Development of *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

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A phosphatase secreted by *Staphylococcus aureus* 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, 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 (*sapS*) gene 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 the host strains. 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

1 protease promoter in *B. subtilis* and the *B. halodurans* σ^D promoter in *B. halodurans*.
2 This is the first report on the development of a Class C acid phosphatase gene as a
3 reporter gene with the advantage of being able to function in both Gram-positive and
4 Gram-negative host strains.
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6 **INTRODUCTION**
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8 Methods for the direct measurement of gene expression include mRNA detection using
9 polynucleotide probes (Northern blots) or reverse transcriptase-polymerase chain reaction
10 (RT-PCR) methods, as well as protein detection methods using antibodies (Western
11 blots) or biological activities (Wood et al., 1995; Ding and Cantor, 2004). However,
12 these methods are in many cases time-consuming and costly. Reporter genes provide an
13 alternative method of genetic analysis that is faster and more convenient. Typically,
14 reporter genes encode proteins that have a unique enzymatic activity or that are otherwise
15 easily distinguishable from the mixture of intra- and extracellular proteins (Biran, 1994;
16 Schenborn and Groskreutz, 1999). They have frequently been used to identify regulatory
17 sequences, to monitor gene expression and function, and to characterize promoter
18 strength and regulation (Pedraza-Reyes et al., 1994; Wang et al., 2004; Serrano-Heras et
19 al., 2005; Koga et al., 2006).
20
21 The choice of a reporter system is determined by a number of important criteria. These
22 include the absence of similar activities to the reporter protein in the host organism and
23 the availability of simple, rapid and sensitive methods for the qualitative and quantitative

1 assay of reporter protein activity. These methods should preferably allow assaying of the
2 reporter protein activity in the presence of cellular components, thus obviating the need
3 for purification steps prior to assay (Jefferson, 1987; Naylor, 1999). The most widely
4 used reporter systems employ genes encoding β-galactosidase (*lacZ*) (Poyart and Trieu-
5 Cuot, 1997; Talukder et al., 2005), chloramphenicol acetyltransferase (*cat*) (Palmano et
6 al., 2001; Cao et al., 2001) and different sugar hydrolases, e.g. β-glucuronidase (*gus*)
7 (Jefferson et al. 1986; Kim et al., 2006). Although these reporter systems are convenient
8 tools for semi-quantitative plate-based assessment of promoter activities, more accurate
9 quantification of promoter strength usually requires enzymatic assays, which typically
10 involve bacterial cell disruption and addition of a substrate to drive the enzymatic
11 reaction followed by measurement of the optical density (Biran, 1994). Another group of
12 reporter systems is based on the emission of light (Wood, 1998; Southward and Surette,
13 2002). In addition to the wild-type green fluorescent protein (GFP) from *Aequorea*
14 *victoria*, many derivatives of GFP have been produced and subsequently used to monitor
15 promoter activity in both in the laboratory and in natural environments (Southward and
16 Surette, 2002; Serrano-Heras et al., 2005; Chary et al., 2005). However, naturally
17 occurring fluorescence can lead to high background levels during *in vitro* and *in vivo*
18 measurements. Alternative strategies have thus involved the luciferase-encoding *luxAB*
19 genes, typically derived from *Vibrio fischeri*, *Vibrio harveyi* and *Photorhabdus*
20 *luminescens* (Kirchner, 1989; Meighen, 1991), and more recently the synthetic
21 *luxCDABE* operon which alleviates the requirement for addition of an exogenous
22 aldehyde substrate in the light emission reaction (Greer and Szalay, 2002; Applegate et
23 al., 1998).

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 (Perez-Arellano and Perez-Martinez, 2003; Janatova et al., 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), lichinase from *Clostridium thermocellum* (Piruzian et al., 2002), and α-galactosidase from *Saccharopolyspora erytraea* (Post and Luebeke, 2005).

