45**: 1191-1196.

Stewart, G.A.A.B. and Williams, P. (1992). *lux* genes and the application of bacterial luminescence. J. Gen. Microbiol. **138**: 1289-1300.

Stock, J.B., Surette, M.G., Levit, M. and Park, P. (1995). Two-component signal transduction systems: Structure-function relationships and mechanism of catalysis, pp. 25-51. In: Two component signal transduction (J.A. Hoch, T.J. Silhavy, eds.), Washington DC, ASM Press.

Sun, J., Smets, I., Bernaerts, K., Van Impe, J., Vanderleyden, K. and Marchal, K. (2001). Quantitative analysis of bacterial gene expression using the *gusA* reporter gene system. Appl. Environ. Microbiol. **67**: 3350-3357.

Talukder, A.A., Yanai, S. and Yamada, M. (2005). Analysis of reading frame and expressional regulation of randomly-proximal genes in *Escherichia coli.* J. Gen. Appl. Microbiol. **51**: 93-103.

Thaller, M.C., Berlutti, F., Pantanella, F., Pompei, R. and Satta, G. (1992). Modified MacConkey medium which allows simple and reliable identification of *Providencia stuartii*. J. Clin. Microbiol. **30**: 2054-2057.

Thaller, M.C., Berlutti, F., Schippa, S., Iori, P., Passarriello, C. and Rossoloni, G.M. (1995a). Heterogeneous patterns of acid phosphatases containing low-molecular-mass polypeptides in members of the family *Enterobacteriaceae*. Int. J. Syst. Bacteriol. **45**: 255-261.

Thaller, M.C., Berlutti, F., Schippa, S., Lombardi, G. and Rossolini, G.M. (1994). Characterization and sequence of PhoC, the principal phosphate-irrepressible acid phosphatase of *Morganella morganii*. Microbiol. **140**: 1341-1350.

Thaller, M.C., Berlutti, F., Schippa, S., Selan, L. and Rossolini, G.M. (1998a). Bacterial acid phosphatase gene fusions useful as targets for cloning-dependent insertional inactivation. Biotechnol. Prog. **14**: 241-247.

Thaller, M .C., L ombardi, G ., B erlutti, F., S chippa, S . an d R ossolini, G .M. (1995b). Cloning and characterization of the NapA acid phosphatase/phosphotransferase of *Morganella morganii*: Identification of a new family of bacterial acid phosphatase-encoding genes. Microbiol. **141**: 147-154.

Thaller, M.C., Schippa, S., Boci, A., Cresti, S. and Rossolini, G.M. (1997a). Identification of the gene (*aphA*) encoding the class B acid phosphatase/phosphotransferase of *Escherichia coli* MG1655 and characterization of its product. FEMS Microbiol. Lett. **146**: 191-198.

Thaller, M.C., Schippa, S., Iori, P., Berlutti, F. and Rossolini, G.M. (1997b). Cloning of a *Chryseobacterium m eningosepticum* acid phosphatase-encoding gene: Identification of a family of outer membrane bacterial phosphatases. Abstract 97th General Meeting of American Society for Microbiology, Miami Beach, FL. USA, 4-8 May, p.286.b

Thaller, M.C., Scippa, S. and Rossolini, G.M. (1998b). Conserved sequence motifs among bacterial, eukaryotic, and archaeal phosphatases that define a new phosphohydrolase superfamily. Prot. Sci. **7**: 1647-1652.

Thibodeau, S .A., F ang, R . an d Jou ng, J.K . (2004). High-throughput beta-galactosidase assay for bacterial cell-base reporter systems. BioTech. **36**: 410-415.

Tomb, J-F., White, O., Kerlavage, A.R., Clayton, R.A., Sutton, G.G., Fleischmann, R.D., Ketchum, K .A., K lenk, H .P., Gill, S ., D ougherty, B .A., N elson, K., Quackenbush, J., Zhou, L ., Kirkness, E .F., Peterson, S ., L oftus, B ., Richardson, D ., D odson, R ., K halak, H.G., Glodek, A., Mckenney, K., Fitzegerald, L.M., Lee, N., Adams, M.D., Hickey, E.K., Berg, D .E., Gocayne, J .D., U tterback, T .R., Peterson, J. D., Kelley, J .M., Karp, P.D., Smith, H.O., Fraser, C.M. and Venter, J.C. (1997). The complete genome sequence of the gastric pathogen *Helicobacter pylori.* Nature **388**: 539-547.

Tombolini, R ., U nge, A., D avy, M .E., D e B ruin, F .J. an d Jan sson, J. (1997). Flow cytometric and microscopic analysis of GFP-tagged *Pseudomonas f luorescens* bacteria. FEMS Microbiol. Ecol. **22**: 17-28.

Tresse, O ., E rrampalli, D ., K ostrzynska, M ., Leung, K .T., L ee, H ., Trevors, J.T . an d Van E lsas, J.D . 1998. Green fluorescent protein as a visual marker in a *p*-nitrophenol degrading *Moraxella* sp. FEMS Microbiol. Lett. **164**: 187-193.

Tsien, R.Y. (1998). The green fluorescent protein. Annu. Rev. Biochem. **67**: 509-544.

Tsien, R .Y. an d M iyawaki, A . (1998). Seeing the machinery of live cells. Science **280**: 1954-1955.

Uchiya, K-I., Tohsuji, M., Nikai, T., Sughiara, H. and Sasakawa, C. (1996). Identification and characterization of *phoN*, a gene on the large plasmid of *Shigella flexneri* 2a encoding a non-specific phosphatase. J. Bacteriol. **178**: 4548-4554.

Uerkvitz, W. (1988). Periplasmic non-specific acid phosphatase II from *Salmonella typhimurium* LT2. J. Biol. Chem. **263**: 15823-15830.

Uerkvitz, W. and Beck, C.F. (1981). Periplasmic phosphatases in *Salmonella typhimurium* LT2. A biochemical, physiological and partial gene analysis of three nucleoside monophosphate dephosphorylating enzymes. J. Biol. Chem. **256:** 382-389.

Ugorcakova, J., Bukovsa, G. and Timko, J. (2000). Construction of promoter-probe shuttle vectors for *Escherichia coli* and *Corynebacteria* on the basis of promoterless alpha-amylase gene. Folia Microbiol. (Praha) **25**:114-120.

Urbain, J-L.C. (2001). Reporter genes and imaging. J. Nucl. Med. **42**: 106-109.

van Ommen Kloeke, F., Baty, A.M., Eastburn, C.C., Diwu, Z. and Geesey, G.G. (1999). Novel method for screening bacterial colonies for phosphatase activity. J. Microbiol. Methods **38**: 25-31.

Veening, J-W., Smits, W.K., Hamoen, L.W., Jongbloed, J.D.H. and Kuipers, O.P. (2004).
Visualization of differential gene expression by improved cyan fluorescent protein and yellow fluorescent protein production in *Bacillus subtilis*. Appl. Environ. Microbiol. **70**: 6809-6815.

Vincent, J.B., Crowder, M.W. and Averill, B. (1992). Hydrolysis of phosphate monoesters: A biological problem with multiple chemical solutions. Trends Biochem. Sci. **17**: 105-110.

Voorhorst, W. G.B., Eggen, R.I.L., Luesink, E.J. and de Vos, W.M. (1995). Characterization of the *celB* gene coding for β-glucosidase from the hyperthermophilic archaeon *Pyrococcus furiosus* and its expression and mutation analysis in *Escherichia coli*. J. Bacteriol. **177**: 7105-7111.

Ward, W.W. (1998). Biochemical and physical properties of green fluorescent protein, pp. 45-75. In: GFP: Properties, applications and protocols (M. Chalfie and S. Kain, eds.), Wiley-Liss, New York.

Welsh, S. and Kay, S.A. (1997). Reporter gene expression for monitoring gene transfer. Curr. Opin. Biotechnol. **8**: 617-622.

Weppelman, R., Kier, L.D. and Ames, B.N. (1977). Properties of two phosphatases and a cyclic phosphodiesterase of *Salmonella typhimurium*. J. Bacteriol. **130**: 411-419.

Wiles, S., Ferguson, K., Stefanidou, M., Young, D.B. and Robertson, B.D. (2005). Alternative luciferase for monitoring bacterial cells under adverse conditions. Appl. Environ. Microbiol. **71**: 3427-3432.

Wilson, K.J., Hughes, S.G. and Jefferson, R.A. (1992). The *Escherichia coli gus* operon, induction and expression of the *gus* operon in *E. coli* and the occurrence and use of GUS in other bacteria, pp. 7-23. In: GUS Protocols: Using the GUS gene as a reporter of gene expression (S. Gallagher, ed.), New York, Academic Press.

Wilson, T. and Hastings, J.W. (1998). Bioluminescence. Annu. Rev. Cell Dev. Biol. **14**: 197-230.

Wood, K.V. (1995). Marker proteins for gene expression. Curr. Opin. Biotechnol. **6**: 50-58.

CHAPTER TWO*

CHARACTERIZATION OF A PHOSPHATASE SECRETED BY *Staphylococcus aureus* strain 154 AND CLASSIFICATION AS A NEW MEMBER OF THE BACTERIAL CLASS C FAMILY OF NON-SPECIFIC PHOSPHATASES

ABSTRACT

An acid phosphatase, designated SapS, hydrolyzing *p*-nitrophenyl phosphate (*p*NPP), was identified and characterized from the culture supernatant of a *Staphylococcus aureus* strain isolated from vegetables. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the protein indicated an estimated molecular mass of 30 kDa. The enzyme displayed optimum activity at 40°C and pH 5. Characterization of the phosphatase in a reconstitution assay showed that $MgCl_2$ and TritonX-100, respectively, restored maximal activity, but not $CaCl_2$. The phosphatase activity was negatively affected by EDTA as determmined with in vitro enzyme assays. The DNA sequence encoding SapS was cloned and sequenced. The putative acid phosphatase gene encodes a protein of 296 amino acids with a 31-residue signal peptide. Database searches revealed significant amino acid sequence homology of SapS to several proteins belonging to the bacterial class C family of non-specific acid phosphatases. Comparison of the sequences indicated that despite a low level of overall conservation between the proteins, four conserved sequence motifs could be identified.

*** This chapter has been published:**

du Plessis, E.M., Theron, J., Joubert, L., Lotter, T. and Watson, T.G. (2002). Characterization of a phosphatase secreted by *Staphylococcus aureus* strain 154, a new member of the bacterial class C family of non-specific phosphatases. *Systematic and Applied Microbiology* 25: 21-30.

2.1 INTRODUCTION

Phosphatases are ubiquitous among prokaryotes and eukaryotes, and catalyze the dephosphorylation of various substrates by hydrolysis of phosphoester or phosphoanhydride bonds (Vincent *et al*., 1992). Each cell is normally equipped with several different enzymes, which play various essential or accessory roles in cell biology. Some of these enzymes are secreted outside the plasma membrane, where they are either released in a soluble form or retained as membrane-bound proteins. These secreted phosphatases are believed to function essentially in scavenging organic phosphoesters (such as nucleotides, sugar phosphates and phytic acid) that cannot cross the cytoplasmic membrane. Inorganic orthophosphate (P_i) and organic by-products are released, that can be transported across the membrane, thus providing the cell with essential nutrients (Oliver, 1996). Some secreted phosphatases have evolved specialized functions relevant to microbial virulence (Bliska *et al*., 1996; Kaniga *et al*., 1996; Reilly *et al* ., 1996; Chhatwal *et al* ., 1997), whilst other phosphatases are found in the cytosolic compartment where they may be involved in dephosphorylating reactions occurring in signal transduction, as well as in several metabolic pathways (Stock *et al*., 1995).

The interest in bacterial phosphatases is, however, not only related to their multiple roles in the biology of the prokaryotic cell and to their involvement in microbial pathogenecity, but also to the possibility of exploiting these enzymes as investigative tools in regulation of gene expression (Makino *et al*., 1994), paradigms for molecular evolution (Thaller *et al*., 1998b), markers for bacterial taxonomy and identification (Pompei *et al* ., 1990), reporters in molecular biology (Manoil and Beckwith, 1985; Thaller *et al* ., 1998a), and tools for bioremediation in environmental microbiology (Macaskie, 1990; Basnakova *et al*., 1998).

Classification of phosphatases was initially based on the functional and biophysical properties of the enzyme. As molecular sequence data became available, it was evident that phosphatases could be grouped into molecular families on the basis of amino acid sequence similarity. The structural criterion has led to the definition of various molecular families of phosphatases for which signature sequence motifs have been defined (Thaller *et al*., 1998b). Current knowledge on bacterial phosphatases is, however, far from complete. Most of the available information is derived from studies performed in the enterobacterial family (Satta *et al*., 1988; Thaller *et al* ., 1995a, 1995b; 1997a) and information on phosphatases of other bacterial species is limited. The study of microbial phosphatases, therefore, remains an active

investigational field, with relevance to various aspects of microbial physiology and biotechnology.

A bacterial strain producing acid phosphatase was isolated from vegetables and identified as a strain of *Staphylococcus aureus*. In this Chapter, the biochemical characterization of the extracellular phosphatase, as well as the cloning and characterization of the phosphatase gene encoding a type C non-specific acid phosphatase are reported. This constitutes the first report on the characterization of an acid phosphatase from *S. aureus* at the molecular level.

2.2 MATERIALS AND METHODS

2.2.1 Bacterial strain and characterization of the bacterium

S. aureus strain 154, used in this study, was isolated from rotting vegetables while screening bacterial isolates for the presence of extracellular phosphatases. The production of an extracellular phosphatase by the purified bacterial isolate on solid medium was verified by activity staining (Golovan *et al*., 2000) and the bacterium was subsequently identified as 99% identical to *S. aureus* by the API system (bio-Mérieux, France). To confirm its identity, the 16S rDNA gene of the bacterium was PCR-amplified using the universal primers fD1 and rP2 (Weisburg *et al*., 1991). The purified PCR product was directly sequenced and databases were then searched, as described below, for sequences similar to the 16S rRNA gene sequence.

2.2.2 Culture conditions and concentration of extracellular proteins

The bacterial strain was routinely cultured in LB medium (1% [w/v] tryptone, 0.5% [w/v] yeast extract, 1% [w/v] NaCl; pH 7). A single bacterial colony was inoculated into 250-ml Erlenmeyer flasks containing 50 ml of sterile LB medium and incubated at 37°C for 24 h on a rotary shaker (175 rpm). Bacterial cells were removed from the culture supernatant by centrifugation at 10 000 × *g* for 15 min at 4°C. The culture supernatant was then directly assayed for phosphatase activity or following concentration by mixing the culture supernatant with four volumes of ice-cold acetone and incubating at -70°C for 1 h. The precipitate was collected by centrifugation at 12 000 × *g* for 30 min, air-dried and suspended in 0.1 M sodium acetate (pH 5).

2.2.3 Enzyme activity assay

Extracellular phosphatase activity toward *p*-nitrophenyl phosphate (*p*NPP) was determined according to the method of Golovan *et al*. (2000), with the following modifications. Under standard conditions, the assays were performed by incubating 100 µl of enzyme preparation with 300 µl of *p*NPP substrate (Roche Diagnostics, Mannheim, Germany), at a final concentration of 25 mM, in 0.1 M sodium acetate (pH 5). Following incubation at 37°C for 30 min, the reaction was terminated by the addition of 1 ml of 1 M NaOH and the liberated *p*-nitrophenol (*p*NP) was measured at 405 nm. The extinction coefficient of *p*-nitrophenyl was taken to be 18.5 $cm^2.\mu mol^{-1}$ (Walter and Schütt, 1974), and one unit of enzyme activity was defined as the amount of enzyme able to release 1 µmol of *p*-nitrophenol per min under the assay conditions.

2.2.4 Influence of pH and temperature on enzyme activity

The influence of pH on phosphatase activity was determined at 37°C in the following buffers: pH 3.5 to 6, 0.1 M sodium acetate-acetic acid; pH 7 to 9, 0.1 M Tris-HCl; pH 9 to 10, 0.1 M glycine-NaOH. To determine the influence of temperature on phosphatase activity, samples were incubated in 0.1 M sodium acetate buffer (pH 5) at various temperatures ranging from 20 to 70°C. Thermostability was investigated by measuring the residual activities after incubation of the enzyme for 30 min at different temperatures between 37 and 60°C in 0.1 M sodium acetate buffer (pH 5). In all cases, the enzyme activity was determined as described above.

2.2.5 Protein determination

Protein concentration of enzyme samples was determined by the method of Bradford (1976), with the BioRad protein assay kit (BioRad, Hercules, CA), and with bovine serum albumin as standard.

2.2.6 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and zymographic detection of phosphatase activity

SDS-PAGE was performed with 12% polyacrylamide gels by the method of Laemmli (1970) after the samples had been heated at 37ºC for 30 min. Molecular weight markers (Roche Diagnostics) were included in each gel. Following electrophoresis, the gels were either

stained with Coomassie brilliant blue R-250 to visualize the extracellular protein bands or incubated for 16 h at room temperature in several changes of renaturation buffer for zymographic analysis (Hamilton *et al* ., 2000). After renaturation treatment, gels were equilibrated for 1 h at 37°C in 0.1 M sodium acetate buffer (pH 5) and then developed for phosphatase activity. For development, the gels were incubated at 37°C for 15 min to 1 h in 0.1 M sodium acetate (pH 5) with 0.1% (w/v) α-naphtyl phosphate and 0.2% (w/v) Fast Garnet GBC salt (Sigma-Aldrich, Aston Manor, South Africa) (Gabriel, 1971; Golovan *et al*., 2000). Phosphatase activity was indicated by the presence of black-stained bands.

2.2.7 Effects of metal ions and other reagents on phosphatase activity

To examine the effects of various substances on phosphatase activity, SDS-PAGE gels were subjected to renaturation treatment in 0.1 M Tris-HCl (pH 7) containing $MgCl_2$, $CaCl_2$, $ZnCl_2$ or TritonX-100. Phosphatase inhibition tests were performed by adding EDTA, sodium molybdate or SDS to the equilibration buffer after renaturation treatment of the gels. The phosphatase activity was assayed by the staining procedure described above. Alternatively, the effect of the inhibitors on enzyme activity was assayed, as described under enzyme activity assays, by including the inhibitors in the assay buffers across a range of pH values from 3.5 to 10.

2.2.8 Amino-terminal amino acid sequencing

The extracellular protein preparation was subjected to SDS-PAGE and then electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore Inc., Freehold, NJ, USA). After transfer, the membrane was stained with 0.1% Coomassie brilliant blue R-250 for 5 min, and the appropriate band was excised and analyzed by automated Edman degradation performed with a Procise 492 automatic sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA).

2.2.9 Recombinant DNA methodology

High-molecular mass chromosomal DNA was extracted from *S. aur eus* strain 154 as described by Lovett and Keggins (1979), but 10 mg/ml lysozyme was added for effective lysis of the cells. To obtain the phosphatase gene, the following oligonucleotides primers were designed: ERF (5'-GGCATGAATAAAATTTCAAAG-3', sense primer) and ERR (5'-GGCTGCAGTTATTTAACTTCGCCTGT-3', antisense primer). The polymerase chain

reaction (PCR) mixtures contained 25 ng of chromosomal DNA, 1 × *Taq* buffer, 2 mM $MgCl_2$, 200 µM of each deoxynucleotide triphoshate (dNTP), 0.5 µM of each the sense and antisense primers, and 1 U Biotaq DNA polymerase (Bioline USA Inc., Randolph, MA) in a total reaction volume of 100 µl. The thermocycling was performed by using a Progene thermocycler (Techne, Burlington, NJ) and incubation was for 1 cycle at 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min. The final elongation step at 72°C was for 5 min. The amplified product was purified from a 0.8% (w/v) agarose gel with the Geneclean kit (Bio 101 Inc.,Vista, CA), ligated to T-tailed plasmid pBluescript II Sk (+) and then electroporated into *E. coli* DH10B. Plasmid DNA was isolated by using a Plasmid Midiprep Kit (QIAGEN, Hilden, Germany), characterized by restriction endonuclease digestion and recombinant plasmid DNA was subsequently sequenced.

2.2.10 DNA sequencing and sequence analysis

Nucleotide sequencing was performed with an Applied Biosystems 377 DNA sequencer using the ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction kit v3.0 (Applied Biosystems), in accordance with the manufacturer's instructions. Sequencing primers M13 forward and reverse were used to sequence both DNA strands. The sequence data was edited with the programmes Sequencing Analysis 3.1 and Sequencing Navigator 1.0.1 included in the ABI PRISM™ software package (Applied Biosystems). The BLAST algorithm was used to search the databases (Altschul *et al*., 1990), while multiple alignments were carried out with the CLUSTALW algorithm (Thompson *et al*., 1994). Signal sequence prediction was carried out using the SIGNALP program (Nielsen *et al.*, 1997).

2.2.11 Sequence accession number

The nucleotide sequence of the acid phosphatase gene from *S. aureus* strain 154 and the encoded amino acid sequence have been deposited in the GenBank nucleotide database under accession number AY061973.

2.3 RESULTS

2.3.1 *Staphylococcus* strain identification

Strain 154 was initially identified on the basis of its phenotypic, physiological and biochemical characteristics. In order to confirm the identity, a partial 16S rDNA sequence (1444 bp) was determined. A search of the databases for similar sequences pointed to the genus *Staphylococcus.* The 16S rDNA sequence of strain 154 differed from the 16S rDNA sequences of other *S. aureus* strains by only 3 nucleotides (99.7% similarity), and from the sequences of other *Staphylococcus* species by 23 nucleotides (*S. epidermidis*; 98% similarity), 25 nucleotides (*S. capitis*; 98% similarity), 30 nucleotides (*S. warneri*; 97%) and more than 34 nucleotides (other *Staphylococcus* species). It was therefore concluded that strain 154 is a strain of *Staphylococcus aureus.*

2.3.2 Characterization of the phosphatase enzyme

When *S. aureus* strain 154 was cultured in LB medium for 24 h, high levels of growth were obtained and the extracellular phosphatase was detected at 1.85 U/mg of protein in the culture supernatant. The number and approximate molecular weight of the extracellular phosphatase(s) were determined by SDS-PAGE and activity staining (Fig. 2.1). The SDS-PAGE revealed that the culture supernatant contained one major and several minor bands. Following staining of a duplicate SDS-PAGE gel for phosphatase activity, a single band with an apparent molecular weight of approximately 30 kDa was obtained. This corresponded with the size of the major protein band, indicating that the phosphatase was a major extracellular enzyme of the bacterium. The *S. aureus* strain 154 was therefore routinely cultured in LB medium and after centrifugation of the culture broth, the supernatant was used as a source of phosphatase to study some basic properties of the enzyme.

2.3.3 Properties of the phosphatase

The activity of the phosphatase at various pH values was measured, following adjustment of the reaction pHs from 3.5 to 10 with various buffers. The enzyme showed a broad pH activity profile with maximal activity at pH 5 (Fig. 2.2A). However, more than 80% of the maximal activity remained from pH 4.5 to 6. The optimum temperature of the enzyme was determined by varying the reaction temperature at pH 5. The enzyme had an optimum temperature of 40°C. The enzyme remained active over a range of temperatures varying

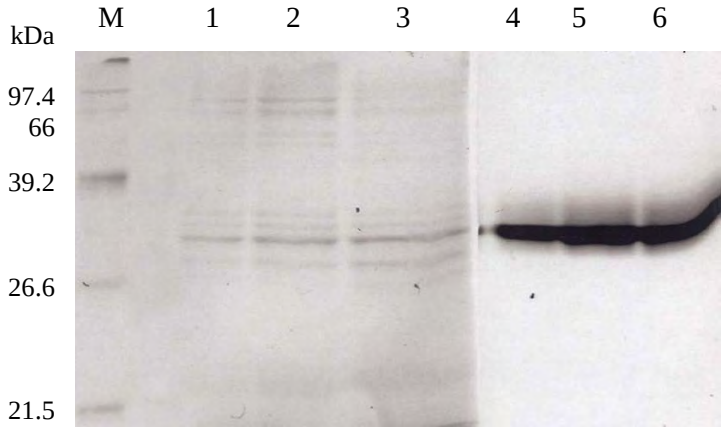


Fig. 2.1 Analysis of the presence of acid phosphatases in cell-free supernatants of *S. aureus* strain 154. Aliquots of culture supernatants were separated by SDS-PAGE and stained with Coomassie blue to visualize the extracellular protein bands (lanes 1-3), or for acid phosphatase activity following renaturation treatment (lanes 4-6). Lane M, low-range protein molecular weight marker; lanes 1 and 4, unconcentrated culture supernatant; lanes 2 and 5, 10× concentrated culture supernatant; lanes 3 and 6, 20× concentrated culture supernatant. The sizes of the molecular weight standards (in kilodaltons) are indicated to the left.

from 20-65°C, with approximately 60% and 83% relative activity at 30 and 50°C, respectively. From 50°C onwards, the activity declined sharply, and it was finally undetectable at 70°C (Fig. 2.2B). The thermal stability of the enzyme was determined by incubating it at pH 5 for 30 min at different temperatures and then detecting the residual activity. Heat treatment at 37°C did not result in significant loss of acid phosphatase activity. However, incubation at higher temperatures induced activity loss. About 56% of the initial activity was lost after incubation at 50°C and almost all enzyme activity was lost after incubation at 60°C (results not shown).

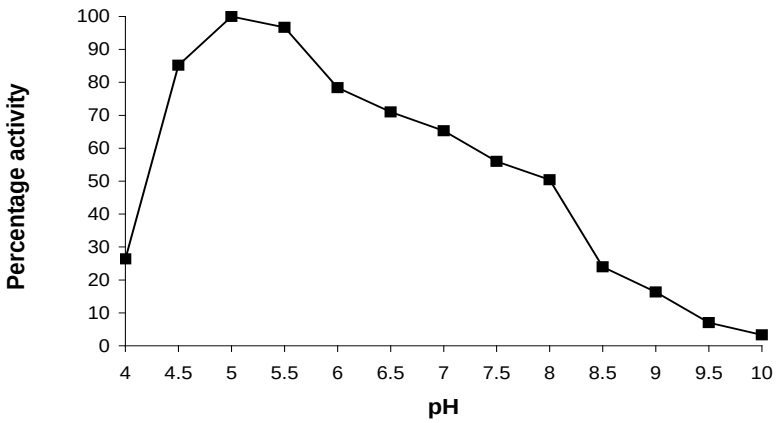
2.3.4 Effect of additives on acid phosphatase activity

The nature of the metal ion(s) required for acid phosphatase activity was studied by using a reconstitution assay. The acid phosphatase enzyme was found to lack activity in 0.1 M Tris-HCl (pH 7) buffer with no additives. However, the full activity was apparently restored with the addition of $MgCl_2$ (0.05 mM) or TritonX-100 (2% [v/v]). Although acid phosphatase activity was partially restored by using $ZnCl_2$ (0.05 mM), $CaCl_2$ did not restore the enzyme activity. Using a similar approach, the acid phosphatase did not appear to be inhibited by EDTA (20 mM), sodium molybdate (25 μM) or SDS (0.01% [w/v]). However, data obtained by quantitative *in vitro* enzyme activity assays indicated that EDTA at a final concentration of 15 mM reduced the enzyme activity in both the acid pH and alkaline pH range. Although enzyme activity was reduced by 40% at pH 6, 50% at pH 7, and 76% at pH 8, the activity at pH 5 was less affected (30%). Similar results were obtained for the other inhibitors tested in this assay (results not shown).

2.3.5 Sequence analysis of the gene and acid phosphatase from *S. aureus* 154

To confirm the identity of the putative acid phosphatase, N-terminal sequencing of the extracelluar enzyme was performed. This yielded an amino acid sequence of NH_2-KSSAEVQQ. A search of the GenBank database revealed that the sequence corresponded to amino acids 32 to 39 of a hypothetical outer membrane protein in *S. aureus* subsp. *aureus* N315 (Kuroda *et al.*, 2001; GenBank accession no. BAB41519). Based on the genomic DNA sequence, an oligonucleotide primer was consequently designed for a region containing the presumed start of the coding sequence of the gene and another was designed for the region containing the putative stop. The primers were compared to other sequences in the GenBank

A



B

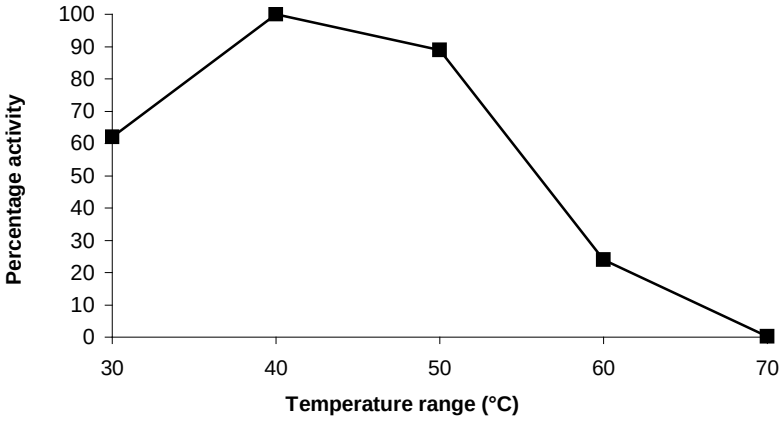


Fig. 2.2 Influence of (A) pH and (B) temperature on the activity of extracellular enzyme preparations prepared from the culture supernatant of *S. aureus* strain 154. For the pH profile, activity was measured at 40°C in buffers of different pH values. For the temperature profile, activity was measured in 0.1 M sodium acetate buffer (pH 5) at different temperatures. Values are the means of results of duplicate experiments.

database to verify their identity and specificity to the targeted sequence. PCR using these oligonucleotides with *S. aureus* strain 154 genomic DNA resulted in amplification of a fragment of approximately 900 bp. The fragment was cloned in pBluescript II SK (+) and sequenced in both directions.

Analysis of the deduced amino acid sequence indicated that the putative acid phosphatase gene encodes a protein of 296 amino acids with a predicted molecular mass of 33 352 Da. The first 31 amino acids in the deduced sequence possessed characteristics of a typical prokaryotic signal peptide, with the cleavage site predicted to be between Ala_{31} and Lys_{32} in the sequence $_{27}$STAFA↓KSSAEVQQ$_{39}$ (Nielsen *et al*., 1997). Cleavage at this point would yield a mature polypeptide with a theoretical molecular mass of 30 056 Da, a value similar to that obtained by SDS-PAGE. Additionally, amino acids 32 to 39 in the deduced sequence matched the N-terminal sequence of the 30-kDa phosphatase in the supernatant from the *S. aureus* strain 154 culture. Thus, the 30-kDa acid phosphatase was designated SapS, for secreted acid phosphatase of *S. aureus.*

2.3.6 Identification of SapS homologues by sequence comparisons

A BLAST search of the *S. aureus* subsp. *aureus* N315 genome (Kuroda *et al*., 2001) revealed that the deduced amino acid sequence of the *S. aureus* strain 154 SapS enzyme displayed 100% sequence identity to a hypothetical protein encoded by an open reading frame (SA0295) located at nucleotides 350 405 - 351 295 of the *S. aureus* N315 genome. A subsequent BLAST search against all protein sequences contained in the GenBank database, revealed similarity between SapS and prokaryotic and eukaryotic phosphatases. Significant levels of similarity (32 to 43% sequence identity) were found with acid phosphatases from *Streptococcus dysgalactiae* subsp. *equismilis* (lppC) (Gase *et al*., 1997), *Streptococcuss pyogenes* (lppC) (Ferretti *et al*., 2001), *Chryseobacterium meningosepticum* (OlpA) (Thaller *et al*., 1997b), *Porphyromonas gingivalis* (OlpA) (unpublished, GenBank accesion no. CAB40970), *Haemophilus influenzae* [*e*(P4)] (Green *et al*., 1991) and a potential acid phosphatase from *Helicobacter pylori* (HP1285) (Tomb *et al*., 1997). A significant level of similarity (35% sequence identity) was also found with a hypothetical protein of unknown function encoded by an open reading frame (PM1064) of the *Pasteurella multocida* chromosome (May *et al*., 2001). The sequence pair distances amongst the above proteins were determined (Person *et al*., 1997) and are presented in Table 2.1.

Multiple alignment of the amino acid sequences of the above-mentioned bacterial phosphatases (Fig. 2.3) revealed that there is little overall homology among all of the proteins. The greatest degree of deviation was located at the ends of both termini of the proteins. However, SapS did possess two sequence motifs which have been described by Thaller *et al*. (1998b) to be shared signature motifs in a family of proteins belonging to bacterial class C non-specific acid phosphatases. The first one is [IV]-[VAL]-D-[IL]-D-E-T-[VM]-L-X-[NT]-X(2)-Y (amino acids 101 to 114) and the second one is [IV]-[LM]-X(2)-G-D-[NT]-L-X-D-F (amino acids 213 to 224). The overall levels of sequence homology within these domains were 57% (29% amino acid sequence identity) and 58% (42% amino acid sequence identity), respectively. In addition to these two domains, a further two regions displaying a high degree of similarity among all of the proteins were identified. These were located towards the N- and C-terminal side of the proteins. The N-terminal 19-amino-acid-region, corresponding to SapS amino acids 63 to 81, shared 32% amino acid sequence identity (53% similarity). The C-terminal 17-amino-acid region, corresponding to amino acids 246 to 262 of SapS, was the most conserved region in the proteins and shared 53% amino acid sequence identity (71% similarity).

2.4 DISCUSSION

Staphylococci are known to have phosphatase activity (Malveaux and San Clemente, 1967) and previous studies on staphylococcal phosphatases have recorded pH optima of pH 5.25 and pH 6.9, respectively (Tirunarayanan, 1968). The purification of a surface-bound neutral phosphatase from *Staphylococcus aureus* that had an optimal pH range of 7.2 to 7.5 has subsequently been reported by Yousif *et al*. (1994). It may therefore be possible that two distinct staphylococcal phosphatases exist, a neutral phosphatase described by Yousif *et al*. (1994) and an acid phosphatase (This study, Tirunarayanan, 1968). Analysis of the pH optima of *Streptococcus equi* and *Streptococcus zooepidemicus* phosphatase activities by whole-cell enzyme assays indicated that both organisms possess two distinct acid phosphatases with activity optima at pH 5 and pH 6-6.5, respectively (Hamilton *et al.*, 2000). The production of different phosphatases in the same bacterial species may be indicative of different physiological roles played by the respective phosphatases.

Table 2.1: Pairwise identity between the bacterial class C acid phosphatase amino acid sequences

Class C acid phosphatase sequence[a] from:	Similarity value % for class C acid phosphatase sequence[a] from							
	S. aureus strain 154	*S. equismilis*	*S. pyogenes*	*P. gingivalis*	*C. meningosepticum*	*H. influenzae*	*H. pylori*	*P. multocida*
Staphylococcus aureus strain 154 (SapS)	100	42.6	42.7	32	35	32.1	31.6	32.9
Streptococcus equismilis (lppC)		100	82.5	35.9	41	28.9	30.4	31.1
Streptococcus pyogenes (lppC)			100	35.7	40.3	27.1	29.9	29.4
Porphyromonas gingivalis (OlpA)				100	37.4	33.9	26.7	30.5
Chryseobacterium meningosepticum (OlpA)					100	33.6	30.4	31.9
Haemophilus influenzae [*e*(P4)]						100	30.8	58.4
Helicobacter pylori (HP1285)							100	31.8
Pasteurella multocida (PM1064)								100

[a] The sequences are from the following sources: *S. aureus* strain 154 SapS (this study), *Streptococcus dysgalactiae* subsp. *equismilis* lppC (GeneBank accession no. CAA73175), *Streptococcuss pyogenes* lppC (GenBank accession no. AAK34595), *Chryseobacterium meningosepticum* OlpA (GenBank accession no. CAB 40970), *Porphyromonas gingivalis* (OlpA) (GenBank acccesion no CAB40970), *Haemophilus influenzae e*(P4) (GenBank accession no. B64087), *Helicobacter pylori* HP1285 (GenBank accession no. AAD08330) and *Pasteurella multocida* PM1064 (GenBank accession no. AAK03148).

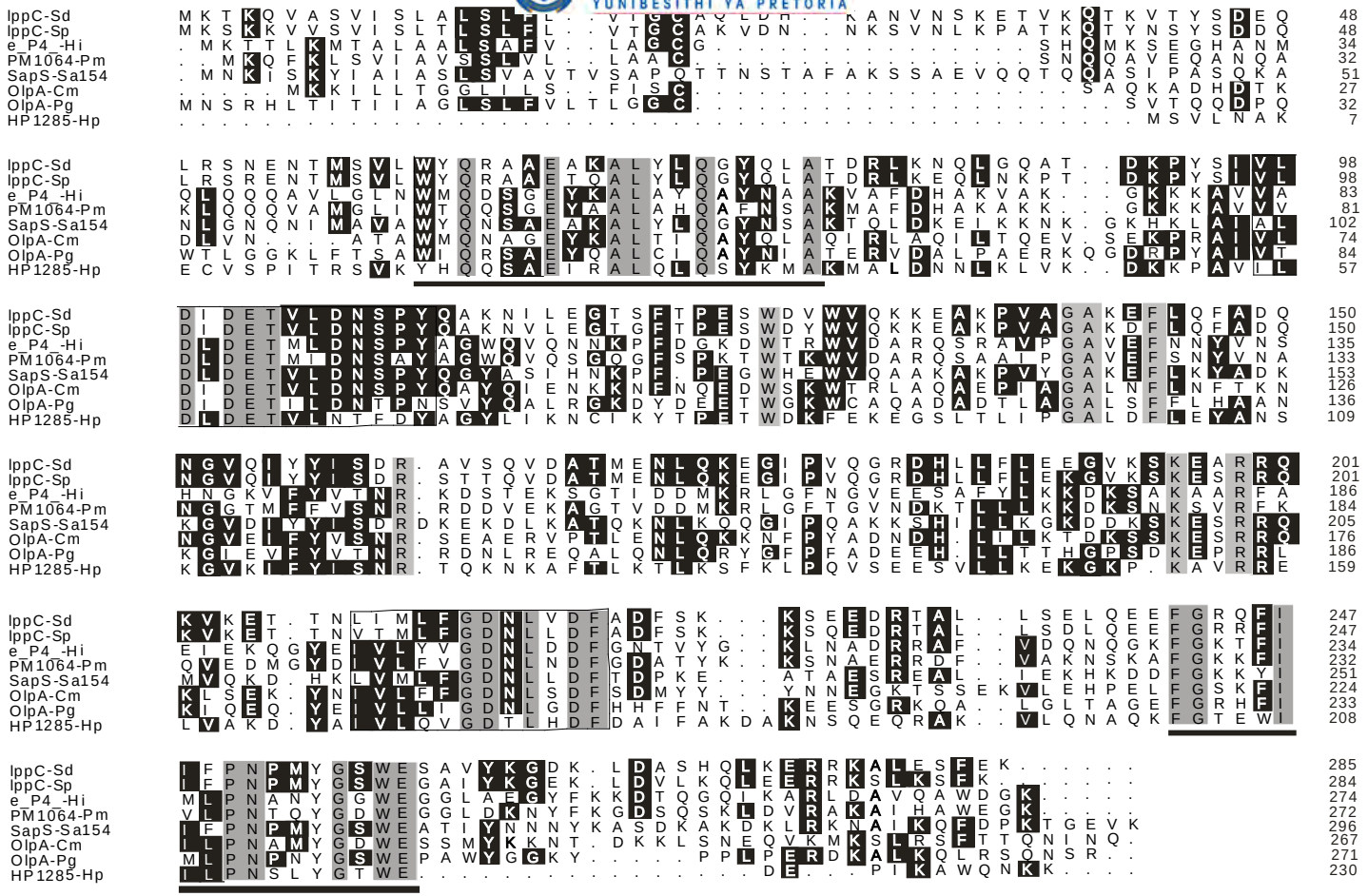


Fig. 2.3 Alignment of the amino acid sequences of *S. aureus* strain 154 acid phosphatase with the amino acid sequences of other bacterial class C non-specific acid phosphatases. Amino acid residues are indicated by single-letter codes. Alignment was maximized by introducing gaps, which are indicated by dots. The numbers indicate the multiple alignment positions from the N terminus. Identical amino acid residues are shaded, while identical amino acids present in at least four of the proteins are shown in inverted text. The regions corresponding to the fingerprint patterns for class C acid phosphatases are boxed. The solid bars indicate two newly identified regions conserved in this family of phosphatases. Abbreviations: lppc-Sd, *S. dysgalactiae* subsp. *equismilis* LppC; lppc-Sp, *S. pyogenes* LppC; ep4-Hi, *H. influenzae e*(P4); PM1064-Pm, *P. multocida* PM1064; SapS-Sa154, *S. aureus* strain 154 SapS; OlpA-Cm, *C. meningosepticum* OlpA; OlpA-Pg, *P. gingivalis* OlpA; HP1285-Hp, *H. pylori* HP1285. The GenBank accession numbers are as indicated in Table 2.1.

S. aureus strain 154, used in this study, to examine the acid phosphatase activities of *S. aureus* was isolated from vegetables and its identity confirmed by 16S rRNA gene analysis. By making use of SDS-PAGE and zymography, the acid phosphatase was observed to predominate under the culture conditions employed in this study (Fig. 2.1). The present results characterized the 30-kDa SapS enzyme as an extracellular acid phosphatase of which the enzymatic activity remained considerable in the acid pH range and showed maximum activity at pH 5 and at 40°C. Enzymatic activity could also be observed in the pH range between 6 to 8, which might indicate a second distinct phosphatase activity (Fig. 2.2A). However, in subsequent zymography assays performed at pH values ranging from pH 5 to 8, only the activity attributed to SapS was detected zymographically. In addition, the SapS acid phosphatase was found to be EDTA-sensitive. In contrast to *S. equi* and *S. zooepidemicus* of which the acid phosphatase with optimum activity at pH 6-6.5 is sensitive to EDTA, but not the acid phosphatase displaying peak activity at pH 5, the activity of the SapS acid phosphatase was reduced in both the acid and the alkaline pH range. Based on the above results, it is unlikely that a second distinct phosphatase may be responsible for the activity observed between pH 6-8. The SapS acid phosphatase thus resembles the *e*(P4) lipoprotein of *H. influenzae*, which also has a pH optimum at 5, but displays significant activity up to pH 6.5 (Reilly *et al*., 1999). In addition, the *e*(P4) lipoprotein protein could also be eluted during anion-exchange chromatography at pH 6 to 8, and is EDTA-sensitive. In this study, attempts at detecting acid phosphatase activity using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as a substrate and nitro blue tetrazolium (NBT) as an enhancer were unsuccessful (results not shown). The obtained data suggests that there is heterogeneity within the bacterial class C acid phosphatases with regards to their ability to hydrolyse BCIP and resistance to inhibitory agents. In contrast to SapS of *S. aureus* and the *e*(P4) lipoprotein of *H. influenzae* (Reilly *et al*., 1999), the LppC acid phosphatase of *S. equi* (Hamilton *et al*., 2000) and *S. equisimilis* (Malke, 1998) are resistant to EDTA and both are able to hydrolyse BCIP utilizing NBT as an enhancer.

To provide genetic evidence that the *S. aureus* strain 154 possesses a gene encoding an acid phosphatase, PCR was used to amplify a product from the *S. aureus* strain 154 genomic DNA that was subsequently cloned and sequenced. This yielded a translated product sequence that displayed homology with bacterial and eukaryotic proteins following sequence database searching. The N-terminal sequence of SapS contained amino acids that exhibited identity with a 35-amino-acid sequence reported by Yousif *et al*. (1994). However, the lack of any

other sequence data and limited phosphatase inhibition studies precluded definitive comparisons to be made. Thus, it remains a possibility that *S. aureus* may contain two distinct phosphatase enzymes. Alternatively, *S. aureus* may possess a single enzyme showing different pH optima, depending upon metal ion activator or inhibitor, as suggested by Tirunarayanan (1968). The deduced amino acid sequence of SapS and the observed acid phosphatase activity in zymogram and enzyme activity assays are nevertheless consistent with its classification as a class C phosphatase. Significant levels of sequence similarity were observed between SapS from *S. aureus* strain 154 and bacterial proteins belonging to the class C acid phosphatases (Table 2.1). Weak homology (16 to 19% sequence identity) was observed between SapS and a family of plant proteins including the APS1 tomato acid phosphatase (GenBank accession no. 445121), a soybean acid phosphatase (GenBank accession no. T07086) and an *Arabidopsis thaliana* protein (GenBank accession no. AAC67358) for which acid phosphatase activity has not yet been reported but whose primary structure is similar to the tomato and soybean enzymes. Low levels of similarity to AphA of *E. coli* (GenBank accession no. P32697) and NapA of *Morganella morganii* (GenBank accession no. Q59544) were also observed (19 and 21% sequence identity, respectively). Both of these enzymes have been classified as class B acid phosphatases (Thaller *et al*., 1995b; 1997a).

By multiple analyses, the amino acid sequence of SapS was compared with the structural homologues of bacterial origin. Of the eight proteins shown in Fig. 2.3, data are available for *S. equisimilis* (Malke, 1998), *H. influenzae* (Thaller *et al*., 1997b), *C. meningosepticum* (Thaller *et al*., 1997b) and, as shown here, *S. aureus*, that establish their functioning as acid phosphatases. The lack of available data on SapS homologues is not surprising given that the investigation of bacterial non-specific acid phosphatases is still maturing and that the class C non-specific acid phosphatases were recognized only recently as a distinct group (Thaller *et al.*, 1998b). Furthermore, evidence for the lipoprotein nature of the proteins has been published for *e*(P4) of *H. influenzae* (Green *et al*., 1991), LppC of *Streptococcus* spp. (Gase *et al*., 1997) and OlpA of *C. meningosepticum* (Thaller *et al*., 1997b). Lipoprotein modification provides a versatile mechanism by which diverse proteins may be anchored within the Gram-positive cell envelope (Sutcliffe and Russell, 1995). In this regard, hydrolysis of phosphate esters, particularly when localized to cell surface structures, may be linked to signal transduction processes. In contrast to PM1064 of *P. multocida,* proteins HP1285 of *H. pylori* and SapS of *S. aureus* lack the consensus ([LV]-[AST]-[GA]↓C) of the

lipoprotein signal sequence cleavage site (Sutcliffe and Russel, 1995). This suggests that, unlike lipoproteins, SapS is not tightly associated with the cell envelope and can be readily released from the periplasmic space. Previous reports have suggested that the minimal catalytic domains of these phosphatases may comprise a common core of about 14 and 11 amino acids, respectively (Thaller *et al* ., 1998b). The SapS enzyme also showed both domains and the position of aspartic acid (D) residues is perfectly conserved within these domains in the respective enzymes. However, two additional conserved domains that were located towards the N- and C-terminal ends of the bacterial class C acid phosphatases were identified. A scan of the Swiss-Prot and Swiss-TrEMBL databases at the ExPASy server (Appel *et al* ., 1994), using either signature pattern, specifically returned the above class C phosphatase proteins. In addition, comparison of the corresponding amino acid sequences (amino acids 63 to 81 and 246 to 262) to other peptides in the databases did not reveal a possible function for these regions. Thus, the functional significance of these domains has yet to be determined. It is nevertheless interesting that there is a strong selective pressure to retain these motifs in enzymes which have little overall homology. Reversible phosphorylation of a protein plays an important role in a wide variety of cellular processes, including regulation of metabolic pathways, cell differentiation and signal transduction (Stock *et al* ., 1995). Signal transduction, involving phosphorylation of histidine and aspartate, is well established in bacteria, but serine, threonine and tyrosine residues are also major targets for reversible phosphorylation in bacteria (Cozonne, 1993). None of the four conserved regions, however, contained sequence motifs of phosphothreonine, -serine and protein tyrosine phosphatases (Barford *et al*., 1998), metallophosphoesterases (Mullaney and Ullah, 1998) and histidine phosphatases (Ostanin *et al*., 1992).

Although the number of SapS homologues proposed here is still small, it is remarkable that those recognized occur in pathogenic or potentially pathogenic species (Table 2.1, Fig. 2.3). *S. aureus* itself is an important pathogen in human disease and the cause of infections ranging from mild, such as skin infections and food poisoning, to life-threatening, such as pneumonia, sepsis, osteomyelitis, and infective endocarditis (Projan and Novick, 1997). However, most of the enzymes, including SapS, require further functional characterization to provide insight into their possible physiological role. It remains to be seen whether this role is limited to serving nutritional functions by scavenging organic phosphoesters (Stock *et al* ., 1995) or extends to pathogenic functions. To this end, detailed mechanistic studies and site-directed mutagenesis are needed to determine the mode of action of SapS. Similarly, SapS deletion

mutants are required to elucidate the precise physiological function of the enzyme and whether it contributes to the pathogenecity of *S. aureus*.

2.5 ACKNOWLEDGEMENTS

We would like to thank Pascal Cossette for amino terminal sequencing of the acid phosphatase enzyme and Jacques Smit and Ilze Meyer for expert technical assistance.

2.6 REFERENCES

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990). Basic local alignment tool. J. Mol. Biol. **215**: 403-410.

Appel, R.D., Bairoch, A. and Hochstrasser, D.F. (1994). A new generation of information retrieval tools for biologists: The example of the ExPASy WWW server. Trends Biochem. Sci. **19**: 258-260.