We have previously isolated and characterized a novel class C non-specific acid phosphatase secreted by *Staphylococcus aureus* strain 154 (Du Plessis et al., 2002). The enzyme, designated SapS, encoded by *sapS*, is a stable monomeric protein of moderate size (296 amino acids, 30 kDa), which undergoes proteolytic cleavage of the N-terminal 31-amino-acid signal peptide to yield the mature protein. Based on its moderate size and the ease by which enzymatic activity tests may be performed (Du Plessis et al., 2002), 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 Gram-negative (*E. coli*), as well as in mesophilic and moderately thermophilic Gram-positive (*B. subtilis*

1 and *B. halodurans*, respectively) hosts. *E. coli* and members of the species *Bacillus* are
2 the most frequently used prokaryotes for the production of heterologous proteins
3 (Westers *et al*., 2004, Lam *et al*., 1998) and were therefore included in this study to
4 evaluate the *sapS* reporter system. The use of the reporter gene was evaluated in *B.*
5 *halodurans* since it harbours the *lac* operon (Ikura and Horikoshi, 1979), and the
6 commonly used *LacZ* reporter system can therefore not be used in this host organism.
7 Furthermore, *B. halodurans* is currenly being evaluated as a surface display expression
8 system (Crampton et al., 2007).
9

10 **MATERIALS AND METHODS**

11 **Bacterial strains and growth conditions**. *E. coli* DH10B (F$^-$ *mcr*A Δ(*mrr-hsd*RMS-
12 *mcr*BC) (φ80d*lac*ZΔM15) Δ*lac*X74 *end*A1 *rec*A1*deo*R Δ(*ara-leu*)7697 *ara*D139 *gal*U
13 *gal*K *nup*G *rps*L λ$^-$), obtained from Invitrogen, was used as intermediary cloning host.
14 Expression studies were done in *E. coli* CU1867, a BL21 (DE3) strain with the
15 chromosomal acid phosphatase *appA* gene disrupted (Ostanin et al., 1992), *B. subtilis* 154
16 (Δ*apr*, Δ*npr*, amy, spo) (Quax and Broekhuizen, 1994) and *B. halodurans* BhFC04
17 (Δ*wprA,* Δ*hag*) (Du Plessis, PhD thesis). *E. coli* and *B. subtilis* were cultured at 37°C in
18 Luria-Bertani medium (LB: 0.5% [w/v] yeast extract, 1% [w/v] tryptone, 1% [w/v] NaCl;
19 pH 7). When appropriate, *E. coli* growth media were supplemented with ampicillin (100
20 μg/ml), chloramphenicol (20 μg/ml) or erythromycin (300 μg/ml), and *B. subtilis* growth
21 media were supplemented with chloramphenicol (5 μg/ml) or erythromycin (10 μg/ml).
22 *B. halodurans* was grown at 37°C in LB medium (pH 8.5) and chloramphenicol (5 μg/ml)
23 was added when appropriate.

Recombinant DNA techniques. Plasmid DNA was extracted using 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 et al. (1989). Enzymes were obtained from Fermentas (St. Leon-Rot, Germany) and Roche Diagnostics (Mannheim, Germany) and were used according to the manufacturer's 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 et al. 1976) and *B. halodurans* (Crampton et al., 2007). PCR was performed using 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 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.

1 **Plasmids.** Plasmid pNW33N, an *E. coli-Bacillus-Geobacillus* shuttle vector obtained
2 from the *Bacillus* Genetic Stock Centre (Ohio USA) and pMG36e, a *Lactocccus*
3 expression vector (van de Guchte *et al.*, 1989), which also replicates in *E. coli* and *B.*
4 *subtilis*, was used to express the *S. aureus* acid phosphatase gene (*SapS*) in the Gram-
5 positive and Gram-negative host strains. In all instances, except for pMG36e-SapS,
6 pNW33N served as genetic backbone into which different transcriptional and
7 translational fusions were inserted to evaluate the *saps* gene as a reporter (Fig. 1). The
8 SapS enzyme was processed differently in *E. coli* and *Bacillus* stains (31 and 43 N-
9 terminal amino acids deleted, respectively, to produce the mature SapS protein).
10 Translational fusions were therefore made with both deletions to determine whether the
11 truncated gene (Δ43) could be used as a reporter in *E. coli* and *Bacillus* strains.
12 Oligonucleotides used in this study are listed in Table 1. *S. aureus* strain 154
13 chromosomal DNA was used for amplification of the *SapS* gene and its derivatives. *B.*
14 *halodurans* BhFC04 chromosomal DNA was used as template for the PCR amplification
15 of the β-glucanase and alkaline protease promoter and signal sequences and the σ^D
16 promoter. The SPO2 promoter was obtained from plasmid pPL608 as a 300bp *Eco*RI
17 DNA fragment (Schoner et al., 1983). The following vectors were constructed:
18 pNW33-SapS: The 1.140-kb full-length *sapS* gene was PCR-amplified using primers
19 Sap-F/ Sap-R1.
20 pNW33N1: The 227-bp β-glucanase promoter (P*gluc*) fragment was PCR-amplified
21 using primers Glu-F/Glu-R and the 888-bp *sapS* gene fragment using primers Δ_{ATG}Sap-
22 F1/Sap-R2.