Barford, D., Das, A.K. and Egloff, M-P. (1998). The structure and mechanism of protein phosphatases: Insights into catalysis and regulation. Annu. Rev. Biomol. Struct. **27**: 133-164.

Basnakova, G., Stephens, E.R., Thaller, M.C., Rossolini, G.M. and Macaskie, L.E. (1998). The use of *Escherichia coli* bearing a *phoN* gene for the removal of uranium and nickel from aqueous flows. Appl. Microbiol. Biotechnol. **50**: 266-272.

Bliska, J.B., Guan, K., Dixon, J.E. and Falkow, S. (1991). Tyrosine phosphate hydrolysis of host proteins by an essential *Yersinia* virulence determinant. Proc. Natl. Acad. Sci. USA **88**: 1187-1191.

Bradford, M. (1976). A rapid and sensitive method for quantification of microgram quantities of protein utilising the principle of protein-dye binding. Anal. Biochem. **72**: 248-254.

Chhatwal, G.S., Walker, M.J., Yan, H., Timmis, K.N. and Guzman, C.A. (1997). Temperature-dependent expression of an acid phosphatase by *Bordetella bronchiseptica*: Role in intracellular survival. Microb. Pathog. **22**: 257-264.

Cozzone, A.J. (1993). ATP-dependent protein kinases in bacteria. J. Cell. Biochem. **51**: 7-13.

Ferretti, J.J., Mcshan, W.M., Adjic, D., Savic, D., Savic, G., Lyon, K., Primeaux, C., Sezate, S.S., Surorov, A.N., Kenton, S., Lai, H., Lin, S., Qian, Y., Jia, H.G., Najar, F.Z., Ren, Q., Zhu, H., Song, L., White, J., Yuan, X., Clifton, S.W., Roe, B.A., McLaughlin, R.E. (2001). Complete

genome sequence of an M1 strain of *Streptococcus pyogenes.* Proc. Natl. Acad. Sci. USA **98**: 4658-4663.

Gabriel, O. (1971). Locating enzymes on gels. Methods Enzymol. **17A**: 578-604.

Gase, K., Liu, G., Bruckmann, A., Steiner, K., Ozegowski, J. and Malke, H. (1997). The *lppC* gene of *Streptococcus e quisimilis* encodes a lipoprotein that is homologous to the *e*(P4) outer membrane protein from *Haemophilus influenzae*. Med. Microbiol. Immunol. **186**: 63-73.

Golovan, S., Wang, G., Zhang, J. and Forsberg, C.W. (2000). Characterization and overproduction of the *Escherichia c oli a ppA* encoded bifunctional enzyme that exhibits both phytase and acid phosphatase activities. Can. J. Microbiol. **46**: 59-71.

Green, B.A., Farley, J.E., Quinn-Dey, T., Deich, R.A. and Zlotnick, G.W. (1991). The *e*(P4) outer membrane protein of *Haemophilus influenzae:* Biologic activity of anti-*e* serum and cloning and sequencing of the structural gene. Infect. Immun. **59**: 3191-3198.

Hamilton, A., Harrington, D. and Sutcliffe, I.C. (2000). Characterization of acid phosphatase activities in the equine pathogen *Streptococcus equi*. System. Appl. Microbiol. **23**: 325-329.

Kaniga, K., Uralil, J.B. and Galan, J. (1996). A secreted protein tyrosine phosphatase with modular effector domains in the bacterial pathogen *Salmonella typhimurium*. Mol. Microbiol. 21: 663-641.

Kuroda, M., Ohta, T., Uchiyama, T., Baba, T., Yuzawa, H., Kobayashi, I., Cui, L., Oguchi, A., Aoki, K., Nagai, Y., Lian, J., Ito, T., Kanamori, M., Matsumaru, H., Maruyama, A., Murakami, H., Hosoyama, A., Mizutani-Ui, Y., Kobayashi, N., Tanaka, T., Sawano, T., Inoue, R., Kaito, C., Sekimizu, K., Hirakawa, H., Kuhara, S., Goto, S., Yabuzaki, J., Kanehisa, M., Yamashita, A., Oshima, K., Furuya, K., Yoshino, C., Shiba, T., Hattori, M., Ogasawara, N., Hayashi, H., Hiramatsu, K. (2001). Whole genome sequencing of methicillin-resistant *Staphylococcus aureus*. Lancet **357**: 1225-1240.

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

Lovett, P.S. and Keggins, K.M. (1979). *B. s ubtilis* as a host for molecular cloning. Methods Enzymol. **68**: 342-357.

Macaskie, L. (1990). An immobilized cell bioprocess for the removal of heavy metals from aqueous flows. J. Chem. Technol. Biotechnol. **49**: 357-379.

Makino, K., Amemura, M., Kim, S-K., Nakata, A. and Shinagawa, H. (1994). Mechanism of transcriptional activation of the phosphate regulon in *Escherichia coli*, pp. 5-12. In: Phosphate in microorganisms (A. M. Torriani-Gorini, E. Yagil, S. Silver, eds.), Washington DC, ASM Press.

Malke, H. (1998). Cytoplasmic membrane lipoprotein LppC of *Streptococcus equisimilis* functions as an acid phosphatase. Appl. Environ. Microbiol. **64**: 2439-2442.

Malveaux, F.J. and San Clemente, C.L. (1967). Elution of loosely bound acid phosphatase from *Staphylococcus aureus.* Appl. Environ. Microbiol. **15**: 738-743.

Manoil, C. and Beckwith, J. (1985). Tn*phoA*: A transposon probe for protein export signals. Proc. Natl. Acad. Sci. USA **82**: 8129-8133.

May, B.J., Zhang, Q., Li, L.L., Paustian, M.L., Whittam, T.S. and Kapur, V. (2001). Complete genomic sequence of *Pasteurella multocida*, Pm70. Proc. Natl. Acad. Sci. USA **98**: 3460-3465.

Mullaney, E.J. and Ullah, A.H.J. (1998). Conservation of the active site motif in *Aspergillus niger* (*ficuum*) pH 6.0 optimum acid phosphatase and kidney bean purple acid phosphatase. Biochem. Biophys. Res. Commun. **243**: 471-473.

Nielsen, H., Engelbrecht, J., Brunak, S. and Von Heijne, G. (1997). Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Prot. Eng. **10**: 1-6.

Oliver, D.B. (1996). Periplasm, pp. 88-103. In: *Escherichia coli* and *Salmonella* (F.C. Neidhardt, R. Curtiss III, E.C.C. Lin, J.L. Ingraham, K.B. Low, B. Magasanik, W. Reznikoff, M. Riley, M. Schaechter, H.E. Umbarger, eds.) 2nd ed., Washington DC, ASM Press.

Ostanin, K., Harms, E.H., Srevis, P.E., Kuciel, R., Zhou, M.M. and Van Etten, R.L. (1992). Overexpression, site-directed mutagenesis, and mechanism of *Escherichia coli* acid phosphatase. J. Biol. Chem. 267: 22830-22836.

Person, W.R., Wood, T., Zhang, Z. and Miller, W. (1997). Comparison of DNA sequences with protein sequences. Genomics **46**: 24-36.

Pompei, R., Cornaglia, G.M., Ingianni, A. and Satta, G. (1990). Use of a novel phosphatase test for simplified identification of the tribe *Proteae*. J. Clin. Microbiol. **28**: 1214-1218.

Projan, S.J. and Novick, R.P. (1997). The staphylococci in human diseases, pp. 55-81. In: The molecular basis of pathogenicity (K.B. Crossley, G.L. Archer, eds.), New York, Churchill Livingstone.

Reilly, T.J., B aron, G.S., Nano, F. and Kuhlenschmidt, M.S. (1996). Characterization and sequencing of a respiratory burst-inhibiting acid phosphatase from *Francisella tularensis*. J. Biol. Chem. **271**: 10973-10983.

Reilly, T.J., Chance, D.L. and Smith, A. (1999). Outer membrane lipoprotein *e*(P4) of *Haemophilus influenzae* is a novel phosphomonoesterase. J. Bacteriol. **181**: 6797 6805.

Satta, G., P ompei, R., Grazi, G. and Cornaglia, G. (1988). Phosphatase activity is a constant feature of all major species of the family *Enterobacteriaceae*. J. Clin. Microbiol. **26:** 2637-2641.

Stock, J.B., S urette, M.G., L evit, M. and Park, P. (1995). Two-component signal transduction systems: Structure-function relationships and mechanism of catalysis, pp. 25-51. In: Two component signal transduction (J.A. Hoch, T.J. Silhavy, eds.), Washington DC, ASM Press.

Sutcliffe, I.C. and Russell, R.R.B. (1995). Lipoproteins of Gram-positive bacteria. J. Bacteriol. **177**: 1123-1128.

Thaller, M.C., Berlutti, F., S chippa, S., Iori, P., P assariello, C. and Rossolini, G.M. (1995a). Heterogeneous patterns of acid phosphatases containing low-molecular-mass polypeptides in members of the family *Enterobacteriaceae*. Int. J. Syst. Bacteriol. **45**: 255-261.

Thaller, M.C., Berlutti, F., Schippa, S., S elan, L. and Rossolini, G.M. (1998a). Bacterial acid phosphatase gene fusions useful as targets for cloning-dependent insertional inactivation. Biotechnol. Prog. **14**: 241-247.

Thaller, M.C., Lombardi, G., Berlutti, F., Schippa, S. and Rossolini, G.M. (1995b). Cloning and characterization of the NapA acid phosphatase/phosphotransferase of *Morganella m organii*: Identification of a new family of bacterial acid phosphatase encoding genes. Microbiol **141**: 147-154.

Thaller, M.C., S chippa, S., Boci, A., Cresti, S. and Rossolini, G.M. (1997a). Identificationof the gene (*aphA)* encoding the class B acid phosphatase/phosphotransferase of *Escherichia coli* MG1655 and characterization of its product. FEMS Microbiol. Lett. **146**: 191-198.

Thaller, M.C., S chippa, S., Iori, P., B erlutti, F. and Rossolini, G.M. (1997b). Cloning of a *Chryseobacterium m eningosepticum* acid phosphatase-encoding gene: Identification of a family of outer membrane bacterial phosphatases. Abstract 97th General Meeting of American Society for Microbiology, Miami Beach, FL. USA, 4-8 May, p.286.

Thaller, M.C., Schippa, S. and Rossolini, G.M. (1998b). Conserved sequence motifs among bacterial, eukaryotic, and archaeal phosphatases that define a new phosphohydrolase superfamily. Prot. Sci. 7: 1647-1652.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994). ClustalW: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl. Acids Res. **22**: 4673-4680.

Tirunarayanan, M.O. (1968). Investigations on the enzymes and toxins of Staphylococci: Study of phosphatase using *p*-nitrophenyl phosphate as substrate. Acta Path. Microbiol. Scand. **74**: 573-590.

Tomb, J-F., White, O., Kerlavage, A.R., Clayton, R.A., Sutton, G.G., Fleischmann, R.D., Ketchum, K.A., Klenk, H.P., Gill, S., Dougherty, B.A., Nelson, K., Quackenbush, J., Zhou, L., Kirkness, E.F., Peterson, S., Loftus, B., Richardson, D., Dodson, R., Khalak, H.G., Glodek, A., Mckenney, K., Fitzegerald, L.M., Lee, N., Adams, M.D., Hickey, E.K., Berg, D.E., Gocayne, J.D., Utterback, T.R., Peterson, J.D., Kelley, J.M., Karp, P.D., Smith, H.O., Fraser, C.M. and Venter, J.C. (1997). The complete genome sequence of the gastric pathogen *Helicobacter pylori.* Nature **388**: 539-547.

Vincent, J.B., Crowder, M.W. and Averill, B. (1992). Hydrolysis of phosphate monoesters: A biological problem with multiple chemical solutions. Trends Biochem. Sci. **17**: 105-110.

Walter, K. and Schütt, C. (1974). Acid and alkaline phosphatase in serum, pp. 856-860. In: Methods of Enzymatic Analysis (H.U. Bergmeyer, ed.), New York, Academic Press.

Weisburg, W.G., Barns, S.M., Pelletier, D.A. and Lane, D.J. (1991). 16S Ribosomal DNA amplification for phylogenetic study. J. Bacteriol. **173**: 697-703.

Yousif, Y., Schiltz, E., Okada, K., Batsford, S. and Vogt, A. (1994). Staphylococcal neutral phosphatase. Apmis **102**: 891-900.

CHAPTER THREE

INACTIVATION OF THE MAJOR CELL WALL PROTEASE GENE (*wprA*) OF *Bacillus halodurans* BhFC01 AND EVALUATION OF *sapS* AS A REPORTER GENE FOR HOST STRAIN EVALUATION AND CELL SURFACE DISPLAY

ABSTRACT

In our laboratory the alkalophilic, thermotolerant Gram-positive strain *Bacillus halodurans* BhFC01 is currently being developed as an expression strain for surface display purposes (Crampton *et al*., 2007). With a view to improving the cell surface display and extracellular production of heterologous proteins and peptides, the major cell wall protease gene (*wprA*) of *B. halodurans* BhFC01 was inactivated to generate strain BhFC04. The *Staphylococcus aureus* acid phosphatase gene (*sapS*) was used as a reporter to evaluate the ability of the C-terminal cell wall-binding domain of the *B. halodurans* peptidoglycan hydrolase gene (*cwlC*) to function as an anchoring motif, and to target the reporter gene product to the cell wall of the host strain. Furthermore, it was also determined whether the extracellular levels of the SapS acid phosphatase enzyme was indeed improved in the *B. halodurans* BhFC04 strain. Zymographic analysis of the *sapS-cwlC* gene fusion product showed that it was targeted to the cell wall of the host strain. However, proteolytic cleavage of the fusion protein remained a problem in *B. halodurans* BhFC04, as was determined through zymographic analysis of the SapS reporter. Nevertheless, increased extracellular levels of the SapS acid phosphatase were obtained with the mutant host strain, as determined by enzymatic assays.

3.1 INTRODUCTION

The capacity of selected *Bacillus* strains to produce and secrete high quantities (20-25 g/L) of extracellular enzymes has placed them amongst the most important industrial enzyme producers (Schallmey *et al*., 2004; Westers *et al*., 2004). The secreted proteins usually remain in biologically active forms and the downstream processing is thus greatly simplified. Despite being used extensively for the large-scale production of native *Bacillus* enzymes such as α-amylases, attempts to optimize *Bacillus subtilis* for the high-level secretion of heterologous proteins have been disappointing (Stephenson *et al*., 1999; Westers *et al*., 2004).

An important factor limiting the use of *B. subtilis* and its relatives for the production of heterologous proteins is the synthesis of high levels of extracellular and cell wall-associated proteases, which degrade heterologous proteins during secretion (Wu *et al*., 1991; Braun *et al*., 1999). In general, native *Bacillus* secretory proteins have co-evolved with these proteases to be resistant to their activity, at least when in their native conformation (Stephenson *et al*., 1999). Although strains deficient in extracellular and cell wall-associated proteases have improved the productivity of *B. subtilis* for the production of heterologous proteins (Wu *et al*., 1991; Lee *et al*., 2000; Olmos-Soto and Contreras-Flores, 2003), the problem of low extracellular yields have only been overcome partially (Stephenson and Harwood, 1998; Murashima *et al*., 2002). Significant amounts of secretory proteins have been reported to be degraded within minutes of being synthesized in *B. subtilis* (Meens *et al*., 1997; Stephenson and Harwood, 1998). Degradation was observed even for *Bacillus* proteins that are highly resistant to proteases in the culture medium, suggesting that at least one component involved in degradation is cell wall-associated (Stephenson and Harwood, 1998). Recent studies have revealed that the slow folding of heterologous proteins at the membrane-cell wall interface of Gram-positive bacteria renders them vulnerable to attack by cell wall-associated proteases (Braun *et al*., 1999; Schallmey *et al*., 2004). In an attempt to improve *B. subtilis* as a production host, the major cell wall protease gene (*wprA*) has been inactivated and was reported to improve extracellular production of heterologous proteins (Stephenson and Harwood, 1998; Lee *et al*., 2000), as well as improve cell surface display of homologous and heterologous proteins (Tsuchiya *et al*., 1999; Kobayashi *et al*., 2000a, 2000b; Kobayashi *et al*., 2002).

Display of heterologous proteins on the surface of microorganisms, enabled by means of recombinant DNA technology, has become an increasingly used strategy in the fields of live vaccine development (Kwak *et al*., 1999; Chen and Georgiou, 2002; Ferreira *et al*., 2005), whole-cell biocatalysis (Jung *et al*., 2006), antibody production (Chen and Georgiou, 2002; Kang *et al*., 2003), environmental bioadsorbents (Xu and Lee, 1999; Samuelson *et al*., 2000; Cho *et al*., 2002), peptide library screening (Turner *et al*., 2003; Majander *et al*., 2005; Dong et *al*., 2006), and novel biosensor development (Wernerus *et al*., 2001). Cell surface display allows heterologous peptides and proteins to be displayed on the surface of microbial cells by fusing them with anchoring motifs of cell surface proteins (Samuelson *et al*., 2002). The protein to be displayed (passenger protein) can be fused to an anchoring motif (carrier protein) by N-terminal, C-terminal or sandwich fusion (Samuelson *et al*., 2002; Lee *et al*., 2003). The Staphylococcal protein A (SpA) has often been used as a model system to study anchoring mechanisms of surface proteins in Gram-positive bacteria (Lee *et al*., 2003). Although *B. subtilis* does not have an anchoring protein similar to the SpA protein (Navarre and Schneewind, 1999; Nguyen and Schumann, 2006), there are, however, many proteins bound non-covalently on the *B. subtilis* cell surface (Kobayashi *et al*., 2000a; Samuelson *et al*., 2002). Cell wall hydrolases are typical of such proteins and some of them consist of two domain structures, *i.e.* an N-terminal catalytic domain and a C-terminal cell wall-binding (CWB) domain (Navarre and Schneewind, 1999; Kobayashi *et al*., 2002).

Various cell wall hydrolases of the genus *Bacillus* have been isolated and characterized, including, amongst other, endo-β-N-acetylglucosaminidase, endo-β-N-acetylmuramidase, endopeptidases and N-acetylmuramoyl-L-alanine amidase, and some of their genes have been cloned (Kuroda *et al*., 1993; Rashid *et al*., 1995; Ishikawa *et al*., 1998). Amidases are the most common cell wall hydrolases and, based on amino acid sequence similarity in the catalytic domain of these enzymes, they have been divided into three classes. Whereas class I amidases comprises of CwlA, CwlL, XlyA, XylB and BlyA, class II amidases comprises of CwlB (LytC), CwlC, CwlD and CwlM, and class III enzymes of SleBs and CwlJ (Ishikawa *et al*., 1998). The class II *cwlB* and *cwlC* genes have been used for cell surface localization of lipases. Tsuchiya *et a l*. (1999) reported the surface localization in *B. subtilis* of the extracellular *B. subtilis* lipase B when fused to the CWB portion of the major autolysin CwlB from *B. subtilis*. The CwlB cell wall-binding domain has also been used for the successful surface localization of the heterologous *Aspergillus or yzae* lipase CutL in *B. subtilis* (Kobayashi *et al*., 2000a). Kobayashi *et al*. (2002) evaluated the cell wall-binding domain of

CwlC for its potential as an anchoring motif and reported that it was useful for displaying lipase B on the *B. subtilis* cell surface.

Our laboratory has a strong interest in developing the moderately thermophilic *B. halodurans* as an expression host and as a surface display system for heterologous proteins (Crampton *et al*., 2007). With the above in mind, the sequenced *B. halodurans* C125 genome (available at: http://www.jamstec.go.jp/genomebase/micrHome_bha.html) was analyzed for the presence of class II amidases that may harbour suitable cell wall-binding domains for surface display purposes. With the exception of *cwlM*, a homologous gene for each of the *B. subtilis* class II amidase-encoding genes could be identified. Since the CwlC cell wall-binding domain had been used successfully in *B. subtilis* for surface localization, the *B. halodurans* peptidoglycan hydrolase gene (*cwlC*) gene was therefore selected for use in this study. The *B. halodurans* CwlC protein displayed 51% amino acid sequence similarity to the CwlC protein of *B. subtilis*, and harbours two repeat motifs in the C-terminal region of the gene, *i.e.* LXKVQXGAFXQRXNAXAL$(X)_7$GY where X is any amino acid. Moreover, since it has been reported that inactivation of the *wprA* cell wall protease gene improved surface display of enzymes and extracellular production of heterologous proteins in *B. subtilis*, the sequenced *B. halodurans* C125 genome was also analyzed for the presence of cell wall protease genes, and one major cell wall protease gene, designated *wprA*, was identified.

This study therefore focused on investigating the use of the *S. aureus sapS* reporter gene for surface display purposes, and for determining heterologous protein production levels in the *B. halodurans* BhFC01 expression strain of which the *wprA* gene had been inactivated. The C-terminal cell wall-binding domain of the *B. halodurans cwlC* gene was used as an anchoring motif for surface display purposes.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids are listed in Table 3.1. *E. coli* was cultured at 37°C in Luria-Bertani medium (LB: 0.5% [w/v] yeast extract, 1% [w/v] tryptone, 1% [w/v] NaCl; pH 7). When appropriate, *E. coli* growth medium was supplemented with ampicillin (100 µg/ml)

or chloramphenicol (20 µg/ml). *B. halodurans* strains were grown at 37°C in LB medium (pH 8.5), and chloramphenicol (5 µg/ml) was added when appropriate.

3.2.2 Recombinant DNA techniques

Plasmid DNA was extracted with a Plasmid Midiprep Kit (QIAGEN, Hilden, Germany) and Perfectprep® Plasmid Mini Kit (Eppendorf, Hamburg, Germany). DNA fragments were purified from agarose gels by use of a DNA Extraction Kit (Fermentas, St. Leon-Rot, Germany). PCR products were cloned into the pGEM®-T Easy vector system (Promega, Madison, USA). Procedures for DNA manipulations were carried out as described by Sambrook and Russell (2001). Enzymes were obtained from Fermentas (St. Leon-Rot, Germany) and Roche Diagnostics (Mannheim, Germany) and were used according to the manufacturers' protocols. Plasmid constructions were first established in *E. coli* DH10B and then transferred to *B. halodurans* BhFC01 and BhFC04, respectively. Transformation of bacteria was performed by electroporation for *E. coli* (Dower *et al*., 1988), and by protoplasting for the *B. halodurans* strains (Crampton *et al*., 2007). PCR was performed with Biotaq DNA polymerase (Bioline USA Inc., Randolph, MA) and a Progene thermocycler (Techne, Burlington, NJ). The oligonucleotides used in this study were obtained from Inqaba Biotechnical Industries, Pretoria, South Africa. Chromosomal DNA was extracted from *S. aureus* 154 and the *B. halodurans* strains according to method of Lovett and Keggins (1979), except that lysozyme was added to a final concentration of 10 mg/ml. Nucleotide sequencing of PCR products and cloned insert DNA was performed with the ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction kit v3.0 (Applied Biosystems, Foster City, CA), followed by resolution on an ABI PRISM™ 310 Genetic Analyser (Applied Biosystems), in accordance with the manufacturer's instructions. All plasmid constructions were verified by restriction endonuclease digestion using agarose gel electrophoresis.