1 pNW33N2: The 315-bp β-glucanase promoter and signal peptide (P*gluc*+sp) was PCR-
2 amplified using primers Glu-F/GluS-R and the 798-bp Δ_{31}*sapS* DNA fragment using
3 primers Δ_{31}Sap-F/Sap-R3.
4 pNW33N3: The 762-bp Δ_{43}*saps*S fragment was PCR-amplified using primers Δ_{43}Sap-
5 F/Sap-R3 and ligated to the 315-bp β-glucanase promoter and signal peptide (P*gluc*+sp)
6 from pNW33N2.
7 pNW33N4: The 280-bp alkaline protease promoter (P*apr*) was PCR-amplified using
8 primers Apr-F1/Apr-R and the 888-bp *sapS* DNA fragment using primers Δ_{ATG}Sap-
9 F2/Sap-R2.
10 pNW33N5: The 327-bp alkaline protease promoter and signal peptide (P*apr*+sp) was
11 PCR-amplified using primers Apr-F2/AprS-R and ligated to the 798-bp Δ_{31}*sapS* fragment
12 from pNW33N2.
13 pNW33N6: The 327-bp alkaline protease promoter and signal peptide (P*apr*+sp) from
14 pNW33N5 was ligated to the 762-bp Δ_{43}*sapS* DNA fragment from pNW33N3.
15 pNW33N7: The SPO2 promoter was cloned into pNW33N and the plasmid designated
16 pNWSpo. The 891-bp full-length *sapS* gene, inclusive of its ATG initiation codon, was
17 amplified by PCR using primers $_{ATG}$Sap-F/Sap-R2 and ligated to pNWSpo to generate
18 pNW33N7.

19 pNW33N8: The 230-bp sigma D promoter (Pσ^{D}) fragment was PCR-amplified using
20 primers Sig-F/Sig-R. The 888-bp *sapS* gene fragment, lacking the ATG initiation codon
21 (Δ_{ATG}*sapS*), was generated by PCR using primers Δ_{ATG}Sap-F3/Sap-R4.

22 pNW33N9: The 905-bp *sapS* gene, including the putative ribosome binding site (RBS),
23 was obtained by PCR using primers RBS-Sap-F/Sap-R3 and ligated to pNW33N. The

1 resulting plasmid pNW33N9 harboured the vector-borne *lacZ* promoter (P_{lacZ})
2 translationally fused to the *sapS* reporter gene.

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

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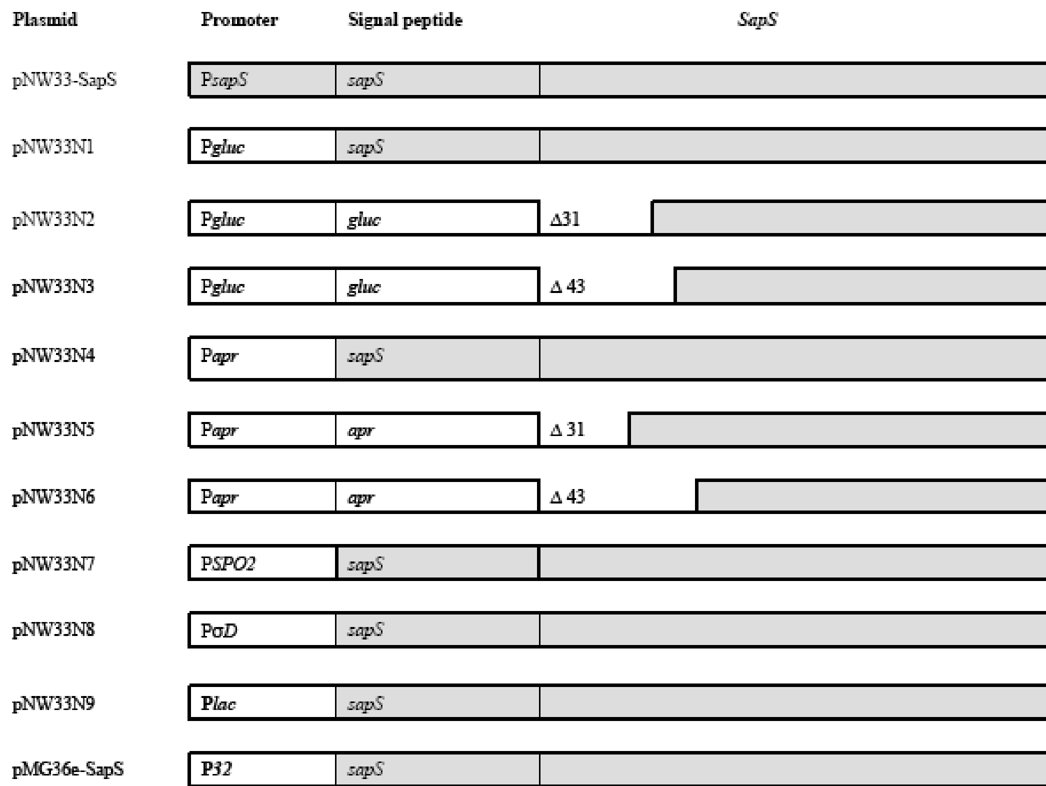


FIG. 1. Schematic presentation of the vector constructs harbouring the *S. aureus sapS* gene for expression in *E. coli* Cu1867, *B. subtilis* 154 and *B. halodurans* BhFC04. *Abbreviations:* P*gluc, B. halodurans β*-glucanase promoter; P*apr*, *B. halodurans* alkaline protease promoter; P*SPO2*, *Bacillus* temperature phage SPO2 promoter; Pσ^{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; Δ_{31}*sapS, sapS* gene fragment lacking the N-terminal signal peptide of 31 amino acids; Δ_{43}*sapS, sapS* lacking the N-terminal signal peptide of 43 amino acids identified in *B. haldurans* BhFC04.

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).

1 Protein samples from the cultures were prepared as described by Van der Vaart *et al.*
2 (1997) with the following modifications. For cell fractionation, 40 ml of the respective
3 cultures was harvested after a 15-min centrifugation at 12 000 × *g* at 4°C. The cell-free
4 supernatants (extracellular fraction) were retained and the proteins precipitated with ice-
5 cold acetone prior to being suspended in 0.1 M sodium acetate buffer (pH 5). The cell
6 pellets were washed once with sterile distilled water, resuspended in 5 ml 0.1 M sodium
7 acetate buffer (pH 5) and sonicated on ice for 20 min using a Model HD2070 Sonoplus
8 Ultrasonic Homogenizer (Bandelin Electronic, Berlin, Germany). The cell lysate was
9 clarified by centrifugation at 12 000 × *g* for 15 min and the supernatant, considered the
10 intracellular fraction, was recovered. The pellet (cell wall fraction) was washed once
11 with sterile distilled water and resuspended in 5 ml 0.1 M sodium acetate buffer (pH 5).
12 Whole cell protein samples were prepared by harvesting the cells from 5 ml of the
13 respective cultures by centrifugation, as described above. The cell pellets were washed
14 with sterile distilled water and resuspended in 5 ml 0.1 M sodium acetate buffer (pH 5).
15 The protein concentration of samples was determined by the method of Bradford (1976),
16 using the BioRad protein assay kit (BioRad, Hercules, CA), with bovine serum albumin
17 as standard.
18 **Qualitative, quantitative and zymographic detection of acid phosphatase activity.**
19 Colonies were grown on LB agar supplemented with the appropriate antibiotic and
20 screened for acid phosphatase activity by flooding the surface with 0.1 M sodium acetate
21 (pH 5), containing 0.1% α-naphtyl phosphate (Roche Diagnostics) and 0.2% Fast Garnett
22 GBC salt (Sigma-Aldrich, Aston Manor, South Africa). Acid phosphatase-positive
23 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 ρNPP 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 1 M NaOH and the liberated ρ-nitrophenol (ρNP) was measured at 405 nm. The extinction coefficient of ρ-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 ρ-nitrophenol per min under the assay conditions. All assays were performed in triplicate and the results are expressed as means ± standard deviation (SD).