3.2.3 Inactivation of the *B. halodurans* BhFC01 major cell wall protease gene (*wprA*)

3.2.3.1 Construction of integrative plasmid pSEC-WprA⁻

To construct a mutant allele of the 3.159-kb *wprA* gene of *B. halodurans* BhFC01, 5' and 3' gene fragments were PCR amplified, digested and subsequently ligated, thereby deleting 1.056 kb of the internal coding sequence. Whereas primers WprA1-F (containing a *Sac*I site) and WprA1-R (containing a *Bam*HI site) were used to PCR-amplify a 1170-bp DNA fragment corresponding to the 5'-end of the gene, primers WprA2-F (containing a *Bam*HI

Table 3.1: Bacterial strains, plasmids and oligonucleotide primers used in this study

Strain, plasmid or primer	Relevant properties	Source or Reference
Strains:		
E. coli DH10B	(*F⁻ mcrA* Δ*(mrr -hsdRMS-mcrBC) (ᵠ80dlacZΔM15) ΔlacX74 endA1 r ecA1deoR*Δ*(ara -leu)7697 ar aD139 gal U gal K nupG rpsL λ⁻*)	Invitrogen
B. halodurans BhFC01	(Δ*hag*)	Crampton *et al*. (2007)
B. halodurans BhFC04	(Δ*wprA* Δ*hag*)	This study
S. aureus 154	SapS acid phosphatase producer	Du Plessis *et al*. (2002)
Plasmids:		
pSEC194	*E. coli-Bacillus* shuttle vector, temperature-sensitive pE194 *ori*, ColE1 *ori*, Amp^r, Chl^r	Crampton *et al*. (2007)
pSEC194-WprA$^-$	pSEC194 containing the *wprA*$^-$ gene of which the internal 1.056 kb has been deleted, Amp^r, Chl^r	This study
pGEM®-T Easy	Cloning vector for PCR products, ColE1, Amp^r, LacZα peptide	Promega
pGEM-CwlC	pGEM®-T Easy containing the cell wall-binding domain of the *cwlC* gene from *B. halodurans* BhFC01, Amp^r	This study
pGEM-SapS	pGEM®-T Easy containing the full-length *S. aureus* 154 *sapS* gene, Amp^r	This study
pNW33N	*E. coli-Bacillus-Geobacillus* shuttle vector, Chl^r	BGSC [a]
pNW33-SapS	pNW33N containing the full-length *sapS* gene from *S. aureus* 154, inclusive of the promoter, signal sequence and mature *sapS* gene sequence, Chl^r	This thesis
pNW33-SC	pNW33N containing the full-length *sapS* gene fused in-frame at the 3' end with the cell-wall binding domain sequence of the *cwlC* gene, Chl^r	This study
Primers [b]:		
WprA1-F [c]	5' - GCGAGCTCTGCAGCGTACTACAACCA - 3'	*Sac*I site incorporated
WprA1-R	5' - GCGGATCCAGCTGATAACGCTACGTA - 3'	*Bam*HI site incorporated
WprA2-F	5' - GCGGATCCTAGCGGACCTGTAGATGT - 3'	*Bam*HI site incorporated
WprA2-R	5' - GGTCTAGATGCCTTGTCCTTCGCTGTA - 3'	*Xba*I site incorporated
WprA-ChromF	5' - CAGCTCGATACGGAGCGG - 3'	
WprA-ChromR	5' - CGACACCCTCTTTACGTATAGTC - 3'	
cwlC/sapS-F5	5' - GGCTCTAGATACATTCATTCTAGCCTC - 3'	*Xba*I site incorporated
cwlC/sapS-R3	5' - GCCTGCAGCTTCGTCTCCTCTTGAACCAA - 3'	*Pst*I site incorporated
sapS/cwlC-F1	5' - GCTGGATCCAATGCTACGTGGATGAG - 3'	*Bam*HI site incorporated
saps/cwlC-R4	5' - GCCTTTATCTAGATTTAACTTCGCCTGT - 3'	*Xba*I site incorporated
M13F	5' - GTAAAACGACGGCCAGT - 3'	

[a] BGSC – *Bacillus* Genetic Stock Centre, Columbus, OH, USA

[b] Relevant restriction sites are underlined

[c] Primers used for amplification of 5' and 3' gene fragments of the *wprA* gene and for amplification of the CWB domain of the *cwlC* gene from *B. halodurans* BhFC01 were designed on the basis of the complete genome sequence of *B. halodurans* C125 (http://www.jamstec.go.jp/genomebase/micrHome_bha.html)

site) and WprA2-R (containing a *Xba*I site) were used to PCR-amplify a 837-bp DNA fragment corresponding to the 3'-end of the gene. Each of the PCR reactions (100 µl) contained 20 ng of chromosomal DNA as template, 1 × *Taq* buffer, 2 mM $MgCl_2$, 200 µM dNTPs, 0.5 µM of each of the sense and antisense primers (Table 3.1), and 1 U of Bioline Biotaq DNA polymerase. Following initial denaturation at 94°C for 3 min, the samples were subjected to 35 cycles of denaturation at 94°C for 1 min, primer annealing at 56°C for 1 min, and primer extension at 72°C for 1 min. After the last cycle, a final extension was performed at 72°C for 5 min. The resulting amplicons were purified by agarose gel electrophoresis, digested with the appropriate restriction endonucleases and then ligated into the *Sac*I and *Xba*I sites of the thermosensitive plasmid pSEC194 in a 3-way ligation reaction to generate the integrative plasmid pSEC-WprA$^-$.

3.2.3.2 Generation of mutant *B. halodurans* BhFC01 strains by *in vivo* recombination

Integration of the mutant *wprA*$^-$ allele into the chromosome of *B. halodurans* BhFC01 was achieved by a two-step procedure, according to the methods described by Biswas *et al*. (1993) and Poncet *et al*. (1997). The pSEC-WprA$^-$ construct was introduced into *B. halodurans* BhFC01 by protoplasting and transformants were selected on selective agar medium (LB agar supplemented with chloramphenicol) at the permissive temperature of 30°C. To allow for chromosomal integration of the recombinant plasmid, transformants were inoculated into 5 ml of selective LB medium and incubated overnight at the non-permissive temperature of 52°C. The cultures were then diluted serially, plated onto selective agar medium and incubated overnight at 52°C. Colony PCR was performed to verify single crossover integration events in selected chloramphenicol-resistant colonies (corresponding to *B halodurans* BhFC01::pSEC-WprA$^-$). For this purpose, primers M13F and WprA-ChromR (Table 3.1), which anneal to the plasmid DNA and genomic sequences downstream of the integration site, respectively, were used. Colonies from which an amplicon of the expected size (2.1 kb) was obtained were subsequently selected, inoculated into 5 ml of non-selective LB medium and incubated overnight at 30°C to allow for excision of the plasmid vector backbone. The cultures were plated onto non-selective LB agar medium and screened for sensitivity to chloramphencol. To confirm that a second crossover event had eliminated the vector DNA from chloramphenicol-sensitive colonies (corresponding to *B hal odurans* BhFC01::*wprA*$^-$), PCR analyses, as described above, were performed. For this purpose, primers WprA-ChromF and WprA-ChromR (Table 3.1), which anneal to genomic sequences

upstream and downstream of the ORF in which the mutant allele was integrated, was used. Consequently, one of the mutant strains, designated *B. halodurans* BhFC04, was selected and used in all subsequent experiments.

3.2.4 Construction of surface display reporter plasmid pNW33-SC

The 1.140-kb *sapS* gene of *S. aureus* 154, inclusive of its promoter and signal sequence, was obtained by PCR amplification using chromosomal DNA as template together with primers sapS/cwlC-F1 and sapS/cwlC-R4 (Table 3.1), which contain a *BamH*I and *Xba*I site, respectively. Primers cwlC/sapS-F5 and cwlC/sapS-R3 (Table 3.1), which contain a *Xba*I and *Pst*I site, respectively, were used with chromosomal DNA from *B. halodurans* BhFC01 as template to PCR-amplify the 630-bp cell wall-binding domain of the *cwlC* gene, starting from amino acid 271 from the N-terminus of the gene. The amplicons were cloned into pGEM®-T Easy (Promega), according to the manufacturer's instructions, to generate pGEM-SapS and pGEM-CwlC, respectively. The cloned insert DNA was sequenced to verify the absence of extraneous mutations. The cloned DNA fragments were subsequently excised by restriction enzyme digestion and ligated into the *Bam*HI and *Pst*I sites of pNW33N in a 3-way ligation reaction, yielding reporter plasmid pNW33-SC, in which the *sapS* gene was fused in-frame with the *cwlC* coding sequence.

3.2.5 Zymographic detection of protease activity

3.2.5.1 Protein sample preparation

B. halodurans strains BhFC01 and BhFC04 were inoculated into 60 ml of LB medium with the appropriate antibiotic and incubated at 37°C for 24 h on a rotary shaker (175 rpm). Following incubation, bacterial cells were harvested by centrifugation at 12 000 × *g* for 20 min at 4°C, and the cell-free culture supernatants were retained for zymography as described below. The cell pellets were suspended in 5 ml of glycine buffer (pH 8) and sonicated for 20 min on ice using a Model HD 2070 Ultrasonic Homogenizer (Bandelin Electronic, Berlin, Germany). The cell lysates were clarified by centrifugation (12 000 × *g*, 10 min) and the pellets, consisting mostly of cell wall proteins, were washed once with sterile distilled water before being suspended in 300 µl of glycine buffer (pH 8).

3.2.5.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and zymography

SDS-PAGE was performed with 12% polyacrylamide gels by the method of Laemmli (1970) after the samples had been heated at 37ºC for 30 min. Low-range molecular weight markers (BioRad, Hercules, CA) were included in each gel. For zymogram analysis, 0.1% (w/v) gelatin co-polymerized with the gels was used. Following electrophoresis at 4ºC and 35 mA constant current, the gels were either stained with Coomassie brilliant blue R-250 to visualize protein bands or processed for zymographic analysis, essentially as described by Karbalei-Heidari *et al.* (2007). Briefly, proteins were renatured by incubating the gels overnight at 4ºC in 2.5% (v/v) TritonX-100 and were then equilibrated for 3.5 h at 37°C in 0.1 M glycine buffer (pH 8). Subsequently, the gels were stained with 1% (w/v) amido black (MP Biomedicals, Eschwege, Germany) for at least 1 h and destained in methanol:acetic acid:water (30:10:60) to reveal zones of substrate hydrolysis.

3.2.6 Amino-terminal amino acid sequencing

The cell wall protein fraction from *B. halodurans* BhFC01 was subjected to SDS-PAGE and then electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore Inc., Freehold, NJ, USA). After transfer, the membrane was stained with 0.1% Coomassie brilliant blue R-250 for 5 min, and the appropriate band was excised and analyzed by automated Edman degradation performed with a Procise 492 automatic sequencer (Perkin-Elmer Applied Biosystems).

3.2.7 Acid phosphatase enzyme activity assays

3.2.7.1 Protein sample preparation and protein concentration determination

B. halodurans strains BhFC01 and BhFC04, each transformed with reporter plasmid pNW33-SapS, were inoculated into 60 ml of LB medium with the appropriate antibiotic and incubated at 37°C for 8, 24 and 48 h, respectively, on a rotary shaker (175 rpm). Protein samples from the cultures were prepared as described by Van der Vaart *et al*. (1997), with the following modifications. For cell fractionation, 40 ml of the respective cultures was harvested by centrifugation at 12 000 × *g* for 15 min at 4°C. The cell-free supernatants (extracellular fraction) were retained. The cell pellets were washed once with sterile distilled water, suspended in 5 ml of 0.1 M sodium acetate buffer (pH 5) and sonicated on ice for 20 min using a Model HD2070 Sonoplus Ultrasonic Homogenizer. The cell lysate was clarified by

centrifugation (12 000 × *g*, 15 min) and the supernatant, considered the intracellular fraction, was recovered. The pellet (cell wall fraction) was washed once with sterile distilled water and suspended in 5 ml of 0.1 M sodium acetate buffer (pH 5). The protein concentration of samples was determined by the method of Bradford (1976), with the BioRad protein assay kit and with bovine serum albumin as standard.

3.2.7.2 *In vitro* enzyme activity assays

Acid phosphatase activity in the prepared protein fractions was determined according to the method of Golovan *et al* . (2000), with the following modifications. The assays were performed by incubating 100 µl of enzyme preparation with 300 µl of *p*-nitrophenyl phosphate (*p*NPP) substrate (Roche Diagnostics), at a final concentration of 25 mM, in 0.1 M sodium acetate (pH 5). Following incubation at 37°C for 30 min, the reaction was terminated by the addition of 1 ml of 1 M NaOH and the liberated *p*-nitrophenol (*p*NP) was measured at 405 nm. The extinction coefficient of *p*NP was taken to be 18.5 $cm^2.\mu mol^{-1}$ (Walter and Schütt, 1974), and 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. All assays were performed in triplicate, and the results are expressed as means ± standard deviations.

3.2.8 Zymographic detection of acid phosphatase activity

3.2.8.1 Protein sample preparation

Overnight cultures of *B. halodurans* strains BhFC01 and BhFC04, each transformed with reporter plasmid pNW33-SC, were used to inoculate 60 ml of LB medium to an OD_{540} of 0.1. The cultures were grown at 37°C on a rotary shaker (175 rpm) to an OD_{540} of 2.0 (approximately 8 h). Following incubation, the bacterial cells were collected by centrifugation at 12 000 × *g* for 10 min at 4°C. The cell-free culture supernatants (extracellular fractions) were retained and the proteins were precipitated with 5% (w/v) TCA (Merck, Modderfontein, South Africa), prior to being suspended in 200 µl of 0.1 M sodium acetate buffer (pH 5). To prepare cell surface protein fractions, the cell pellets were suspended in a 5 M LiCl solution (prepared in 25 mM Tris-HCl [pH 7.2]) and incubated on ice for 30 min with agitation (Tsuchiya *et al*., 1999). The cells were then centrifuged, as described before, and the supernatants and cell pellets were retained. Proteins were precipitated from the supernatant (cell surface fraction) with 5% (w/v) TCA and suspended in

200 µl of 0.1 M sodium acetate buffer (pH 5). The cell pellets were processed as described before (Section 3.2.7.1) to obtain intracellular and cell wall fractions.

3.2.8.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and zymography

SDS-PAGE was performed with 12% polyacrylamide gels by the method of Laemmli (1970) after the samples had been heated at 37ºC for 30 min. Molecular weight markers (Roche Diagnostics) were included in each gel. Following electrophoresis, the gels were either stained with Coomassie brilliant blue R-250 to visualize the protein bands or incubated for 16 h at room temperature in several changes of renaturation buffer for zymographic analysis (Hamilton *et al*., 2000). After renaturation treatment, gels were equilibrated for 1 h at 37°C in 0.1 M sodium acetate buffer (pH 5) and then developed for phosphatase activity. For development, the gels were incubated at 37°C for 15 min to 1 h in 0.1 M sodium acetate (pH 5) with 0.1% (w/v) α-naphtyl phosphate and 0.2% (w/v) Fast Garnet GBC salt (Sigma-Aldrich, Aston Manor, South Africa) (Gabriel, 1971). Phosphatase activity was indicated by the presence of black-stained bands.

3.3 RESULTS

3.3.1 Inactivation of the *wprA* cell wall protease gene of *B. halodurans* BhFC01

Towards determining whether inactivation of the *wprA* gene, encoding a cell wall protease, may improve extracellular production and surface display of heterologous proteins, an integrative plasmid was constructed to enable integration of a mutant *wprA*$^{-}$ allele into the chromosome of *B. halodurans* BhFC01 by *in vivo* recombination. The *wprA*$^{-}$ gene, of which the internal 1.056 kb region was deleted by PCR-based methods, was cloned into the thermosentive plasmid pSEC194 and used to transform *B. halodurans* BhFC01. Integration of the entire pSEC-wprA^{-} plasmid was obtained at the non-permissive temperature. Colonies were subsequently chosen, cultured at the permissive temperature in non-selective medium and the resulting chloramphenicol-sensitive colonies, which had lost the pSEC194 vector backbone by a second crossover event, were screened for the presence of *wprA*$^{-}$ by PCR analysis. Oligonucleotide primers WprA-ChromF and WprA-ChromR (Table 3.1), which anneal to genomic sequences upstream and downstream of the integrated mutant allele, amplified an expected 2.007-kb product when chromosomal DNA of mutant strains were used as template. As a control, template DNA from *B. halodurans* BhFC01 generated a

3.159-kb product that corresponds in size to the full-length *wprA* gene (results not shown). A mutant strain, designated *B. halodurans* BhFC04, was used in all subsequent analyses.

To confirm the absence of WprA protease activity in *B. halodurans* BhFC04, cell-free culture supernatants and cell wall fractions were prepared, and analyzed by SDS-PAGE and zymography. Analysis of the Coomassie blue-stained gel indicated the presence of two distinct protein bands of approximately 70 and 72 kDa, respectively, in the cell wall fraction prepared from the wild-type *B. halodurans* BhFC01 strain (Fig. 3.1, lane 4). In contrast, the larger of the two protein bands was not present in the cell wall fraction of the mutant *B. halodurans* BhFC04 strain (Fig. 3.1, lane 3). Furthermore, no protease activity was observed for cell wall fraction of the mutant *B. halodurans* BhFC04 strain (Fig. 3.1, lane 7). Thus, to determine the identity of the larger 72 kDa protein, N-terminal sequencing of the protein from the wild-type BhFC01 strain was performed. This yielded an amino acid sequence of NH_2-SADIHYSD. A search of the sequenced *B. halodurans* C125 genome (http://www.jamstec.go.jp/genomebase/micrHome_bha.html) revealed that the sequence corresponded to amino acids 408 to 415 of the major WprA cell wall protease in *B. halodurans* strain 125. Based on the absence of a similarly sized protein in the cell wall fraction of *B. halodurans* BhFC04, these results therefore confirmed that the *wprA* gene was inactivated successfully in the mutant strain.

3.3.2 Evaluation of heterologous protein production in *B. halodurans* strains BhFC01 and BhFC04 with *sapS* as a reporter gene

To determine if heterologous protein production was indeed improved in *B. halodurans*, following inactivation of the *wprA* major cell wall protease gene, reporter plasmid pNW33-SapS, which contains the promoter and coding region of the *S. aureus* SapS preprotein, was transformed into *B. halodurans* strains BhFC01 and BhFC04. The acid phosphatase activity of intracellular, cell wall and extracellular fractions from both *B. halodurans* BhFC01(pNW33-SapS) and *B. halodurans* BhFC04(pNW33-SapS) was determined quantitatively with *in vitro* enzyme assays after at 8, 24 and 48 h of culturing. The results of these assays are presented in Fig. 3.2.

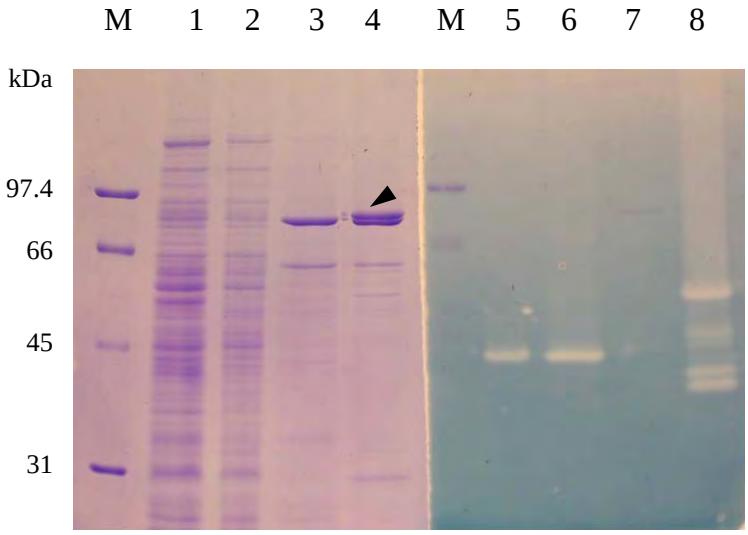
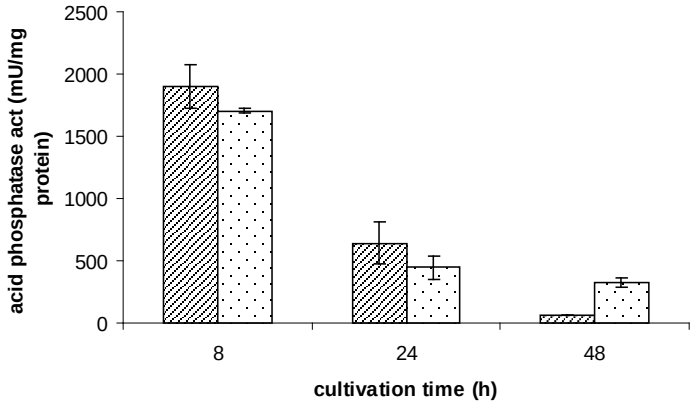


Fig. 3.1 Analysis of the presence of proteases in cell-free culture supernatants and cell wall fractions of the wild-type *B. ha lodurans* BhFC01 and mutant *B. ha lodurans* BhFC04 strains. Proteins in the respective fractions were resolved by SDS-PAGE and the gel stained with Coomassie blue to visualize the protein bands (lanes 1-4), or for protease activity following renaturation treatment (lanes 5-8). Lanes M, low-range protein molecular weight marker; lanes 1 and 5, *B. ha lodurans* BhFC04 cell-free culture supernatant; lanes 2 and 6, *B. halodurans* BhFC01 cell-free culture supernatant, lanes 3 and 7, *B. ha lodurans* BhFC04 cell wall fraction; lanes 4 and 8, *B. ha lodurans* BhFC01 cell wall fraction. The arrow indicates the WprA protein band, while the sizes of the molecular weight standards (in kilodaltons) are indicated to the left.

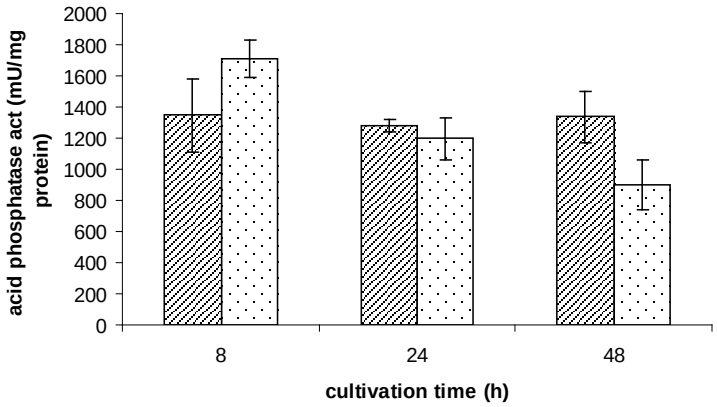
The acid phosphatase enzyme activity in intracellular fractions prepared from 8-h cultures of *B. hal odurans* BhFC01(pNW33-SapS) and BhFC04(pNW33-SapS) was 1898 and 1709 mU/mg of protein, respectively. A significant reduction in enzyme activity was observed in the intracellular fractions of 24- and 48-h cultures of both these strains (Fig. 3.2A). In contrast, no similar reduction in enzyme activities was observed in the cell wall fractions prepared from *B. hal odurans* BhFC01(pNW33-SapS) and BhFC04(pNW33-SapS) after 24 and 48 h of culturing (Fig. 3.2B). The acid phosphatase enzyme activity in the cell wall fraction of 8-h cultures of *B. halodurans* BhFC01(pNW33-SapS) was 1347 mU/mg of protein and comparable enzyme activities was assayed in the cell wall fractions from 24-h (1279 mU/mg) and 48-h (1337 mU/mg) cultures. For cell wall fractions prepared from *B. halodurans* BhFC04(pNW33-SapS), the enzyme activity decreased gradually from 1706 mU/mg of protein in 8-h cultures to 1195 mU/mg in 24-h cultures, and 898 mU/mg in 48-h cultures (Fig. 3.2B).

Although the acid phosphatase enzyme activities determined in extracellular fractions prepared from *B. hal odurans* BhFC01(pNW33-SapS) and BhFC04(pNW33-SapS) were lower compared to that determined in the intracellular and cell wall fractions, a clear trend could be observed (Fig. 3.2C). Whereas the enzyme activity in extracellular fractions prepared from *B. hal odurans* BhFC01(pNW33-SapS) decreased with an increase in cultivation time, the inverse was observed for *B. hal odurans* BhFC04(pNW33-SapS). The enzyme activity decreased from 154 mU/mg of protein in extracellular fractions prepared from 8-h cultures of *B. halodurans* BhFC01(pNW33-SapS) to 138 and 74 mU/mg in fractions prepared from 24-h and 48-h cultures, respectively. In contrast, for *B. hal odurans* BhFC04(pNW33-SapS) the enzyme activity increased from 126 mU/mg of protein after 8 h of culturing to 401 and 440 mU/mg after 24 and 48 h of culturing, respectively (Fig. 3.2C). These results therefore indicated that extracellular production of the SapS reporter enzyme was increased 3.5-fold in *B. hal odurans* BhFC04, of which the gene encoding the WprA major cell wall protease had been inactivated.

A



B



C

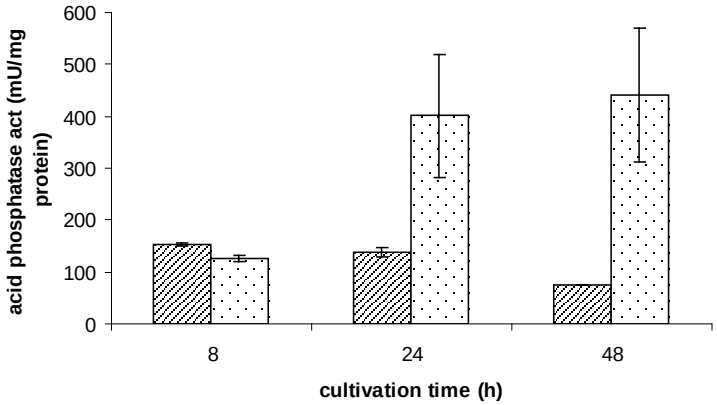


Fig. 3.2 Acid phosphatase enzyme activity (act) in the (A) intracellular, (B) cell wall and (C) extracellular fractions of *B. halodurans* strains BhFC01 and BhFC04, harbouring reporter construct pNW33-SapS. The respective fractions were prepared and assayed after 8, 24 and 48 h of culturing. BhFC01 ▨ ; BhFC04 ⁘

3.3.3 Evaluation of surface display in *B. halodurans* strains BhFC01 and BhFC04 with *sapS* as a reporter gene

To determine whether the SapS reporter enzyme fused with the CwlC cell wall-binding domain was displayed on the surface of *B. halodurans* strains BhFC01 and BhFC04, the host strains were transformed with plasmid pNW33-SC, which directs expression of a SapS-CwlC fusion protein. The SapS protein comprises 297 amino acids, while the mature SapS protein without the 31-amino-acid signal peptide, comprises 266 amino acids and has a predicted molecular mass of 30 056 Da. The CwlC cell wall-binding domain, used in this study as an anchoring motif, comprises 163 amino acids with a predicted molecular mass of 18 100 Da. Therefore, the fused *sapS-cwlC* gene encodes a protein of 460 amino acids with a molecular mass of 48 156 Da.

Cell surface, cell wall and extracellular fractions were prepared from cultures of *B. halodurans* BhFC01(pNW33-SC) and *B. halodurans* BhFC04(pNW33-SC), and analyzed by SDS-PAGE and zymography of which the results are shown in Fig. 3.3. Analysis of the Coomassie-stained gel did not indicate the presence of overexpressed proteins corresponding in size to the 48-kDa fusion gene product in the respective fractions. However, zymography showed the presence of a 48-kDa activity band in the cell wall fractions from both *B. halodurans* BhFC01(pNW33-SC) and *B. halodurans* BhFC04(pNW33-SC) (Fig. 3.3, lanes 10 and 11), thus indicating the presence of the SapS-CwlC fusion protein. Moreover, the presence of strong activity bands with a molecular weight of 30 kDa in cell wall fractions from *B. halodurans* BhFC01(pNW33-SC) (lane 10) and *B. halodurans* BhFC04(pNW33-SC) (lane 11) suggested that cleavage of the fusion protein into the CwlC and the mature SapS components occurred after transport through the cell membrane. It is likely that the linkage between the two domains is more sensitive to proteolysis and that the resultant CwlC portion is bound to the cell wall. No fusion protein products were observed in the cell surface (lanes 8 and 9) or extracellular fractions (lanes 12 and 13) of either of the host strains harbouring pNW33-SC. However, strong 30-kDa activity bands in the cell surface extracts from both the host strains harbouring pNW33-SC were observed, the size of which is in agreement with that of the mature SapS protein. Coomassie staining of the protein fractions showed the presence of 30-kDa protein bands in the cell surface and cell wall fractions of the two host strains harbouring the fusion construct (Fig. 3.3, lanes 1-4). From these results it was concluded that there was no significant difference in the cell surface display capacity of *B. halodurans* strains BhFC01 and BhFC04, as determined with zymography.

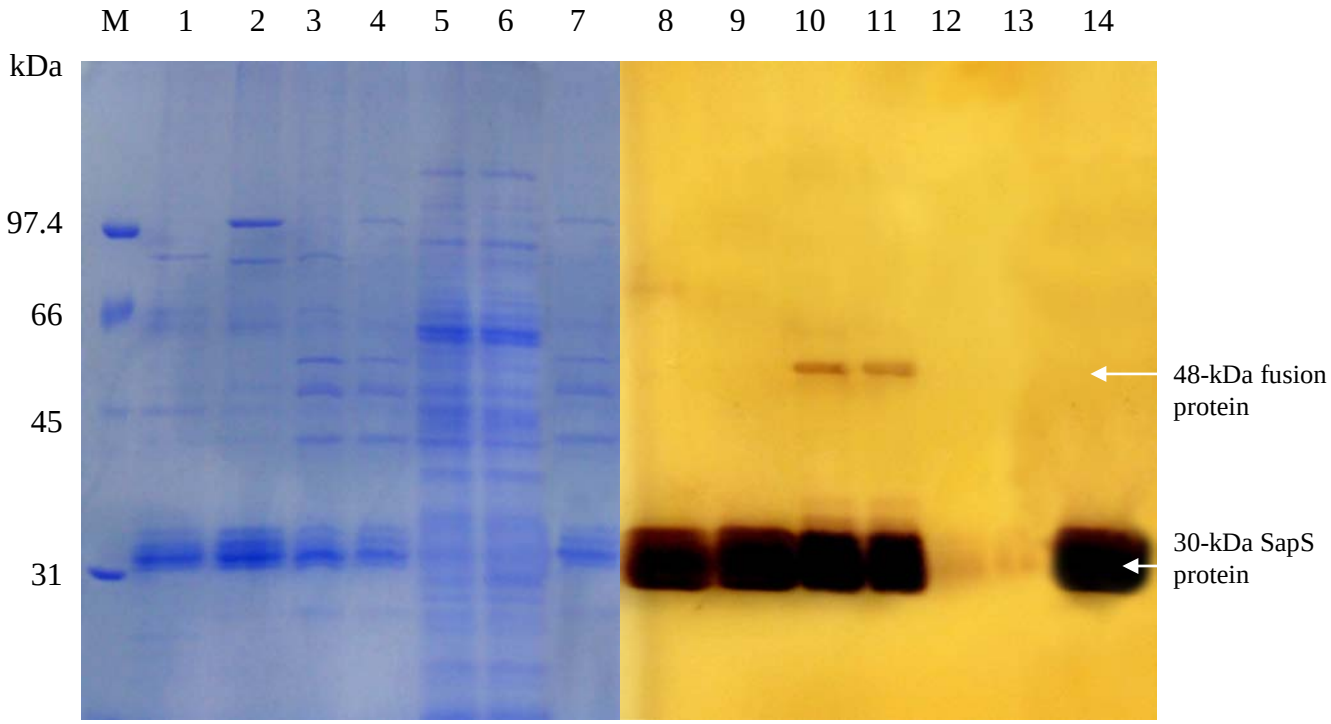


Fig. 3.3 Coomassie-stained SDS-PAGE gel (lanes 1-7) and zymography (lanes 8-14) of the cell surface (Cs), cell wall (Cw) and extracellular (Ex) fractions from *B. halodurans* BhFC01(pNW33-SC) and *B. hal odurans* BhFC04(pNW33-SC). Lane M, low-range protein molecular weight marker; lanes 1 and 8, Cs fraction from BhFC01(pNW33-SC); lanes 2 and 9, Cs fraction from BhFC04(pNW33-SC); lanes 3 and 10, Cw fraction from BhFC01(pNW33-SC); lanes 4 and 11, Cw fraction from BhFC04(pNW33-SC); lanes 5 and 12, Ex fraction from BhFC01(pNW33-SC); lanes 6 and 13, Ex fraction from BhFC04(pNW33-SC). For comparative purposes, the cell wall fraction from BhFC04(pNW-SapS), expressing the 30-kDa SapS protein, is indicated in lanes 7 and 14. The SapS-CwlC fusion protein and SapS protein are indicated by arrows. The sizes of the molecular weight standards (in kilodaltons) are indicated to the left.

3.4 DISCUSSION

In this study the *S. aureus sapS* gene was used as a reporter both to evaluate the ability of the C-terminal cell wall-binding domain of the *B. halodurans cwlC* gene to target the reporter gene product to the cell wall of the host strain, and to determine the efficacy of heterologous protein production in a WprA protease-deficient *B. halodurans* host strain. Initial studies focused on the inactivation of the major cell wall protease (*wprA*) gene. This was achieved successfully, as proved with zymography, giving rise to *B. halodurans* BhFC04.

Enzyme studies performed with *B. halodurans* harbouring the *sapS* reporter gene construct demonstrated that the *sapS* gene can be used as a reporter to determine whether heterologous protein production was increased in strain BhFC04 when compared to strain BhFC01. Results obtained confirmed reports in the literature that extracellular heterologous enzyme production was improved by inactivation of the *wprA* gene (Stephenson and Harwood, 1998; Lee *et al.*, 2000). During secretion of the *B. subtilis* α-amylase (AmyL) it is subjected to considerable cell-associated proteolysis due to WprA activity; however, an increased yield of α-amylase was obtained after inactivation of the *wprA* gene (Stephenson and Harwood 1998). Extracellular production of the heterologous *Staphylococcus aureus* staphylokinase (SAK) enzyme in a *B. subtilis* strain, which is deficient in six extracellular proteases (WB600), was marginal (45 mg/L) (Lee *et al*., 2000). Disruption of the *wprA* gene, however, strongly promoted the production of SAK in the stationary phase (181 mg/ml) (Lee *et al*., 2000). The authors also reported that the extracellular stability of the mature SAK was dramatically enhanced and concluded that the *wprA* gene product plays a significant role in degrading foreign proteins, both during secretion and in the extracellular milieu. In this study, similar SapS activity levels were obtained in the cell wall fractions of *B. halodurans* BhFC01(pNW33-SapS) and BhFC04(pNW33-SapS), indicating that the SapS enzyme was not significantly degraded during translocation in the cell wall due to WprA enzyme activity. Degradation of the SapS enzyme due to WprA activity seemed to be a problem in the extracellular mileu as was clear from the reduced extracellular enzyme activity observed in *B. halodurans* strain BhFC01 when compared to strain BhFC04 after 24 and 48 h of culturing.

The cell wall-binding domain of the *cwlC* gene successfully targeted the *sapS-cwlC* fusion product to the cell wall of *B. halodurans* BhFC04, as determined with zymography. However, when analyzed, the cleaved *sapS* gene product was mostly obtained. This result is

in agreement with the observation by Kobayashi *et al*. (2000a,b; 2002) that instability of surface proteins is generally a serious problem. However, Kobayashi *et al.* (2000b) evaluated a *wprA* mutant as well as a double mutant (*wprA*$^-$ and *sigD*$^-$) for surface display of *B. subtilis* lipase (LipB), using the anchoring motif of the *B. subtilis cwlB* gene. The *wprA* mutant accumulated CwlB-LipB in a greater amount than the wild-type strain in the stationary phase, but with the double mutant strain the highest activity was obtained in the late exponential to early stationary phase. In this study, results obtained with zymography of the 8-h stationary phase culture of *B. halodurans* BhFC04 (pNW33-SC) indicated that the yield of the SapS-CwlC fusion protein were not improved when compared with the results obtained for BhFC01(pNW33-SC). Kobayashi *et al*. (2002) also reported that the use of an extracellular alkaline and neutral protease-deficient mutant derived from the *wprA* and *sigD*-deficient *B. subtilis* strain did not improve the yield of surface displayed lipase.

For many applications, the immobilized protein must be expressed on the outside of the cell wall in such a way that it is accessible for large substrates. For example, lipase from *Humicola lanuginosa* and cutinase from *Fusarium solanii* subsp. *pisi*, immobilized by an anchoring domain of α-glutinin, proved to be immunologically detectable on the surface of intact yeast cells, but displayed dramatically reduced enzymatic activity towards an emulsion of olive oil (Van der Vaart *et al*., 1997). It is possible that some of the immobilized fusion proteins, in this study the SapS-CwlC fusion protein, is not exposed to the outside of the cell wall, but is located at the inner part of the protein layer and will therefore not be able to degrade the substrate. Not only was acid phosphatase activity of the fusion protein detected with *in vitro* enzyme assays, but it was also detected with plate screen assays of *B. halodurans* BhFC01 and BhFC04 harbouring pNW33-SC (results not shown). However, it was not possible to distinguish between the activity obtained from the SapS-CwlC fusion protein and the cleaved off mature *sapS* gene product. Therefore, no conclusions as to the ability of the fusion protein specifically to access the substrate could be made.

The main aim of this study was not the development of a surface display system, but rather the evaluation of the *S. aureus sapS* gene as a reporter gene and the evaluation of its efficacy. The *sapS* reporter system was used successfully for showing that the CwlC anchoring motif could be used to target acid phosphatase to the cell wall. The *in vitro* enzyme assays were used to rapidly and cost-effectively determine in which cell fraction enzyme activity was observed. Furthermore, the activity can be detected with zymography that yields information

on the size of the products obtained, which is not possible when using *in vitro* enzyme assays only. This allowed conclusions to be made regarding the efficacy of surface display. Since it was clear that proteolytic cleavage remained a problem, future strategies to address this problem could be the inactivation of *sigD*, since the use of a strain harbouring an inactivated *sigD* gene was reported to improve surface display in *B. subtilis* (Kobayashi *et al*., 2002).

3.5 REFERENCES

Biswas, I., Gruss, A., Ehrlich, S.D. and Manguin, E. (1993). High-efficiency gene inactivation and replacement system for Gram-positive bacteria. J. Bacteriol. **175**: 3628-3635.

Bradford, M. (1976). A rapid and sensitive method for quantification of microgram quantities of protein utilising the principle of protein-dye binding. Anal. Biochem. **72**: 248-254.

Braun, P., Gerritse, G., Van Dijl, J-M. and Quax, W.J. (1999). Improving protein secretion by engineering components of the bacterial translocation machinery. Curr. Opin. Biotech. **10**: 376-381.

Chen, W. and Georgiou, G. (2002). Cell surface display of heterologous proteins: From high-throughput screening to environmental applications. Biotechnol. Bioeng. **79**: 496-503.

Cho, C.M-H., Mulchandani, A. and Chen, W. (2002). Bacterial surface display of organophosphorous hydrolase for selective screening of improved hydrolysis of organophosphate nerve agents. Appl. Environ. Microbiol. **68**: 2026-2030.

Crampton, M., Berger, E., Reid, S. and Louw, M.E. (2007). The development of a flagellin surface display system in a moderate thermophile, *Bacillus halodurans* Alk36. Appl. Microbiol. Biotechnol. **75**: 599-607.

Dong, J., Liu, C., Zhang, J., Xin, Z-T., Yang, G., Gao, B., Mao, C-Q., Liu, N-L., Wang, F., Shao, N-S., Fan, M. and Xue, Y-N. (2006). Selection of novel nickel-binding peptides from flagella displayed secondary peptide library. Chem. Biol. Drug. Des. **68**: 107-112.

Dower, W.J., Miller, J.F. and Ragsdale, C.W. (1988). High efficiency transformation of *E. coli* by high voltage electroporation. Nucl. Acids Res. **16:** 6127.

Du Plessis, E.M., Theron, J., Joubert, L., Lotter, T. and Watson, T.G. (2002). Characterization of a phosphatase secreted by *Staphylococcus aureus* strain 154, a new member of the bacterial class C family of non-specific phosphatases. Syst. Appl. Microbiol. **25**: 21-30.

Ferreira, L.C.S., Ferreira, R.C.C. and Schumann, W. (2005). *Bacillus subtilis* as a tool for vaccine development: From antigen factories to delivery vectors. Anal. Braz. Acad. Sci. **77**: 113-124.

Gabriel, O. (1971). Locating enzymes on gels. Methods Enzymol. **17A**: 578-604.

Golovan, S., Wang, G., Zhang, J. and Forsberg, C.W. (2000). Characterization and overproduction of the *Escherichia co li appA*-encoded bifunctional enzyme that exhibits both phytase and acid phosphatase activities. Can. J. Microbiol. **46**: 59-71.

Hamilton, A., Harrington, D., Sutcliffe, I.C. (2000). Characterization of acid phosphatase activities in the equine pathogen *Streptococcus equi*. Syst. Appl. Microbiol. **23**: 325-329.

Ishikawa, S., H ara, Y., Ohnishi, R. and Se kiguchi, J. (1998). Regulation of a new cell wall hydrolase gene, *cwlF*, which affects cell separation in *Bacillus subtilis*. J. Bacteriol. **180**: 2549-2555.

Jung, H-C., K won, S-J. and P an, J-G. (2007). Display of thermostable lipase on the surface of a solvent resistant bacterium, *Pseudomonas put ida* GM730, and its applications in whole cell biocatalysis. BMC Biotech. **6**:23.

Kang, S.M., Rhee, J-K., Kim, E-J., Han, K-H. and Oh, J-W. (2003). Bacterial cell surface display for epitope mapping of hepatitis C virus core antigen. FEMS Microbiol. Lett. **226**: 347-353.

Karbalei-Heidari, H.R., Ziaee, A-A., S challer, J. and A moozegar, M.A. (2007). Purification and characterization of an extracellular haloalkaline protease produced by the moderately halophilic bacterium, *Salinivibrio* sp. strain AF-2004. Enzym. Microb. Tech. **40**: 266-272.

Kobayashi, G., Toida, J., A kamatsu, T., Y amamoto, H., Shida, T. and Se kiguchi, J. (2000a). Accumalation of a recombinant *Aspergillus oryzae* lipase artificially localized on the *Bacillus subtilis* cell surface. J. Biosci. Bioeng. **90**: 422-425.

Kobayashi, G., Toida, J., A kamatsu, T., Yamamoto, H., Shida, T. and Se kiguchi, J. (2000b). Accumulation of artificial cell wall-binding lipase by *Bacillus s ubtilis wprA* and/or *sig*D mutants. FEMS Microbiol. Lett. **188**: 165-169.

Kobayashi, G., F ujii, K., Wa, M.S., Yamamoto, H. and Sekiguchi, J. (2002). Simultaneous display of bacterial and fungal lipases on the cell surface of *Bacillus subtilis*. J. Biosci. Bioeng. **93**: 15-19.

Kuroda, A., A sami, Y. and Se kiguchi, J. (1993). Molecular cloning of a sporulation-specific cell wall hydrolase gene of *Bacillus subtilis*. J. Bacteriol. **175**: 6260-6268.

Kwak, Y-D., Yoo, S-K. and Kim, E-J. (1999). Cell surface display of human immunodeficiency virus type 1gp120 on *Escherichia coli* by using ice nucleation protein. Clin. Diagnos. Lab. Immunol. **6**: 499-503.

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

Lee, S.J., Kim, D.M., Bae, K.H., Byun, S.M. and Chung, J.H. (2000). Enhancement of secretion and extracellular stability of staphylokinase in *Bacilllus subtilis* by *wprA* gene disruption. Appl. Environ. Microbiol. **66**: 476-480.

Lee, S.Y., Choi, J.H. and Xu, Z. (2003). Microbial cell surface display. Trends Biotech. **21:** 45-52.

Lovett, P.S. and Keggins, K.M. (1979). *B. subtilis* as a host for molecular cloning. Methods Enzymol. **68**: 342-357.

Majander, K., Korhonen, T.K. and Wikström, W. (2005). Simultaneous display of multiple peptides in the FliD capping and FliC filament proteins of the *E. coli* flagellum. Appl. Environ. Microbiol. **71**: 4263-4268.

Meens, J., Herbort, M., Klein, M. and Freudl, R. (1997). Use of the pre-pro part of *Staphyloccus hyicus* lipase as carrier for the secretion of *Escherichia coli* outer membrane protein A (OmpA) prevent sproteolytic degradation of OmpA by cell-associated proteases in two different Gram-positive bacteria. Appl. Environ. Microbiol. **63**: 2814-2820.

Murashima, K., Chen, C-L., Kosugi, A., Tamary, Y., Doi, R.H. and Wong, S-L. (2002). Heterologous production of *Clostridium cellulovorans engB*, using protease-deficient *Bacillus subtilis*, and preparation of active recombinant cellulosomes. J. Bacteriol. **184**: 76-81.

Navarre, W.W. and Schneewind, O. (1999). Surface proteins of Gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. Microbiol. Mol. Biol. Rev. **63**: 174-229.

Nguyen, H.D. and Schumann, W. (2006). Establishment of an experimental system allowing immobilization of proteins on the surface of *Bacillus subtilis* cells. J. Biotechnol. **122**: 473-482.

Olmos-Soto, J. and Contreras-Flores, R. (2003). Genetic system constructed to overproduce and secrete proinsulin in *Bacillus subtilis*. Appl. Microbiol. Biotechnol. **62**: 369-373.

Poncet, S., Bernard, C., Dervyn, E., Cayley, J., Klier, A. and Rappoport, G. (1997). Improvement of *Bacillus sphaericus* toxicity against dipteran larvae by integration, via homologous recombination,

of the Cry IIA toxin gene from *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. **63**: 4413-4420.

Rashid, M.H., Sato, N. and Sekiguchi, J. (1995). Analysis of the minor autolysins of *Bacillus subtili*s during vegetative growth by zymography. FEMS Microbiol. Lett. **132:** 131-137.

Sambrook, J. an d R ussell, D .W. (2001) Molecular cloning - a laboratory manual, 3rd edn. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York.

Samuelson, P., Gunneriusson, E., N ygren, P-A. and St ahl, S. (2002). Display of proteins on bacteria. J. Biotech. **96**: 129-154.

Samuelson, P., Wernerus, H., Svedberg, M. and Stahl, S. (2000). Staphylococcal surface display of metal-binding polyhistidyl peptides. Appl. Environ. Microbiol. **66**: 1243-1248.

Schallmey, M., S ingh, A. and Wa rd, O.P. (2004). Developments in use of *Bacillus* species for industrial production. Can. J. Microbiol. **50**: 1-17.

Stephenson, K., Bron, S. and Harwood, R.H. (1999). Cellular lysis in *Bacillus subtilts*, the effect of multiple extracellular protease deficiencies. Lett. Appl. Microbiol. **29**: 14-145.

Stephenson, K. and Harwood, R.H. (1998). Influence of cell-wall-associated protease on production of α-amylase by *B. subtilis*. Appl. Environ. Microbiol. **64**: 2875-2881.

Tsuchiya, A., K obayashi, G., Y amamoto, H. and Se kiguchi, J. (1999). Production of a recombinant lipase artificially localized on the *B. subtilis* cell surface. FEMS Microbiol. Lett. **176**: 373-378.

Turner, M.S., Hafner, L.M., Wal sh, T. and G iffard, P.M. (2003). Peptide surface display and secretion using two LPXTG-containing surface proteins from *Lactococcus fermentum* BR11. Appl. Environ. Microbiol. **69**: 5855-5863.

Van der Vaart, J.M., Te Biesebeke, R., Chapman, J.W., Toschka, H.Y., Klis, F.M. and Verrips, T. (1997). Comparison of cell wall proteims of *Saccharomyces cerevisiae* as anchors for cell surface expression of heterologous proteins. Appl. Environ. Microbiol. **63**: 615-620.

Walter, K. and Schütt, C. (1974). Acid and alkaline phosphatase in serum, pp. 856-860. In: Methods of Enzymatic Analysis (H.U. Bergmeyer, ed.), New York, Academic Press.

Wernerus, H., Lehtiö, J., Teeri, T., Nygren, P-A. and St ahl, S. (2001). Generation of metal-binding Staphylococci through surface display of combinatorially engineered cellulose binding domains. Appl. Environ. Microbiol. **67**: 4678-4684.

Westers, L., Westers, H. and Quax, W.J. (2004). *Bacillus subtilis* as cell factory for pharmaceutical proteins: A biotechnological approach to optimize the host organism. Biochim. Biophys. Acta **1694**: 299-310.

Wu, X-C., Lee, W., T ran, L. and Won g, S-L. (1991). Engineering a *Bacillus s ubtilis* expression/secretion system with a strain deficient in six extracellular proteases. J. Bacteriol. **173**: 4952-4958.

Xu, Z. and Lee, S.Y. (1999). Display of polyhistidine peptides on the *Escherichia coli* cell surface by using outer membrane protein C as anchoring motif. Appl. Environ. Microbiol. **65**: 5142-5147.

CHAPTER FOUR*

EVALUATION OF THE *Staphylococcus aureus* CLASS C NON-SPECIFIC ACID PHOSPHATASE (SapS) AS A REPORTER FOR GENE EXPRESSION AND PROTEIN SECRETION IN GRAM-NEGATIVE AND GRAM-POSITIVE BACTERIA

ABSTRACT

A phosphatase secreted by *Staphylococcus au reus* strain 154 has previously been characterized and classified as a new member of the bacterial class C family of non-specific acid phosphatases. As the acid phosphatase activity can be easily detected using a cost-effective plate screen assay, quantitatively measured by a simple enzyme assay, and detected with zymography, its potential use as a reporter system was investigated. The *S. aureus* acid phosphatase gene (*sapS*) has been cloned and expressed from its own regulatory sequences in *Escherichia coli*, *Bacillus subtilis* and *Bacillus halodurans*. Transcriptional and translational fusions of the *sapS* gene with selected heterologous promoters and signal sequences were constructed and expressed in all three of the host strains. From the range of promoters evaluated, the strongest promoter for heterologous protein production in each of the host strains was identified, *i.e.* the *E. c oli lacZ* promoter in *E. c oli*, the *B. h alodurans* alkaline protease promoter in *B. subtilis*, and the *B. halodurans* σ^D promoter in *B. halodurans.* This is the first report on the development of a class C acid phosphatase gene as a reporter gene with the advantage of being able to function in both Gram-positive and Gram-negative host strains.