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 (Bio-Rad) 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 GBG salt (Gabriel, 1971). Phosphatase activity was indicated by the presence of black-stained bands.

Amino-terminal amino acid sequencing. Amino-terminal amino acid sequencing was determined as described previously (Du Plessis et al. 2002).

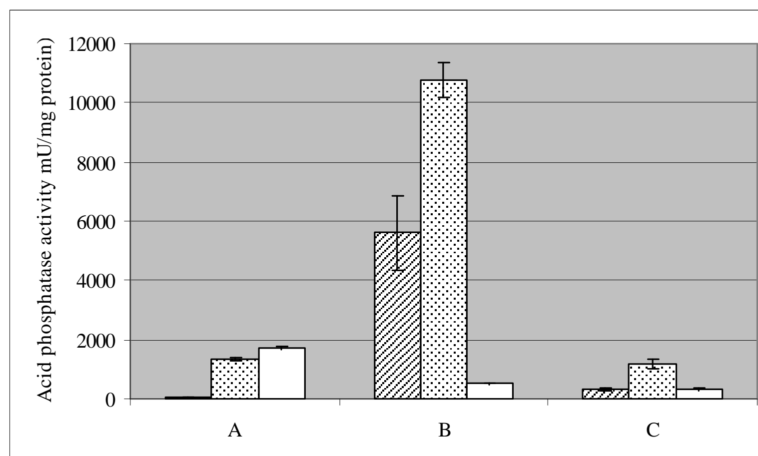
TABLE 1. Oligonucleotides used in this study

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 *sapsS* including initiation ATG
Δ_{ATG}Sap-F1	GGCCTGCAGAATAAAATTTCAAAG	*Pst*I, 5' end of *sapS* lacking initiation ATG
Δ_{ATG}Sap-F2	GGCGTGGACAATAAAATTTCAAAG	*Sal*I, 5' end of *sapS* lacking initiation ATG
Δ_{ATG}Sap-F3	CAGGCATGCAATAAAATTTCAAAGTATATTGC	*Sph*I, 5' end of *sapS* lacking initiation ATG
Δ_{ATG}Sap-F4	GGCCTGCAGGAATAAAATTTCAAAG	*Pst*I, 5' end of *sapS* lacking initiation ATG
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

1 **RESULTS**

2 **Expression of the *S. aureus sapS* gene in *E. coli*, *B. subtilis* and *B. halodurans*.**
3 Plasmid pNW-SapS, harbouring the promoter and coding region of the sapS preprotein
4 (signal sequence and mature protein) was transformed into *E. coli* CU1867, *B. subtilis*
5 154 and *B. halodurans* BhFC04. The acid phosphatase enzyme was successfully
6 expressed in all three host strains as determined with *in vitro* enzyme assays (Fig. 2) and
7 zymography (Fig. 3). No acid phosphatase activity was detected with the *in vitro* enzyme
8 assays of the host strains harbouring the pNW33N vector. In contrast to *Bacillus* sp.
9 where the acid phosphatase activity was mostly cell-asssociated, in *E. coli* it was
10 intracellular (Fig. 2).



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13 FIG. 2. Extracellular, whole cell and intracellular *in vitro* acid phosphatase activity results
14 of the host strains harbouring pNW33-SapS (A) *E. coli* CU1867. (B) *B. subtilis* 154. (C)
15 *B. halodurans* BhFC04.