*** This chapter has been published:**

du Plessis, E., Theron, J., Berger, E. and Louw, M. (2007). Evaluation of the *Staphylococcus aureus* class C non-specific acid phosphatase (SapS) as a reporter for gene expression and protein secretion in Gram-negative and Gram-positive bacteria. *Applied and Environmental Microbiology* 73: 7232-7239.

4.1 INTRODUCTION

Methods for the direct measurement of gene expression include mRNA detection with polynucleotide probes (Northern blot assays) or reverse transcriptase-polymerase chain reaction (RT-PCR) methods, as well as protein detection methods that use antibodies (Western blot assays) or biological activities (Wood *et al*., 1995; Ding and Cantor, 2004). However, these methods are, in many cases, time-consuming and costly. Reporter genes provide an alternative method of genetic analysis that is faster and more convenient. Typically, reporter genes encode proteins that have a unique enzymatic activity or that are otherwise easily distinguishable from the mixture of intra- and extracellular proteins (Biran *et al.*, 1999; Schenborn and Groskreutz, 1999). They have frequently been used to identify regulatory sequences, to monitor gene expression and function, and to characterize promoter strength and regulation (Pedraza-Reyes *et al*., 1994; Wang *et al*., 2004; Serrano-Heras *et al*., 2005; Koga *et al*., 2006).

The choice of a reporter system is determined by a number of important 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 (Jefferson, 1986; Naylor, 1999). The most widely used reporter systems use genes that encode β-galactosidase (*lacZ*) (Poyart and Trieu-Cuot, 1997; Talukder *et al*., 2005), chloramphenicol acetyltransferase (*cat*) (Cao *et al*., 2001; Palmano *et al*., 2001) and different sugar hydrolases, *e.g.*, β-glucuronidase (*gus*) (Jefferson, 1986; Kim *et al*., 2006). Although these reporter systems are convenient tools for semi-quantitative plate-based assessment of promoter activities, more accurate quantification of promoter strength usually requires enzymatic assays, which typically involve bacterial cell disruption and addition of a substrate to drive the enzyme reaction, followed by measurement of optical density (Biran *et al*., 1999). Another group of reporter systems is based on the emission of light (Wood, 1998; Southward and Surette, 2002). In addition to the wild-type green fluorescent protein (GFP) from *Aequorea victoria*, many derivatives of GFP have been produced and subsequently used to monitor promoter activity both in the laboratory and in natural environments (Southward and Surette, 2002; Chary *et al*., 2005; Serrano-Heras *et al*., 2005). However, naturally occurring fluorescence can lead to high background levels during

in vitro and *in vivo* measurements. Alternative strategies have thus involved the luciferase-encoding *luxAB* genes, typically derived from *Vibrio fischeri*, *Vibrio har veyi* and *Photorhabdus luminescens* (Kirchner, 1989; Meighen, 1991), and more recently the synthetic *luxCDABE* operon that alleviates the requirement for the addition of an exogenous aldehyde substrate in the light emission reaction (Applegate *et al*., 1998; Greer and Szalay, 2002).

Since each reporter system has its own advantages and disadvantages that may limit its usefulness in specific host organisms and in specific types of studies, no single reporter gene is universally applicable (Naylor, 1999). It is therefore desirable to have a number of reporter systems available for the same organism (Janatova *et al*., 2003; Perez-Arellano and Perez-Martinez, 2003). Consequently, modification of widely used reporter systems (Hautefort *et al*., 2003; Veening *et al*., 2004; Choe *et al*., 2005), the optimization of methods used for measuring reporter activity (Thibodeau *et al*., 2004; Hampf and Gossen, 2006; Loening *et al*., 2006), and the isolation and evaluation of new reporters are continuing. The relevance of the latter is exemplified by several reports regarding the evaluation of new reporters, amongst other, β-galactosidase from *B. megaterium* (Schmidt *et al*., 2005) and *Thermus thermophilus* (Park and Kilbane, 2004), lichenase from *Clostridium thermocellum* (Piruzian *et al*., 2002), and α-galactosidase from *Saccharopolyspora erytraea* (Post and Luebeke, 2005).

A novel class C non-specific acid phosphatase secreted by *Staphylococcus aureus* strain 154 has previously been isolated and characterized (Du Plessis *et al*., 2002). The enzyme, designated SapS and encoded by *sapS,* is a stable monomeric protein of moderate size (296 amino acids, 30 kDa) that undergoes proteolytic cleavage of the N-terminal 31-amino-acid signal peptide to yield the mature protein. On the basis of its moderate size and the ease with which enzymatic activity tests may be performed, the present study focused on the development and evaluation of the SapS acid phosphatase as a reporter for the characterization of promoters and signal sequences in a Gram-negative host (*E. coli*), as well as in mesophilic and moderately thermophilic Gram-positive hosts (*B. subtilis* and *B. halodurans*, respectively). *E. c oli* and members of the species *Bacillus* are the most frequently used prokaryotes for the production of heterologous proteins (Lam *et al*., 1998; Westers *et al*., 2004) and were therefore included in this study to evaluate the *sapS* reporter system. The use of the reporter gene was evaluated in *B. halodurans* since it harbours the *lac* operon (Ikura and Horikoshi, 1979), and the commonly used *lacZ* reporter system can

therefore not be used in this host organism. Furthermore, *B. halodurans* is currently being evaluated as a surface display expression system (Crampton *et al*., 2007).

4.2 MATERIALS AND METHODS

4.2.1 Bacterial strains and growth conditions

E. coli DH10B (*F⁻ mcrA* Δ(*mrr-hsdRMS-mcrBC*) (*φ80dlacZΔM15*) Δ*lacX74 e ndA1 rec*A1*deo*R Δ(*ara-leu*)*7697 ar aD139 galU galK nupG rpsL λ⁻*), obtained from Invitrogen, was used as an intermediary cloning host. Expression studies were done with *E. coli* CU1867, a BL21(DE3) strain with the chromosomal acid phosphatase *appA* gene disrupted (Ostanin *et al*., 1992), *B. subtilis* 154 (Δ*apr* Δ*npr amy spo*) (Quax and Broekhuizen, 1994), and *B. halodurans* BhFC04 (Δ*wprA* Δ*hag*) (Chapter 3, This thesis). *E. coli* and *B. subtilis* were cultured at 37°C in Luria-Bertani medium (LB: 0.5% [w/v] yeast extract, 1% [w/v] tryptone, 1% [w/v] NaCl; pH 7). When appropriate, *E. coli* growth media were supplemented with ampicillin (100 µg/ml), chloramphenicol (20 µg/ml) or erythromycin (300 µg/ml), and *B. subtilis* growth media were supplemented with chloramphenicol (5 µg/ml) or erythromycin (10 µg/ml). *B. halodurans* was grown at 37°C in LB medium (pH 8.5), and chloramphenicol (5 µg/ml) was added when appropriate.

4.2.2 Recombinant DNA techniques

Plasmid DNA was extracted with a Plasmid Midiprep Kit (QIAGEN, Hilden, Germany) and Perfectprep® Plasmid Mini Kit (Eppendorf, Hamburg, Germany). DNA fragments were purified from agarose gels by use of a DNA Extraction Kit (Fermentas, St. Leon-Rot, Germany). Procedures for DNA manipulations were carried out as described by Sambrook and Russell (2001). Enzymes were obtained from Fermentas (St. Leon-Rot, Germany) and Roche Diagnostics (Mannheim, Germany) and were used according to the manufacturers' protocols. Unless otherwise indicated, plasmid constructions were first established in *E. coli* DH10B and then transferred to *E. coli* CU1867, *B. subtilis* 154 and *B. halodurans* BhFC04. Transformation of bacteria was performed by electroporation for *E. coli* (Dower *et al*., 1988), and by protoplasting according to published procedures for *B. subtilis* (Chang and Cohen, 1979) and *B. halodurans* (Crampton *et al*., 2007). PCR was performed with Biotaq DNA polymerase (Bioline USA Inc., Randolph, MA) and a Progene thermocycler (Techne, Burlington, NJ). The oligonucleotides used in this study were obtained from Inqaba

Biotechnical Industries, Pretoria, South Africa. Chromosomal DNA was extracted from *S. aureus* 154 and *B. halodurans* BhFC04 according to the method of Lovett and Keggins (1979), except that lysozyme was added to a final concentration of 10 mg/ml. Nucleotide sequencing of all PCR products was performed using the ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction kit v3.0 (Applied Biosystems, Foster City, CA), followed by resolution on an ABI PRISM ™ 310 Genetic Analyser (Applied Biosystems), in accordance with the manufacturer's instructions. All plasmid constructions were verified by restriction endonuclease digestion, followed by agarose gel electrophoresis.

4.2.3 Plasmids

Plasmid pNW33N, an *E. coli-Bacillus-Geobacillus* shuttle vector obtained from the *Bacillus* Genetic Stock Centre (Columbus, OH), and pMG36e, a *Lactococcus* expression vector (Van de Guchte *et al*., 1989), which also replicates in *E. coli* and *B. subtilis*, was used to express the *S. aureus* acid phosphatase gene (*sapS*) in the Gram-positive and Gram-negative host strains. In all instances, except for pMG36e-SapS, pNW33N served as the genetic backbone into which different transcriptional and translational fusions were inserted to evaluate the *sapS* gene as a reporter (Fig. 4.1). The SapS enzyme was processed differently in *S. aureus* and *E. coli* compared to the *Bacillus* sp. (31 and 43 N-terminal amino acids were deleted, respectively, to produce the mature SapS protein). Consequently, translational fusions were made with both the mature *sapS* gene determined for *E. coli* and *S. aureus*, as well as the *Bacillus* sp. truncated mature *sapS* gene (Δ12). The oligonucleotides used in this study are listed in Table 4.1. *S. aureus* strain 154 chromosomal DNA was used for amplification of the *sapS* gene and its derivatives. *B. halodurans* BhFC04 chromosomal DNA was used as the template for the PCR amplification of the β-glucanase and alkaline protease promoter and signal sequences, and the σ^D promoter. The SPO2 promoter was obtained from plasmid pPL608 as a 300-bp *Eco*RI DNA fragment (Schoner *et al*., 1983). The following vectors were constructed:

pNW33-SapS: The 1.140-kb full-length *sapS* gene was PCR amplified with primers Sap-F and Sap-R1.

pNW33N1: The 227-bp β-glucanase promoter (P_{gluc}) fragment was PCR amplified with primers Glu-F and Glu-R, and the 888-bp *sapS* gene fragment, lacking the ATG initiation codon (Δ_{ATG}*sapS*), was PCR amplified with primers Δ_{ATG}Sap-F1 and Sap-R2.

pNW33N2: The 315-bp β-glucanase promoter and signal peptide ($P_{gluc+sp}$) was PCR-amplified using primers Glu-F and GluS-R, and the 798-bp *sapS* DNA fragment, lacking the N-terminal 31-amino-acid signal peptide (Δ_{31}*sapS*), was PCR amplified with primers Δ_{31}Sap-F and Sap-R3.

pNW33N3: A 762-bp *sapS* fragment, lacking the N-terminal 43 amino-acid signal peptide as determined for *B. halodurans* BhFC04 (Δ_{12}*sapS*), was PCR amplified with primers Δ_{43}Sap-F and Sap-R3 and ligated to the 315-bp β-glucanase promoter and signal peptide ($P_{gluc+sp}$) from pNW33N2.

pNW33N4: The 280-bp alkaline protease promoter (P_{apr}) was PCR amplified with primers Apr-F1 and Apr-R, and the 888-bp *sapS* DNA fragment, lacking the ATG initiation codon (Δ_{ATG}*sapS*), was PCR amplified with primers Δ_{ATG}Sap-F2 and Sap-R2.

pNW33N5: The 327-bp alkaline protease promoter and signal peptide (P_{apr+sp}) was PCR amplified with primers Apr-F2 and AprS-R and ligated to the 798-bp Δ_{31}*sapS* fragment from pNW33N2.

pNW33N6: The 327-bp alkaline protease promoter and signal peptide (P_{apr+sp}) from pNW33N5 was ligated to the 762-bp Δ_{12}*sapS* DNA fragment from pNW33N3.

pNW33N7: The SPO2 promoter was cloned into pNW33N, and the plasmid designated pNWSpo. The 891-bp *sapS* gene, starting from the ATG initiation codon, was amplified by PCR with primers $_{ATG}$Sap-F and Sap-R2 and ligated to pNWSpo to generate pNW33N7.

pNW33N8: The 230-bp sigma D promoter ($P_{\sigma D}$) fragment was PCR amplified with primers Sig-F and Sig-R. The 888-bp *sapS* gene fragment, lacking the ATG initiation codon (Δ_{ATG}*sapS*), was generated by PCR with primers Δ_{ATG}Sap-F3 and Sap-R4.

pNW33N9: The 905-bp *sapS* gene, including the putative ribosome binding site (RBS), was obtained by PCR with primers RBS-Sap-F and Sap-R3 and ligated to pNW33N. The resulting plasmid, pNW33N9, harboured the vector-borne *lacZ* promoter (P_{lacZ}) translationally fused to the *sapS* reporter gene.

pMG36e-SapS: The 888-bp *sapS* gene fragment, lacking the ATG initiation codon (Δ_{ATG}*sapS*), was generated by PCR using primers Δ_{ATG}Sap-F4 and Sap-R5. The *sapS* gene fragment was ligated into pMG36e, an expression vector that harbours the strong lactococcal P32 promoter and an ATG initiation codon, thus placing the reporter gene fragment in phase with the initiation codon.

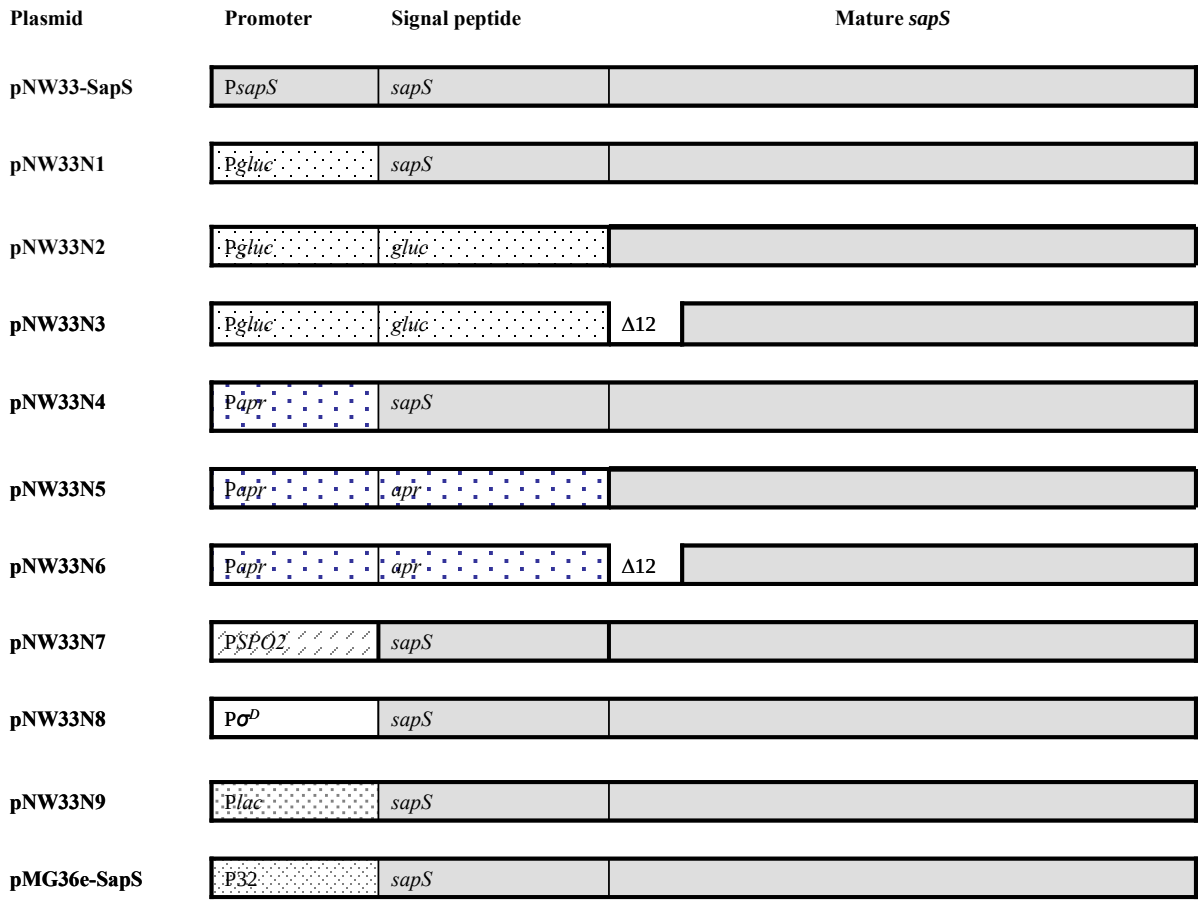


Fig. 4.1 Schematic representation of the vector constructs harbouring the *S. aureus sapS* gene for expression in *E. coli* CU1867, *B. subtilis* 154 and *B. halodurans* BhFC04. **Abbreviations:** P_{gluc}, *B. halodurans* β-glucanase promoter; P_{apr}, *B. halodurans* alkaline protease promoter; P_{SPO2}, *Bacillus* temperate phage SPO2 promoter; $P_{\sigma D}$, the *B. halodurans hag* gene (flagellin protein) promoter; P_{lacZ}, *E. coli lacZ* promoter; P32, strong lactococcal promoter; P_{sapS}, *S. aureus sapS* promoter. In the figure, Δ12 serves to indicate that the N-terminal sequence of the *S. aureus* SapS protein expressed in *B. halodurans* was 12 amino acids shorter than that determined for the native *S. aureus* SapS protein (Du Plessis *et al*., 2002).

Table 4.1: Oligonucleotides used in this study

Primer	Sequence (5' to 3')[a]	Description and location
Sap-F	GCGTCGACAATGCTACGTGGATGAG	*Sal*I, upstream of P_{sapS}
RBS-Sap-F	GGCTGCAGCATGAGGTGATAAG	*Pst*I, 5' end of *sapS* including the RBS
$_{ATG}$Sap-F	GGCGTCGAatgAATAAAATTTCAAAG[b]	*Sal*I, 5' end of *sapS* including ATG initiation codon
Δ_{ATG}Sap-F1	GGCCTGCAGAATAAAATTTCAAAG	*Pst*I, 5' end of *sapS* lacking ATG initiation codon
Δ_{ATG}Sap-F2	GGCGTGGACAATAAAATTTCAAAG	*Sal*I, 5' end of *sapS* lacking ATG initiation codon
Δ_{ATG}Sap-F3	CAGGCATGCAATAAAATTTCAAAGTATATTGC	*Sph*I, 5' end of *sapS* lacking ATG initiation codon
Δ_{ATG}Sap-F4	GGCCTGCAGGAATAAAATTTCAAAG	*Pst*I, 5' end of *sapS* lacking ATG initiation codon
Δ_{31}Sap-F	GCCTGCAGAAAAGTTCTGCTGAAGTT	*Pst*I, 5' end of *sapS* lacking N-terminal 31 amino acids
Δ_{43}Sap-F	GGCTGCAGTCTATACCAGCATCACAAAAG	*Pst*I, 5' end of *sapS* lacking N-terminal 43 amino acids
Sap-R1	GGCTGCAGTTATTTAACTTCGCCTGT	*Pst*I, 3' end of *sapS*
Sap-R2	GGGCATGCTTATTTAACTTCGCCTGT	*Sph*I, 3' end of *sapS*
Sap-R3	GCGTCGACTTATTTAACTTCGCCTGT	*Sal*I, 3' end of *sapS*
Sap-R4	CACGGATCCTTATTTAACTTCGCCTGT	*Bam*HI, 3' end of *sapS*
Sap-R5	GCGGTACCTTATTTAACTTCGCCTGT	*Kpn*I, 3' end of *sapS*
Glu-F	CGTCTAGACTACGCGCTGTATGATAA	*Xba*I, upstream of P_{gluc}
Glu-R	CGCTGCAGCATCTTCCATCCTCCTTATAG	*Pst*I, downstream of P_{gluc}
GluS-R	CACTGCAGAGCTTTTACCCCTTGATGA	*Pst*I, downstream of β-glucanase signal peptide
Apr-F1	GCGAGCTCCTCGTGGAATATCTCCAAGAC	*Sac*I, upstream of P_{apr}
Apr-F2	GGGAAGCTTCTCGTGGAATATCTCCAAGAC	*Hind*III, upstream of P_{apr}
Apr-R	GCGTCGACCAATAGAAACTCCTCCTT	*Sal*I, downstream of P_{apr}
AprS-R	GGCTGCAGATCTGCGAACGTTCCAAC	*Pst*I, downstream of alkaline protease signal sequence
Sig-F	CTCGGTACCCTCGCGTTACGCTCTTTCTGT	*Kpn*I, upstream of $P_{\sigma D}$
Sig-R	GCGCATGCCATTAAAATTTCCTCCTTG	*Sph*I, downstream of $P_{\sigma D}$

[a] Relevant restriction sites are underlined
[b] The introduction of an ATG initiation codon is indicated by lower case letters

4.2.4 Protein sample preparation and protein concentration determination

Bacterial strains harbouring the plasmid constructs were inoculated into LB medium with the appropriate antibiotics and incubated at 37°C for 24 h on a rotary shaker (175 rpm). Protein samples from the cultures were prepared as described by Van der Vaart *et al.* (1997), with the following modifications. For cell fractionation, 40 ml of the respective cultures was harvested after a 15-min centrifugation at 12 000 × *g* at 4°C. The cell-free supernatants (extracellular fraction) were retained and the proteins were precipitated with ice-cold acetone prior to being suspended in 0.1 M sodium acetate buffer (pH 5). The cell pellets were washed once with sterile distilled water, suspended in 5 ml of 0.1 M sodium acetate buffer (pH 5) and sonicated on ice for 20 min with a Model HD2070 Sonoplus Ultrasonic Homogenizer (Bandelin Electronic, Berlin, Germany). The cell lysate was clarified by centrifugation at 12 000 × *g* for 15 min, and the supernatant, considered the intracellular fraction, was recovered. The pellet (cell wall fraction) was washed once with sterile distilled water and suspended in 5 ml of 0.1 M sodium acetate buffer (pH 5). Whole-cell protein samples were prepared by harvesting the cells from 5 ml of the respective cultures by centrifugation, as described above. The cell pellets were washed with sterile distilled water and suspended in 5 ml of 0.1 M sodium acetate buffer (pH 5). The protein concentration of samples was determined by the method of Bradford (1976), with the BioRad protein assay kit (BioRad, Hercules, CA), and with bovine serum albumin as standard.

4.2.5 Qualitative, quantitative and zymographic detection of acid phosphatase activity

Colonies were grown on LB agar supplemented with the appropriate antibiotic and screened for acid phosphatase activity by flooding the surface with 0.1 M sodium acetate (pH 5) containing 0.1% α-naphtyl phosphate (Roche Diagnostics) and 0.2% Fast Garnet GBC salt (Sigma-Aldrich, Aston Manor, South Africa). Acid phosphatase-positive colonies produce a black precipitate.

Acid phosphatase activity was quantified according to the method of Golovan *et al*. (2000), with the following modifications. The assays were performed by incubating 200 µl of enzyme preparation with 200 µl of *p*NPP substrate (Roche Diagnostics), at a final concentration of 25 mM, in 0.1 M sodium acetate (pH 5). Following incubation at 37°C for 30 min, the reaction was terminated by the addition of 1 ml of 1 M NaOH and the liberated *p*-nitrophenol (*p*NP) was measured at 405 nm. The extinction coefficient of *p*-nitrophenyl was

taken to be 18.5 $cm^2.\mu mol^{-1}$ (Walter and Schütt, 1974), and 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. All assays were performed in triplicate, and the results are expressed as means ± standard deviations.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 12% polyacrylamide gels by the method of Laemmli (1970) after the samples had been heated at 37°C for 30 min. Molecular weight markers (BioRad) were included in each gel. Following electrophoresis, the gels were either stained with Coomassie brilliant blue R-250 to visualize the protein bands or incubated for 16 h at room temperature in several changes of renaturation buffer for zymographic analysis (Hamilton *et al*., 2000). After renaturation treatment, gels were equilibrated for 1 h at 37°C in 0.1 M sodium acetate buffer (pH 5) and incubated at 37°C for 15 min to 1 h in 0.1 M sodium acetate (pH 5) containing 0.1% (w/v) α-naphtyl phosphate and 0.2% (w/v) Fast Garnet GBC salt (Gabriel, 1971). Phosphatase activity was indicated by the presence of black-stained bands.

4.2.6 Amino-terminal amino acid sequencing

The extracellular protein preparation was subjected to SDS-PAGE and then electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore Inc., Freehold, NJ, USA). After transfer, the membrane was stained with 0.1% Coomassie brilliant blue R-250 for 5 min, and the appropriate band was excised and analyzed by automated Edman degradation performed with a Procise 492 automatic sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA).