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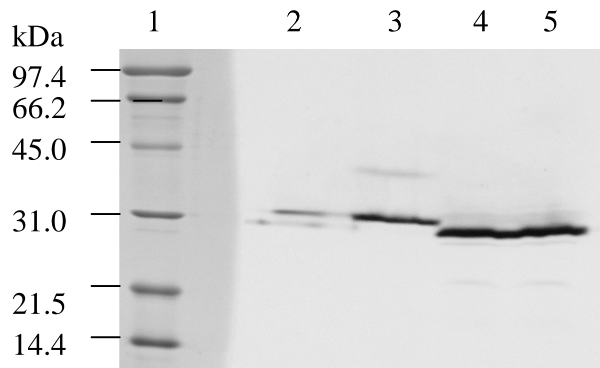

FIG. 3. Zymographic analysis of *S. aureus* sapS acid phosphatase activity in *E. coli* CU1867 (pNW33-SapS), *Bacillus* 154 (pNW33-SapS), and *S. aureus* (wild type). Lane 1, low–range protein molecular weight marker (BioRad); lane 2, *S. aureus* supernatant fraction ; lane 3, *E. coli* CU1867 (pNW33-SapS)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

Zymographic analysis of the cell wall fractions of the three host strains harbouring pNW33N-SapS was performed. 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 mass 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 mass of the acid phosphatase activity bands obtained for the *B. subtilis* and *B. halodurans* whole cell fractions (Fig. 3, lanes 4 and 5) were lower than that obtained for *E. coli* (Fig. 3, lane 3). In order to determine if the protein was processed differently in the Gram-positive *Bacillus* strains, 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 twelve amino acids shorter than the native *S. aureus* SapS protein N-terminal sequence (Du Plessis et al., 2002). Consequently, the coding regions of the *S. aureus* 154 and *B. halodurans* BhFC04 mature SapS proteins respectively, were included in the vector construction.

Evaluation of heterologous promoters/ and signal sequences using *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. 1) was determined qualitatively with the plate screen assay (Fig. 4A) and quantitatively with in vitro enzyme assays (Fig. 4B).

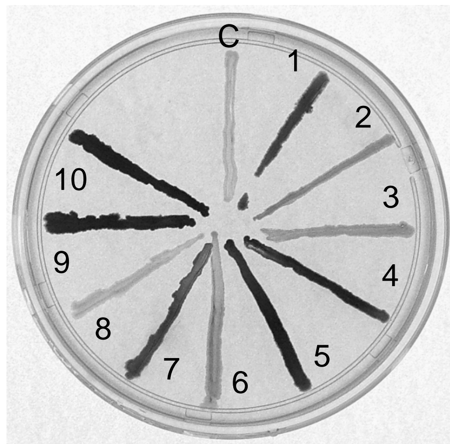

FIG. 4A. Plate screen showing acid phosphatase activity of 24 h cultures of *E. coli* CU1867 harbouring the various constructs. Control (c), pNW33N; 1, pNW33N1; 2, pNW33N2; 3, pNW33N3; 4, pNW33N4; 5, pNW33N5; 6, pNW33N6; 7, pNW33N7; 8, pNw33N8; 9, pNW33N9 and 10, pNW33-SapS.

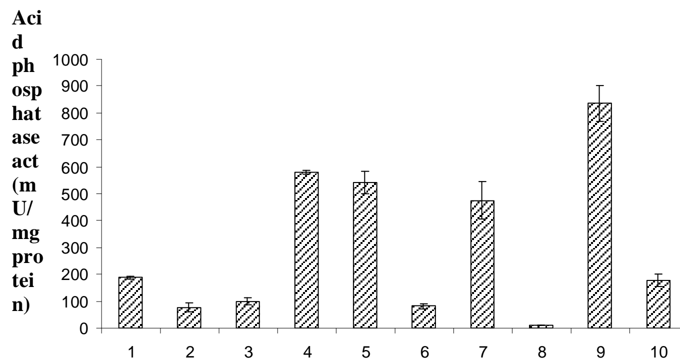

FIG. 4B**.** Whole cell *in vitro* acid phosphatase activity results of *E. coli* CU1867 24 h cultures harbouring different promoter constructs. 1, pNW33N1; 2, pNW33N2; 3, pNW33N3; 4, pNW33N4; 5, pNW33N5; 6, pNW33N6; 7, pNW33N7; 8, pNW33N8; 9, pNW33N9; 10, pMG36e-SapS.

E. coli CU1867 harbouring the pNW33N vector showed no acid phoshatase activity after activity staining (Fig. 4A). The host strain harbouring pNW33-SapS stained pitch black indicating high levels of enzyme activity. Varying levels of brown to black colour development was detected for the host strain harbouring the heterologous promoter-reporter gene constructs pNW33N1-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.

In order to quantify the acid phosphatase activity results the production levels of the *sapS* enzymatic reporter gene from the various heterologous promoters and signal sequences were monitored after 24 h hours by *in vitro* acid phosphatase activity assays. In *E. coli* CU1867 the highest enzyme activity (835 mU/mg protein) was obtained for P*lacZ* ligated to the reporter gene (pNW33N9), followed by (589 mU/mg) obtained for the P*apr*-

reporter gene construct pNW33N4 and (541 mU/mg) obtained for the P*apr+sp*-reporter gene construct (pNW33N5). The activity measured for *Papr+sp* ligated to the truncated reporter gene (pNW33N6*)* was approximately 5-fold less (81 mU/mg) protein. Lower levels of phosphatase activity (189mU/mg) was obtained for the P*gluc*-reporter gene construct pNW33N1 when compared to the activity (589 mU/mg) obtained for P*apr*-reporter gene construct pNW33N4. 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 177mU/mg protein. The enzyme activity determined for the *Bacillus* temperature phage P*SPO2* ligated to the reporter gene (pNW33N7) was 475 mU/mg protein. In accordance with the plate screen assay, no activity was detected from the *B. halodurans* Pσ^{D}-reporter gene construct (pNW33N8) in *E. coli* CU1867. Low levels (52mU/mg) of extracellular enzyme activity was detected for *E. coli* harbouring the P*lacZ* promoter (construct pNW33N9) after 24h. 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.*

Evaluation of heterologous promoters/ and signal sequences using *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. 5A) and quantitatively (Fig. 5B).

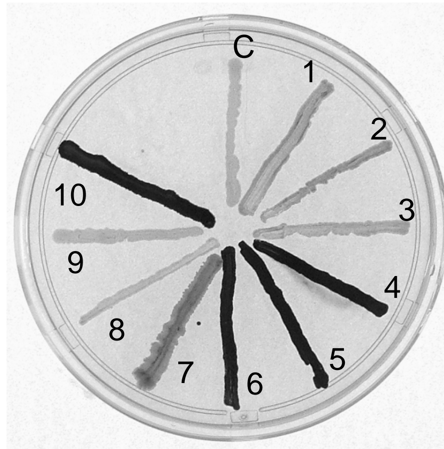

FIG. 5A. Plate screen showing acid phosphatase activity of 24 h cultures of *B. subtilis* strain 154 harbouring the various constructs. Control (c), pNW33N; 1, pNW33N1; 2, pNW33N2; 3, pNW33N3; 4, pNW33N4; 5, pNW33N5; 6, pNW33N6; 7, pNW33N7; 8, pNW33N8; 9, pNW33N9 and 10, pNW33-SapS.

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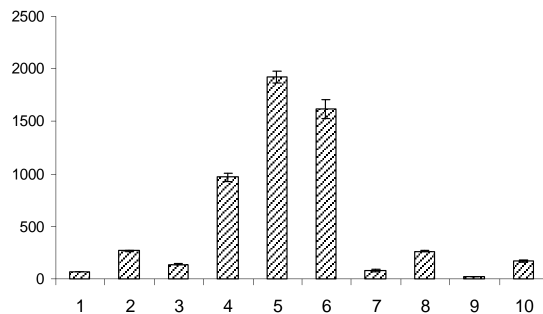

7 FIG. 5B. Whole cell *in vitro* acid phosphatase activity results of *B. subtilis strain* 154 24
8 h cultures harbouring the different promoter constructs. 1, pNW33N1; 2, pNW33N2; 3,
9 pNW33N3; 4, pNW33N4; 5, pNW33N5; 6, pNW33N6; 7, pNW33N7; 8, pNW33N8; 9,
10 pNW33N9; 10, pMG36e-SapS.