4.3 RESULTS

4.3.1 Expression of the *S. aureus sapS* gene in *E. coli*, *B. subtilis* and *B. halodurans*

Plasmid pNW33-SapS, harbouring the promoter and coding region of the *S. aureus* SapS preprotein (signal sequence and mature protein) was transformed into *E. coli* CU1867, *B. subtilis* 154 and *B. halodurans* BhFC04. The acid phosphatase enzyme was successfully expressed in all three host strains as determined by *in vitro* enzyme assays (Fig. 4.2) and zymography (Fig. 4.3). No acid phosphatase activity was detected with the *in vitro* enzyme assays of the host strains harbouring the pNW33N vector. In contrast to *Bacillus* spp., where

the highest acid phosphatase activity was obtained for the whole-cell fractions, in *E. coli* the highest activity was obtained for the intracellular fraction (Fig. 4.2).

Zymographic analysis of the cell wall fractions of the three host strains harbouring pNW33-SapS was performed following SDS-PAGE and protein renaturation (Fig. 4.3). The cell wall fractions were chosen as they gave rise to high activity levels. For *E. coli*, the SapS activity band was found at the molecular weight position of the mature *S. aureus* 154 acid phosphatase protein band, indicating that the processing had occurred at or close to the cleavage site determined previously for the *S. aureus* 154 SapS protein (Du Plessis *et al*., 2002). The molecular weight of the acid phosphatase activity bands obtained for the *B. subtilis* and *B. halodurans* cell wall fractions (Fig. 4.3, lanes 4 and 5) were lower than that obtained for *E. coli* (Fig. 4.3, lane 3). In order to determine if the protein was processed differently in the Gram-positive *Bacillus* spp., N-terminal sequencing of the enzyme was performed. The N-terminal sequence of the *S. aureus* SapS protein expressed in Gram-positive *B. halodurans* was determined to be NH_2-SIPASQKANL, which is 12 amino acids shorter than the native *S. aureus* SapS protein N-terminal sequence (Du Plessis *et al*., 2002). Consequently, the coding regions of both the *S. aureus* 154 and *B. halodurans* BhFC04 mature SapS proteins were included in the vector construction.

4.3.2 Evaluation of heterologous promoters and signal sequences with *sapS* as a reporter gene in *E. coli* CU1867

To ascertain the feasibility of using the *sapS* gene as a reporter gene in *E. coli* CU1867, the acid phosphatase activity of the host strain harbouring the reporter gene constructs (Fig. 4.1) was determined qualitatively with the plate screen assay (Fig. 4.4A) and quantitatively with *in vitro* enzyme assays (Fig. 4.4B).

E. coli CU1867 harbouring the pNW33N vector showed no acid phosphatase activity after activity staining (Fig. 4.4A). The host strain harbouring pNW33-SapS stained pitch black, indicating high levels of enzyme activity. Various levels of brown to black colour development were detected for the host strain harbouring the heterologous promoter-reporter gene constructs pNW33N1 through pNW33N9, indicating differences in promoter strengths. No black colour development was detected for *E. coli* CU1867(pNW33N8), indicating that the *B. halodurans* $P_{\sigma D}$ ligated to the reporter gene was not expressed.

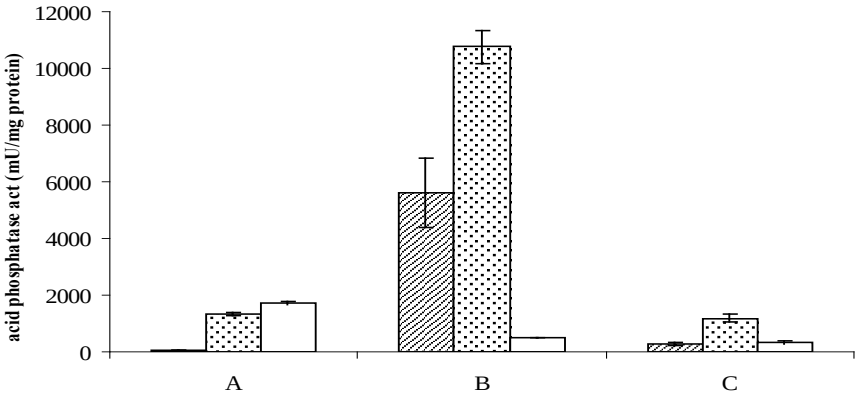


Fig. 4.2 Extracellular (▨), whole-cell (▩) and intracellular (□) *in vitro* acid phosphatase activity (act) results of the host strains harbouring pNW33-SapS. A, *E. coli* CU1867; B, *B. subtilis* 154; C, *B. halodurans* BhFC04.

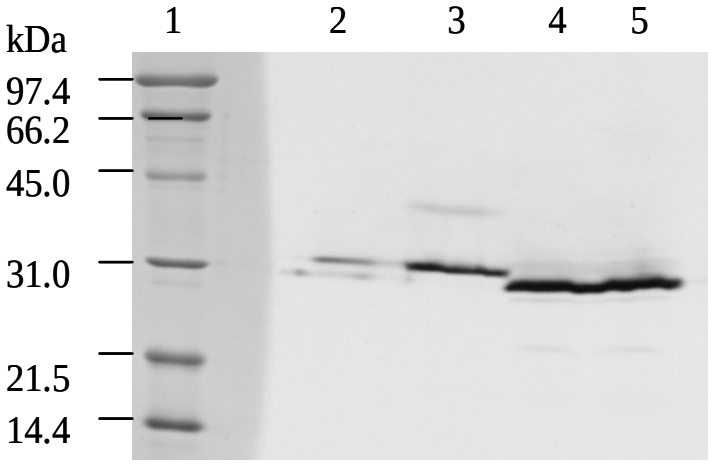


Fig. 4.3 Zymographic analysis of *S. aur eus* SapS acid phosphatase activity in *E. coli* CU1867(pNW33-SapS), *Bacillus* 154(pNW33-SapS), and *S. a ureus*(wild type). Lane 1, low-range protein molecular weight marker; lane 2, *S. aur eus* supernatant fraction; lane 3, *E. coli* CU1867(pNW33-SapS) cell wall fraction; lane 4, *B. subtilis* 154(pNW33-SapS) cell wall fraction; lane 5, *B. halodurans* BhFC04(pNW33-SapS) cell wall fraction.

In order to quantify the acid phosphatase activity results, the production levels of the *sapS* enzymatic reporter genes from the various heterologous promoters and signal sequences were monitored after 24 h by *in vitro* acid phosphatase activity assays. In *E. coli* CU1867, the highest enzyme activity (835 mU/mg of protein) was obtained for P_{lacZ} ligated to the reporter gene (pNW33N9), followed by activities obtained for the P_{apr}-reporter gene construct pNW33N4 (589 mU/mg) and the P_{apr+sp}-reporter gene construct pNW33N5 (541 mU/mg). The activity measured for P_{apr+sp} ligated to the truncated reporter gene (pNW33N6) was approximately five-fold less (81 mU/mg of protein). A lower level of phosphatase activity was obtained for the P_{gluc}-reporter gene construct pNW33N1 (189 mU/mg) than for P_{apr}-reporter gene construct pNW33N4 (589 mU/mg). This result indicated that the *B. halodurans* BhFC04 alkaline protease promoter is a stronger promoter than the β-glucanase promoter. The enzyme activity determined for the lactococcal promoter P32 ligated to the reporter gene (pNW33N10) was 177 mU/mg of protein. The enzyme activity determined for the *Bacillus* temperate phage P_{SPO2} ligated to the reporter gene (pNW33N7) was 475 mU/mg of protein. In accordance with the plate screen assay, no activity was detected from the *B. halodurans* $P_{\sigma D}$-reporter gene construct (pNW33N8) in *E. coli* CU1867. A low level of extracellular enzyme activity (52 mU/mg) was detected for *E. coli* harbouring the P_{lacZ} promoter (pNW33N9) after 24 h. The extracellular activity can probably be ascribed to leakage, as opposed to secretion, in the *E. coli* host strain. No extracellular activity was detected for any of the other constructs expressed in *E. coli* (results not shown).

4.3.3 Evaluation of heterologous promoters and signal sequences with *sapS* as a reporter gene in *B. subtilis* 154

The acid phosphatase activity of *B. subtilis* 154 harbouring the various constructs was determined qualitatively (Fig. 4.5A) and quantitatively (Fig. 4.5B).

B. subtilis 154 harbouring pNW33N showed no acid phosphatase activity with the plate screen assay (Fig. 4.5A). The host strain harbouring pNW33-SapS stained pitch black, indicating high levels of enzyme activity. As for the *E. coli* CU1867 host strain, various levels of brown to black colour development were observed for the heterologous promoter-reporter gene constructs pNW33N1 through pNW33N9. *B. subtilis* 154 harbouring the P_{apr}-reporter gene construct (pNW33N4), the P_{apr+sp}-mature reporter gene construct (pNW33N5) and the P_{apr+sp}-truncated mature reporter gene construct (pNW33N6) stained black, indicating

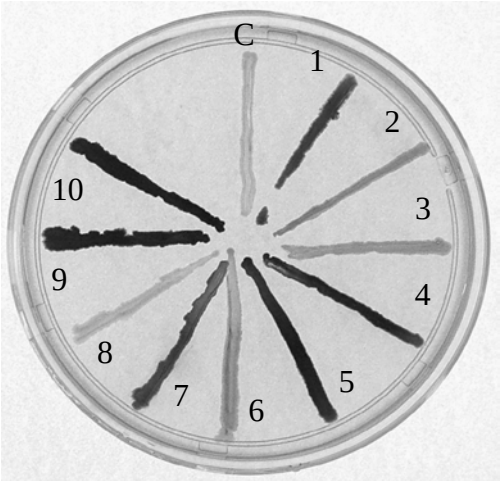


Fig. 4.4A Plate screen showing acid phosphatase activities of 24-h cultures of *E. c oli* CU1867 harbouring the various constructs. Control (C), pNW33N; 1, pNW33N1; 2, pNW33N2; 3, pNW33N3; 4, pNW33N4; 5, pNW33N5; 6, pNW33N6; 7, pNW33N7; 8, pNW33N8; 9, pNW33N9; 10, pNW33-SapS.

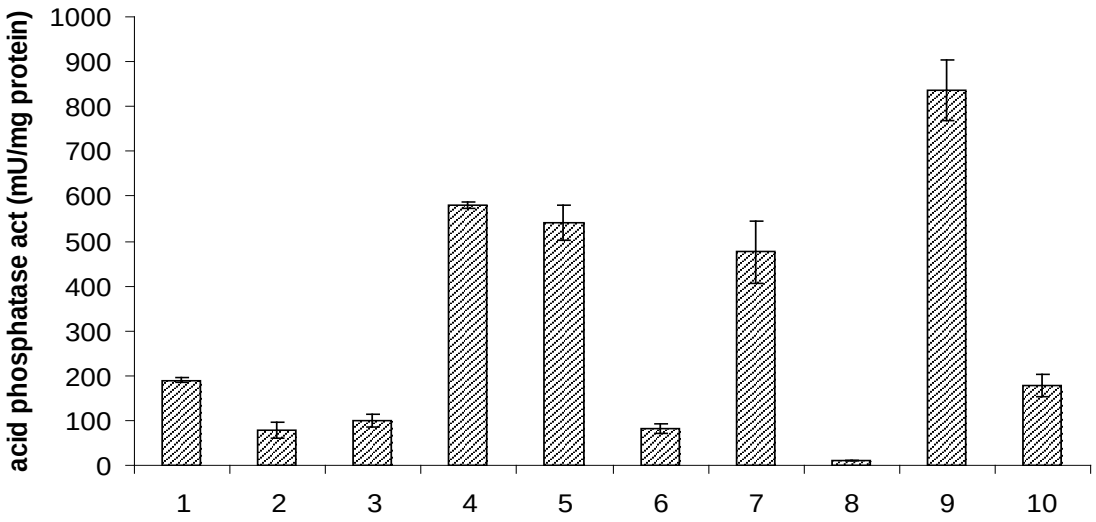


Fig. 4.4B Whole-cell *in vi tro* acid phosphatase activity (act) results of 24-h cultures of *E. coli* CU1867 harbouring different promoter constructs. 1, pNW33N1; 2, pNW33N2; 3, pNW33N3; 4, pNW33N4; 5, pNW33N5; 6, pNW33N6; 7, pNW33N7; 8, pNW33N8; 9, pNW33N9; 10, pMG36e-SapS.

high levels of reporter gene activity. No black colour development was detected for *B. subtilis*(pNW33N8), indicating that $P_{\sigma D}$ ligated to the reporter gene was not expressed in *B. subtilis* 154. Similarly, no activity was observed for *B. subtilis*(pNW33N9), showing that the *E. coli* P_{lacZ}-reporter gene construct was not expressed in the Gram-positive host strain.

In order to quantify the acid phosphatase activity results in *B. subtilis* 154, the production levels of the *sapS* enzymatic reporter gene from the various heterologous promoters and signal sequences were monitored after 24 h by *in vitro* acid phosphatase activity assays (Fig. 4.5B). The highest enzyme activity (1923 mU/mg of protein) was obtained for the P_{apr+sp}-mature reporter gene construct pNW33N5, followed by activities obtained for the P_{apr+sp}-truncated mature reporter gene construct pNW33N6 (1620 mU/mg) and the P_{apr}-reporter gene construct pNW33N4 (968 mU/mg). A lower level of phosphatase activity was obtained for the P_{gluc}-reporter gene construct pNW33N1 (65 mU/mg) than for P_{apr}-reporter gene construct pNW33N4 (968 mU/mg). The *in vitro* assay results correlated with the plate screen assay, *i.e.* the recombinant strains harbouring the β-glucanase promoter-reporter gene constructs (pNW33N1, pNW33N2 and pNW33N3) stained lighter than the recombinant strains harbouring the alkaline protease promoter-reporter gene constructs (pNW33N4, pNW33N5 and pNW33N6). As for *E. coli*, this result indicated that the *B. halodurans* BhFC04 alkaline protease promoter is a stronger promoter than the β-glucanase promoter. The P32 lactococcal promoter was also effectively recognized, since 166 mU/mg enzyme activity was measured with the pNW33N10 construct. No acid phosphatase activity was detected for the P_{lacZ}-reporter gene construct (pNW33N9) in the host strain. This is not surprising since *Bacillus* is very stringent in its recognition of promoters (Patek *et al.*, 2003). Extracellular acid phosphatase activity was detected for the P_{apr+sp}-mature reporter gene construct pNW33N5 (60 mU/mg) and the P_{apr+sp}-truncated mature reporter gene construct pNW33N6 (44 mU/mg). No extracellular activity was detected for any of the other constructs evaluated in this host strain (results not shown).

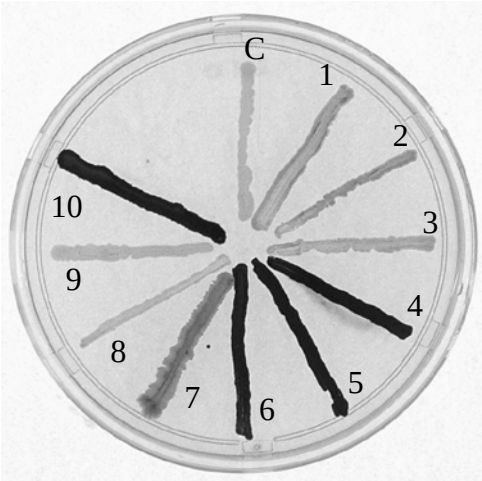


Fig. 4.5A Plate screen assay showing acid phosphatase activities of 24-h cultures of *B. subtilis* strain 154 harbouring the various constructs. Control (C), pNW33N; 1, pNW33N1; 2, pNW33N2; 3, pNW33N3; 4, pNW33N4; 5, pNW33N5; 6, pNW33N6; 7, pNW33N7; 8, pNW33N8; 9, pNW33N9; 10, pNW33-SapS.

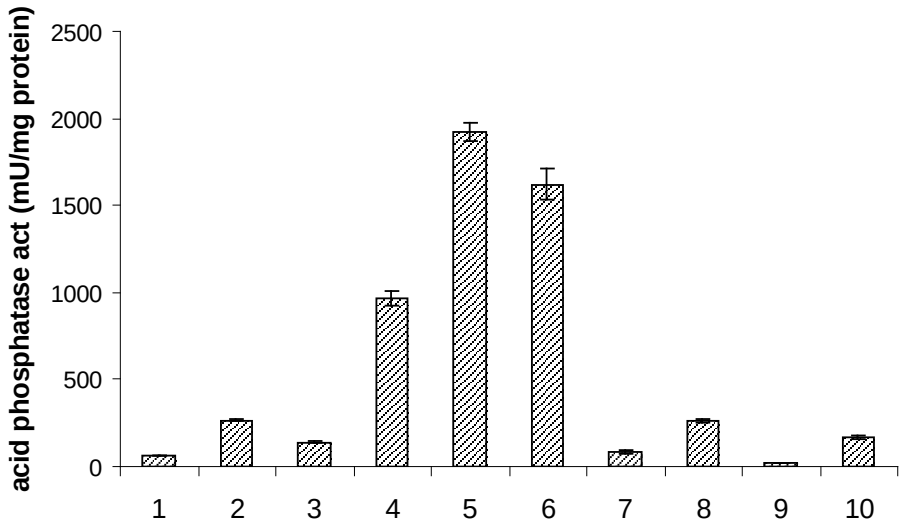


Fig. 4.5B Whole-cell *in vitro* acid phosphatase activity (act) results of 24-h cultures of *B. subtilis strain* 154 harbouring the different promoter constructs. 1, pNW33N1; 2, pNW33N2; 3, pNW33N3; 4, pNW33N4; 5, pNW33N5; 6, pNW33N6; 7, pNW33N7; 8, pNW33N8; 9, pNW33N9; 10, pMG36e-SapS.

4.3.4 Evaluation of heterologous promoters and signal sequences with *sapS* as a reporter gene in *B. halodurans* BhFC04

The acid phosphatase activity of *B. halodurans* BhFC04 harbouring the expression vector constructs was determined qualitatively (Fig. 4.6A) and quantitatively (Fig. 4.6B).

B. halodurans BhFC04 harbouring pNW33N showed no acid phosphatase activity with the plate screen assay (Fig. 4.6A). The host strain harbouring pNW33-SapS stained black, indicating high levels of expression of the *sapS* gene from its own promoter and signal sequence. No colour was detected for the host strain harbouring any of the other promoter-reporter gene constructs (Fig. 4.6A).

Of the heterologous promoter-reporter gene constructs, the best activity (18 mU/mg of protein) was obtained with *B. halodurans* BhFC04 harbouring the $P_{\sigma D}$-reporter gene construct (pNW33N8). Since the acid phosphatase activity measured in this host strain was very low and no enzyme activity could be detected for the extracellular fractions of any of the *B. halodurans* transformants harbouring the various constructs, the reporter gene could not be used for the evaluation of the efficacy of the isolated *B. halodurans gluc* and *apr* signal sequences for the extracellular production of heterologous proteins.

4.4 DISCUSSION

In this study, the *S. aureus* acid phosphatase SapS enzyme was evaluated as a reporter for promoter and signal sequence characterization in *E. coli* CU1867, *B. subtilis* 154 and *B. halodurans* BhFC04. Initial studies focused on determining whether the *sapS* gene was expressed in the three host strains from its own promoter and signal sequence. A zymogram of active acid phosphatase led to the determination of the molecular masses of the *sapS* gene expressed in the three different host strains and showed that the enzyme was processed differently in *E. coli* than in *B. subtilis* and *B. halodurans*. The precursors of secreted proteins from Gram-positive bacteria generally have longer and more hydrophobic signal peptides than those of Gram-negative bacteria (von Heijne, 1990). In *S. aureus*, alanine is at the -1 position and lysine is at the +1 position of the signal peptide of the *sapS* gene, as determined by N-terminal sequencing. In *B. halodurans* BhFC04, alanine was found to be at the -1 position and serine was at the +1 position in the signal peptide of the *sapS* gene.

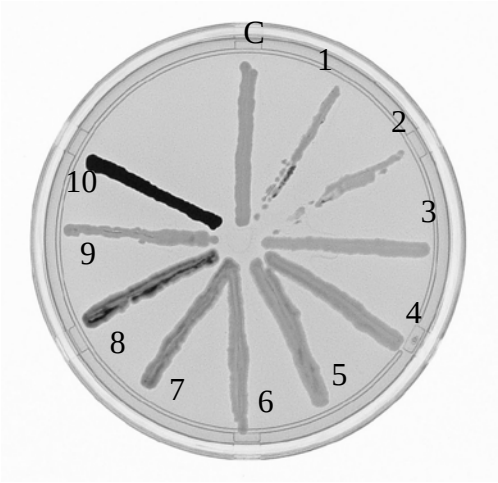


Fig. 4.6A Plate screen assay showing acid phosphatase activities of 24-h cultures of *B. halodurans* BhFC04 harbouring the various constructs. Control (C), pNW33N; 1, pNW33N1; 2, pNW33N2; 3, pNW33N3; 4, pNW33N4; 5, pNW33N5; 6, pNW33N6; 7, pNW33N7; 8, pNw33N8; 9, pNW33N9; 10, pNW33-SapS.

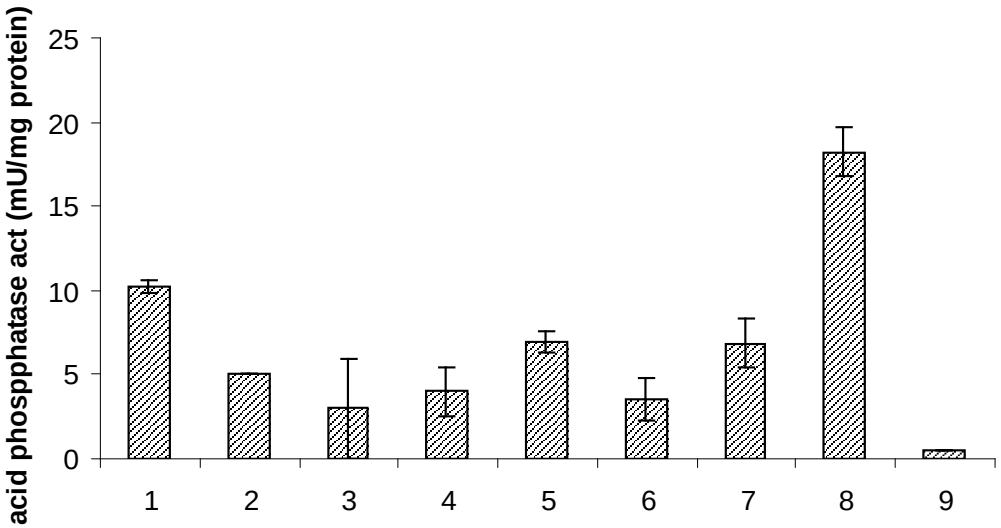


Fig. 4.6B Whole-cell *in vitro* acid phosphatase activity (act) results of 24-h cultures of *B. halodurans* BhFC04 harbouring the different promoter constructs. 1, pNW33N1; 2, pNW33N2; 3, pNW33N3; 4, pNW33N4; 5, pNW33N5; 6, pNW33N6; 7, pNW33N7; 8, pNW33N8; 9, pNW33N9.

In *B. subtilis*, alanine is the predominant residue (>90%) at the -1 and -3 positions of the *Bacillus* signal peptides (Nagarayan, 1993). Furthermore, for *B. subtilis*, the SapS activity band was found at the molecular mass position of the mature *B. halodurans* BhFC04 acid phosphatase protein band, indicating that the processing had occurred at or close to the cleavage site determined for *B. halodurans* BhFC04.

The suitability of the *sapS* gene as an expression reporter system was evaluated by the ligation of a range of heterologous promoters that included both well-known and newly isolated promoters and signal sequences. The expression and secretion abilities of the transcriptional and translational fusion products were estimated by acid phosphatase activity determination. Since Nagarajan *et al*. (1992) reported the successful use of the *Bacillus amyloliquefaciens* neutral and alkaline protease promoter and signal sequences for overexpression of heterologous proteins, the *B. halodurans* alkaline protease gene promoter and signal sequence was isolated and evaluated for its ability to express and secrete the reporter gene. The thermostable endo-(1,3-1,4) β-glucanase-encoding gene from *B. halodurans* has previously been expressed successfully in *E. coli*, *B. subtilis* (Louw *et al*., 1993) and *L. plantarum* (unpublished results). Therefore, the β-glucanase gene promoter and signal sequence was isolated and evaluated for the ability to express and secrete the reporter gene. It has been reported that the *Bacillus* temperate phage SPO2 promoter functions well in *B. subtilis* (Schoner *et al*., 1983) and was included in the range of promoters to be evaluated. The strong lactococcal promoter P32 was used to express genes from prokaryotic and eukaryotic origins in lactococci, *B. subtilis* and *E. coli* (Van de Guchte *et al*., 1989). Thus, s*apS* gene expression from this promoter was also evaluated. The σ^D promoter region of the *B. halodurans hag* gene (flagellin protein) was included in the range of promoters evaluated as it was used in the development of a surface display system in *B. halodurans* Alk36 (Crampton *et al*., 2007).

Enzyme studies performed with *E. coli*, *B. subtilis* and *B. halodurans* harbouring the various transcriptional and translational reporter gene constructs demonstrated that the *sapS* gene can be used as a reporter in all three of the host strains. The enzyme activity obtained for recombinant strains harbouring the heterologous promoter-reporter gene constructs was less than the activity measured for *sapS* expressed from its own promoter and signal sequence. This could be due to the reduction in the quantity of fusion proteins produced depending on the differences in promoter strengths and not necessarily from misfolding. The decrease in

enzymatic activity of fusion proteins containing heterologous promoters was also found for GFP, *lacZ* and luciferase. GFP and luciferase reporters retain approximately 5% of their activity compared to non-fused controls (Strathdee *et al*., 2000). Piruzian *et al*. (2002) reported a decrease in thermostable lichenase (LicB) and GUS activities for cells expressing the fusion constructs compared to the native proteins. SapS activity was obtained with translational fusions of isolated promoter and signal sequences to the native mature 798-bp *sapS* gene sequence, as well as the truncated 762-bp *sapS* gene sequence in all three of the host strains, showing the versatility of the *sapS* gene as an enzymatic reporter gene. The facts that very low levels of extracellular SapS activity were detected for the constructs in the three host strains and the activity was whole-cell associated make it unsuitable for the isolation or evaluation of signal peptides for the extracellular production of heterologous proteins.

From the range of promoters evaluated with this system, the strongest promoter for the expression of heterologous proteins was easily identified in each of the three host strains. These include the *E. coli lacZ* promoter in *E. coli*, the *B. halodurans* alkaline protease promoter in *B. subtilis*, and the *B. halodurans* σ^D promoter in *B. halodurans.* Although the *Bacillus* temperate phage SPO2 promoter was reported to be a strong promoter for heterologous protein production in *B. subtilis* (Schoner *et al*., 1983), similar activity levels were obtained in both *E. coli* CU1867 and *B. subtilis* 154. Although the lactococcal P32 promoter was used for heterologous protein production in *E. coli* and *B. subtilis* (Van de Guchte *et al*., 1989), it was found in this study that the *B. subtilis* temperate phage SPO2 and *B. halodurans* BhFC04 alkaline protease promoters both gave rise to higher levels of enzyme activity in *E. coli* and *B. subtilis,* than did the P32 promoter.

This is the first report on the development of a class C acid phosphatase gene as a reporter gene with the advantage of being able to function in both Gram-positive and Gram-negative host strains. Furthermore, the *sapS* enzymatic reporter gene has shown potential for use in the characterization and evaluation of a range of heterologous promoters that could find application in the development of expression vectors for improved production of industrially important proteins.

4.5 REFERENCES

Applegate, B.M., Kehrmeyer, S.R. and Slayer, G.S. (1998). A chromosomally based *tod-luxCDABE* whole cell reporter for benzene, toluene, ethylbenzene, and xylene (BTEX) sensing. Appl. Environ. Microbiol. **64**: 2730-2735.

Biran, I., Klimently, L., Hengge-Aronis, R., Ron, E.Z. and Rishpon, J. (1999). On-line monitoring of gene expression. Microbiol. **145**: 2129-2133.

Bradford, M.A. (1976). A rapid and sensitive method for quantification of microgram quantities of protein utilising the principle of protein-dye binding. Anal. Biochem. **72**: 248-254.

Cao, Q., Qu, Z., Wan, Y., Zhang, H. and Shen, D. (2001). Cloning, molecular characterization, and application of rice epithytic *Bacillus pimulus* promoter fragments. Curr. Microbiol. **43**: 244-248.

Chang, S. and Cohen, S. (1979). High frequency transformation of *Bacillus subtilis* protoplasts by plasmid DNA. Mol. Gen. Genet. **168**: 111-115.

Chary, V.K., Busui, M., Renye, J.A. and Piggot, P.J. (2005). Vectors that facilitate the replacement of transcriptional *lacZ* fusions in *Streptococcus mutans* and *Bacillus subtilis* with fusions to *gfp* or *gusA.* FEMS Microbiol. Lett. **247**: 171-176.

Choe, J., Guo, H.H., van de n E ngh, G. (2005). A dual-fluorescence reporter system for high-throughput clone characterization and selection by cell sorting. Nucl. Acids Res. **33**: e49.

Crampton, M., Berger, E., Reid, S. and Louw, M.E. (2007). The development of a flagellin surface display system in a moderate thermophile, *Bacillus halodurans* Alk36. Appl. Microbiol. Biotechnol. **75**: 599-607.

Ding, C. and Cantor, C.R. (2004). Quantitative analysis of nucleic acids-the last few years of progress. J. Biochem. Mol. Biol. **37**: 1-10.

Dower, W.J., Miller, J.F. and Ragsdale, C.W. (1988). High efficiency transformation of *E. coli* by high voltage electroporation. Nucl. Acids Res. **16**: 6127.

Du Plessis, E.M., Theron, J., Joubert, L., Lotter, T. and Watson, T.G. (2002). Characterization of a phosphatase secreted by *Staphylococcus aureus* strain 154, a new member of the bacterial class C family of non-specific phosphatases. Syst. Appl. Microbiol. **25**: 21-30.

Gabriel, O. (1971). Locating enzymes on gels. Methods Enzymol. **17A**: 578-604.

Golovan, S., Wang, G., Zhang, J. and Forsberg, C.W. (2000). Characterization and overproduction of the *Escherichia co li appA*-encoded bifunctional enzyme that exhibits both phytase and acid phosphatase activities. Can. J. Microbiol. **46**: 59-71.

Greer, L.F. and Szalay, A.A. (2002). Imaging of light emission from the expression of luciferases in living cells and organisms: A review. Luminescence **17**: 43-74.

Hamilton, A., Harrington, D. and Sut cliffe, I.C. (2000). Characterization of acid phosphatase activities in the equine pathogen *Streptococcus equi.* Syst. Appl. Microbiol. **23**: 325-329.

Hampf, M. and G ossen, M. (2006). A protocol for combined *Photinus* and *Renilla* luciferase quantification compatible with protein assays. Anal. Biochem. **356**: 94-99.

Hautefort, I., Proenca, M.J. and Hinton, J.C.D. (2003). Single-copy green fluorescent protein gene fusions allow accurate measurement of *Salmonella* gene expression *in vitro* and during infection of mammalian cells. Appl. Environ. Microbiol. **69**: 7480-7491.

Ikura, Y. and H orikoshi, K. (1979). Ioslation and some properties of β-galactosidase producing bacteria. Agric. Biol. Chem. **43**: 85-88.

Janatova, I., Costaglioli, P., Wesche, J., Masson, J.M. and Meilhoc, E. (2003). Development of a reporter system for the yeast *Schwanniomyces occidentalis*: Influence of DNA composition and codon usage. Yeast **20**: 687-701.

Jefferson, R.A. (1986). β-Glucoronidase from *E. coli* as a gene-fusion marker. Proc. Natl. Acad. Sci. USA **83**: 8447-8451.

Kim, K.W., Franceschi, V.R., D avin, L.B.V. and L ewis, N.G. (2006). Beta-glucuronidase as reporter gene: Advantages and limitations. Methods Mol. Biol. **323**: 263-273.

Kirchner, G., Roberts, J.L., Gustafson, G.D. and Ingolia, T.D. (1989). Active bacterial luciferase from a used gene: Expression of *Vibrio harveyi luxAB* translational fusion in bacteria, yeast and plant cells. Gene **81**: 349-354.

Koga, K., Ikegami, A., Nakasone, K., Murayama, R., Akanuma, G., Natori, Y., Nanamiya, H. and Kawamura, F. (2006). Construction of *Bacillus subtilis* strains carrying the transcriptional *bgaB* fusion with the promoter region of each *rrn* operon and their differential transcription during spore development. J. Gen. Appl. Microbiol. **52**: 119-124.

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

Lam, K.H.E., Chow, K.C. and Wog, W.K.R. (1998). Construction of an efficient *Bacillus subtilis* system for extracellular production of heterologous proteins. J. Biotechnol. **63**: 167-177.

Loening, A.M., Fenn, T.D., Wu, A.M. and Gambhir, S.S. (2006). Consensus guided mutagenesis of *Renilla* luciferase yields enhanced stability and light output. Protein Eng. Des. Sel. **19**: 391-400.

Louw, M.E., Reid, S.J. and Watson, T.G. (1993). Characterization, cloning and sequencing of a thermostable endo-(1,3-1,4) β-glucanase-encoding gene from an alkophilic *Bacillus brevis*. Appl. Microbiol. Biotechnol. **38**: 507-513.

Lovett, P.S. and Keggins, K.M. (1979). *B. subtilis* as a host for molecular cloning. Methods Enzymol. **68**: 342-357.

Meighen, E.A. (1991). Molecular biology of bacterial luminescence. Microbiol. Rev. **55**: 123-142.

Nagarajan, V., Albertson, H., Chen, M. and Ribbe, J. (1992). Modular expression and secretion vectors for *Bacillus subtilis*. Gene **114**: 121-126.

Nagarayan, V. (1993). Protein secretion, p. 713-726. In: *Bacillus subtilis* and other Gram-positive bacteria (A.L. Sonenshein, J.A. Hoch, R. Losick (eds.), Washington, D.C., ASM Press.

Naylor, L.H. (1999) Reporter gene technology: The future looks bright. Biochem. Pharmacol. **58**: 749-757.

Ostanin, K., Harms, E.H., Stevis, P.E., Kuciel, R., Zhou, M-M. and van Etten, R.L. (1992). Overexpression, site-directed mutagenesis and mechanism of *Escherichia coli* acid phosphatase. J. Biol. Chem. **267**: 22830-22836.

Palmano, S., Kirkpatrick, B.C. and Firrao, G. (2001). Expression of chloramphenical acetyltranferase in *Bacillus subtilis* under the control of a phytoplasma promoter. FEMS Microbiol. Lett. **199**: 177-179.

Park, H.S. and Kilbane, J.J. (2004). Gene expression studies of *Thermus thermophilus* promoters P_{dnaK}, P_{arg} and $P_{scs\text{-}mdh}$. Lett. Appl. Microbiol. **38**: 415-224.

Patek, M., Muth, G. and Wohlleben, W. (2003). Function of *Corynebacterium glutamicum* promoters in *Escherichia coli, Streptomyces lividans,* and *Bacillus subtilis*. J. Biotechnol. **104**: 325-334.

Pedraza-Reyes, M., Gutierrez-Corona, F. and Nicholson, W.L. (1994). Temporal regulation and forespore-specific expression of the spore photoproduct lyase gene by sigma-G RNA polymerase during *Bacillus subtilis* sporulation. J. Bacteriol. **176**: 3983-3991.

Perez-Arellano, I. and Perez-Martinez, G. (2003). Optimization of green fluorescent protein (GFP) expression from a lactose-inducible promoter in *Lactobacillus casei*. FEMS Microbiol. Lett. **222**: 123-127.

Piruzian, E.S., Glodenkova, I.V., Musiychuk, K.A., Kobets, N.S., Arman, I.P., Bobrysheva, I.V., Chekuta, I.A. and Glazkova, D. (2002). A reporter system for prokaryotic and eukaryotic cells based on the thermostable lichinase from *Clostridium thermocellum*. Mol. Genet. Genomics **266**: 778-786.

Post, D.A. and Luebeke, V.E. (2005). Purifucation, cloning, and properties of α-galactosidase from *Saccharomyces erythraea* and its use as a reporter system. Appl. Microbiol. Biotechnol. **67**: 91-96.

Poyart, C. and Trieu-Cuot, P. (1997). A broad-host-range mobalizable shuttle vector for the construction of transcriptional fusions to β-galactosidase in Gram-positive bacteria. FEMS Microbiol. Lett. **156**: 193-198.

Quax, W.J. and Broekhuizen, C.P. (1994). Development of a new *Bacillus* carboxyl esterase for use in the resolution of chiral drugs. Appl. Microbiol. Biotechnol. **41**: 425-431.

Sambrook, J. and Russell, D.W. (2001) Molecular cloning - a laboratory manual, 3rd edn. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York.

Schenborn, E. and Groskreutz, D. (1999). Reporter gene vectors and assays. Mol. Biotech. **13**: 29-44.

Schmidt, S., Wolf, N., Strey, J., Nahrstedt, H., Meinhardt, F. and Waldeck, J. (2005). Test systems to study transcriptional regulation and promoter activity in *Bacillus megaterium*. Appl. Microbiol. Biotechnol. **68**: 647-655.

Schoner, R.G., Williams, D.M. and Lovett, P.S. (1983). Enhanced expression of mouse dihydrofolate reductase in *Bacillus subtilis*. Gene **22**: 47-57.

Serrano-Heras, G., Salas, M. and Bravo, A. (2005). A new plasmid vector for regulated gene expression in *Bacillus subtilis*. Plasmid **54**: 278-282.

Southward, C.M. and Surette, M.G. (2002). The dynamic microbe: Green fluorescent protein brings bacteria to light. Mol. Microbiol. **45**: 1191-1196.

Strathdee, C .A., M cLeod, M .R. and U nderhill, T .M. (2000). Dominant positive and negative selection using luciferase, green fluorescent protein and β-galactosidase reporter gene fusions. BioTech. **28**: 210-214.

Talukder, A .A., Y anai, S. and Y amada, M . (2005). Analysis of reading frame and expressional regulation of randomly-proximal genes in *Escherichia coli*. J. Gen. Appl. Microbiol. **51**: 93-103.

Thibodeau, S.A., Fang, R . and Jou ng, J. K. (2004). High-throughput β-galactosidase assay for bacterial cell-base reporter systems. BioTech. **36**: 410-415.

Van de Guchte, M., Van der Vossen, J.M.B.M., Kok, J. and Benema, G. (1989). Construction of a lactococcal expression vector: Expression of hen egg white lysozyme in *Lactococcus l actis* subsp. *lactis.* Appl. Environ. Microbiol. **55**: 224-228.

Van der Vaart, J.M., Te Biesebeke, R., Chapman, J.W., Toschka, H.Y., Klis, F.M. and Verrips C.T. (1997). Comparison of cell wall proteins of *Saccharomyces cerevisiae* as anchors for cell surface expression of heterologous proteins. Appl. Environ. Microbiol. **63**: 615-620.

Veening, J-W., Smits, W.K ., H amoen, L.W., Jongbloed, J.D .H. and Kuipers, O.P. (2004). Visualization of differential gene expression by improved cyan fluorescent protein and yellow fluorescent protein production in *Bacillus subtilis*. Appl. Environ. Microbiol. **70**: 6809-6815.

von Heijne, G. (1990). The signal peptide. J. Membr. Biol. **115**: 195-201.

Walter, K. and Sc hütt, C . (1974). Acid and alkaline phosphatase in serum, p. 856-860. In: H.U. Bergmeyer (ed.), Methods of enzymatic analysis. New York, Academic Press.

Wang, Z.W., Law, W.S. and Chao, Y.P. (2004). Improvement of the thermoregulated T7 expression system by using the heat-sensitive *lacI*. Biotech. Prog. **20**: 1352-1358.

Westers, L., Westers, H. and Quax, W.J. (2004). *Bacillus subtilis* as cell factory for pharmaceutical proteins: A biotechnological approach to optimize the host organism. Biochim. Biophys. Acta **1694**: 299-310.

Wood, K.V. (1995). Marker proteins for gene expression. Curr. Opin. Biotechnol. **6**: 50-58.

Wood, K.V. (1998). The chemistry of bioluminescent reporter. Promega Notes No. **65**: 14-29.

CHAPTER FIVE

CONCLUDING REMARKS

Reporter gene technology has been applied extensively for the identification and characterization of novel promoters, which may be used towards establishing improved expression systems for high-level production of recombinant proteins (Serrano-Heras *et al.*, 2005; Miksch *et al.*, 2006). This interest, together with the isolation of a secreted acid phosphatase from *Staphylococcus aureus* strain 154 and the availability of simple qualitative and quantitative enzyme assays, has led to the development and evaluation of this enzyme as a reporter for use in molecular genetic research. The details of the results obtained during the course of this investigation have been discussed in the individual chapters. The information that has evolved during this investigation is summarized briefly in the sections below.

The acid phosphatase identified and characterized from the culture supernatant of *S. aureus* strain 154 was designated SapS. Based on biochemical and molecular characterization of the acid phosphatase, the enzyme was classified as a new member of the bacterial class C family of non-specific acid phosphatases (NSAPs) (Chapter 2). In addition to the two catalytic domains described previously for this family of acid phosphatases (Thaller *et al.*, 1998), sequence analysis of SapS revealed a further two regions located towards the N- and C-termini of the protein, respectively, that displayed a high degree of similarity among all of the class C NSAPs. Although the functional importance of these conserved regions has not yet been determined, it is nevertheless interesting to note that there is a strong selective pressure to retain these motifs in enzymes that have little overall homology. The recent publication of the crystal structure of the *e*(P4) acid phosphatase of *H. influenzae*, which is also a member of the class C NSAPs (Ou *et al.*, 2006; Felts *et al.*, 2007), offers a possible explanation for the conservation of these motifs. The *e*(P4) acid phosphatase was shown to consist of a core α/β domain, which contains the residues responsible for catalysis and binding the active site Mg^{2+} ion, and a smaller α domain, which may play roles in substrate binding and dimerization. Moreover, dimerization of the polypeptide was proposed to be important for catalysis as intersubunit contacts appeared to stabilize the active site (Felts *et al.*, 2006). Since the conserved regions identified in SapS correspond to regions within the α domain, it is

tempting to speculate that these regions may be involved in protein-protein interaction that results in dimeric assembly and/or catalytic activity of the enzyme. To investigate whether the identified conserved regions may play a role in dimerization and/or catalytic activity, deletion mutagenesis of the respective regions should be performed and the acid phosphatase activity of the resulting mutants be determined and compared to the native enzyme.

The *S. aureus* acid phosphatase (SapS) was subsequently used as a reporter for strain evaluation and cell surface display. For this purpose, *B. halodurans* of which the major cell wall protease gene (*wprA*) had been inactivated was used as expression host, and the C-terminal cell wall-binding domain of the *B. halodurans* peptidoglycan hydrolase gene (*cwlC*) was used to anchor SapS to the cell wall of the host strain (Chapter 3). Increased extracellular levels of the SapS acid phosphatase were obtained with the mutant host strain, thus confirming reports in the literature that extracellular heterologous enzyme production is improved by inactivation of the *wprA* gene (Lee *et al.*, 2000). Moreover, the SapS reporter was also used successfully for showing that the CwlC anchoring motif could be used to target the SapS acid phosphatase to the cell wall. However, proteolytic cleavage of the fusion protein remained a problem as was determined with zymography Future strategies to address this problem could be the inactivation of *sigD*, since the use of a strain with both the *sigD* and the *wprA* gene's inactivated was reported to improve surface display in *B. subtilis* (Kobayashi *et al.*, 2000a,b).

The SapS acid phosphatase was next evaluated as a reporter for gene expression and protein secretion. Transcriptional and translational fusions of the *sapS* gene with selected heterologous promoters and signal sequences were constructed and expressed in *E. coli*, as well as in *B. subtilis* and *B. halodurans*. The strongest promoter for heterologous protein production in each of the host strains was identified, *i.e.* the *E. coli lacZ* promoter in *E. coli*, the *B. halodurans* alkaline protease promoter in *B. subtilis*, and the *B. halodurans* σ^D promoter in *B. halodurans* (Chapter 4). However, the results also indicated that the *S. aureus* SapS enzyme is not suitable for evaluation of signal peptide sequences, which may find application in improved extracellular production of heterologous proteins. This is due to the expressed acid phosphatase enzyme being mostly cell wall-associated in both *E. coli* and *B. subtilis*. In this regard, the *S. aureus* secreted nuclease (Nuc) is a more appropriate secretion-specific reporter for Gram-positive bacteria since more than 80% of the active *S. aureus* nuclease gene product is secreted into the culture medium (Kovacenic *et al*., 1985; Le Loir *et*

al., 1994; Poquet *et al.*, 1998). Despite not being suitable as an export-specific reporter, the *S. aureus* acid phosphatase was nevertheless shown to be very useful for characterization and comparison of promoter activity in both Gram-positive and Gram-negative bacterial host strains.

In conclusion, the biochemical properties of SapS and the availability of cost-effective qualitative and quantitative assays for acid phosphatase enzyme activity determination contribute to making it a suitable reporter for the analysis of gene expression and the study of individual promoters and their regulation. Although background acid phosphatase activity may represent a problem in some host strains, it can be overcome by using host strains of which the acid phosphatase-encoding gene has been inactivated such as the *E. coli* host strain used in this study (Ostanin *et al.*, 1992). Moreover, no endogenous acid phosphatase activity was observed in the *B. subtilis* and *B. halodurans* host strains used in this study. Therefore, the development of the SapS reporter system, which was shown to be functional in both Gram-negative and Gram-positive bacteria, represents a useful addition to the range of reporter systems currently available. Indeed, the SapS reporter system is currently being used in our laboratory for the isolation and characterization of stress-inducible promoters from *B. halodurans*, since the commonly used *lacZ* reporter gene cannot be used in this organism as it harbors the *lac* operon.

REFERENCES

Felts, R.L., Reilly, T.J., Calcutt, M.J. and Tanner, J.J. (2006). Cloning, purification and crystallization of *Bacillus anthracis* class C acid phosphatase. Acta Cryst.: Struct. Biol. Cryst. Commun. **62**: 705-708.

Kobayashi, G., Toida, J., Akamatsu, T., Yamamoto, H., Shida, T. and Sekiguchi, J. (2000a). Accumalation of a recombinant *Aspergillus oryzae* lipase artificially localized on the *Bacillus subtilis* cell surface. J. Biosci. Bioeng. **90**: 422-425.

Kobayashi, G., Toida, J., Akamatsu, T., Yamamoto, H., Shida, T. and Sekiguchi, J. (2000b). Accumulation of artificial cell wall-binding lipase by *Bacillus subtilis wprA* and/or *sig*D mutants. FEMS Microbiol. Lett. **188**: 165-169.

Kovacevic, S., Veal, L.E., Hsiung, M. and Miller, J. (1985). Secretion of Staphylococcal nuclease by *B. subtilis*. J. Bacteriol. **162**: 521-528.

Lee, S.J., Kim, D.M., Bae, K.H., Byun, S.M. and Chung, J.H. (2000). Enhancement of secretion and extracellular stability of staphylokinase in *Bacilllus subtilis* by *wprA* gene disruption. Appl. Environ. Microbiol. **66**: 476-480.

Le Loir, Y.L., Gruss, A., Ehrlich, S.D. and Langella, P. (1994). Direct screening of recombinants in Gram-positive bacteria using the secreted Staphylococcal nuclease as a reporter. J. Bacteriol. **176:** 5135-5139.

Miksch, G., Bettenworth, F., Friehs, K., Flaschel, E., Saalbach, A. and Nattkemper, T.W. (2006). A rapid reporter system using GFP as a reporter protein for identification and screening of synthetic stationary-phase promoters in *E. coli*. Appl. Microbiol. Biotechnol. **70**: 229-236.

Ostanin, K., Harms, E.H., Srevis, P.E., Kuciel, R., Zhou, M.M. and Van Etten, R.L. (1992). Overexpression, site-directed mutagenesis, and mechanism of *Escherichia coli* acid phosphatase. J. Biol. Chem. 267: 22830-22836.

Ou, Z., Felts, R.L., Reilly, T.J., Nix, J.C. and Tanner, J.J. (2006). Crystallization of recombinant *Haemophilus influenzae e*(P4) acid phosphatase. Acta Cryst. **62**: 464-466.

Poquet, I., Ehrlich, S.D. and Gruss, A. (1998). An export-specific reporter designed for Gram-positive bacteria: Application to *Lactococcus lactis*. J. Bacteriol. **180**: 1904-1912.

Serrano-Heras, G., Salas, M. and Bravo, A. (2005). A new plasmid vector for regulated gene expression in *Bacillus subtilis*. Plasmid **54**: 278-282.

Thaller, M.C., Scippa, S. and Rossolini, G.M. (1998). Conserved sequence motifs among bacterial, eukaryotic, and archaeal phosphatases that define a new phosphohydrolase superfamily. Prot. Sci. **7**: 1647-1652.