11 *B. subtilis* 154 harbouring pNW33N showed no acid phosphatase activity with the plate
12 screen assay (Fig. 5A). The host strain harbouring pNW33-SapS stained pitch black
13 indicating high levels of enzyme activity. As for the *E. coli* CU1867 host strain varying
14 levels of brown to black colour development was observed for the heterologous
15 promoter-reporter gene constructs pNW33N1-pNW33N9. *B. subtilis* 154 harbouring the
16 P*apr*-reporter gene construct pNW33N4, the P*apr+sp*-mature reporter gene construct
17 pNW33N5 and the P*apr+sp*- truncated mature reporter gene construct pNW33N6 stained
18 black indicating high levels of reporter gene activity. No black colour development was
19 detected for *B. subtilis* (pNW33N8) indicating that Pσ^{D} ligated to the reporter gene was
20 not expressed in *B. subtilis* 154. Similarly no activity was observed for *B. subtilis*
21 (pNW33N9) showing that the *E. coli* P*lacZ*-reporter gene construct was not expressed in
22 the Gram-positive host strain.

1 In order to quantify the acid phosphatase activity results in *B. subtilis* 154 the production
2 levels of the *sapS* enzymatic reporter gene from the various heterologous promoters and
3 signal sequences were monitored after 24 h hours by *in vitro* acid phosphatase activity
4 assays. The highest enzyme activity (1923mU/mg protein) was obtained for the
5 *Papr+sp*-mature reporter gene construct pNW33N5, followed by (1620mU/mg protein)
6 obtained for the *Papr+sp*-truncated mature reporter gene construct pNW33N6 and
7 (968mU/mg protein) obtained for the P*apr*-reporter gene construct pNW33N4. Lower
8 levels of phosphatase activity (267 mU/mg) was obtained for the P*gluc*-reporter gene
9 construct pNW33N1 when compared to the activity (968 mU/mg) obtained for P*apr*-
10 reporter gene construct pNW33N4. The in vitro assay results correlated with the plate
11 screen assay i.e. the recombinant strains harbouring the β-glucanase promoter reporter
12 gene constructs (pNW33N1, pNW33N2 and pNW33N3) respectively stained lighter than
13 the recombinant strains harbouring the alkaline protease promoter constructs (pNW33N4,
14 pNW33N5 and pNW33N6). As for *E. coli* this result indicated that the *B. halodurans*
15 BhFC04 alkaline protease promoter is a stronger promoter than the β-glucanase promoter.
16 The P32 lactococcal promoter was also effectively recognized since 166mU enzyme
17 activity was measured with the pNW33N10 construct. No acid phosphatase activity was
18 detected for the P*lacZ*-reporter gene construct (pNW33N9) in the host strain. This is not
19 surprising since *Bacillus* is very stringent in its recognition of promoters (Patel et al.,
20 2003). Extracellular acid phosphatase activity was detected for the P*apr+sp*- mature
21 reporter gene construct pNW33N5 (60 mU/mg protein) and the P*apr+sp*- truncated
22 mature reporter gene construct pNW33N6 (44 mU/mg protein). No extracellular activity

1 was detected for any of the other constructs evaluated in this host strain (results not
2 shown).

3 **Evaluation of heterologous promoters/ and signal sequences using *sapS* as a reporter**
4 **gene in and *B. halodurans* BhFC04.** The acid phosphatase activity of *B. haldodurans*
5 BhFC04 harbouring the expression vector constructs was determined qualitatively (Fig.
6 6A) and quantitatively (Fig. 6B).
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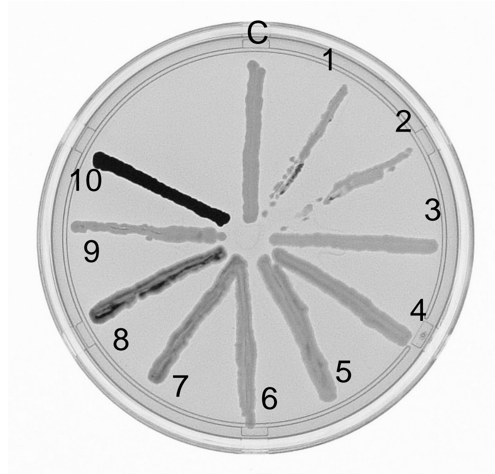

15 FIG. 6A. Plate screen showing acid phosphatase activity of *B. halodurans* BhFC04 24 h
16 cultures harbouring the various constructs. Control (c), pNW33N; 1, pNW33N1; 2,
17 pNW33N2; 3, pNW33N3; 4, pNW33N4; 5, pNW33N5; 6, pNW33N6; 7, pNW33N7; 8, \
18 pNw33N8; 9, pNW33N9 and 10, pNW33-SapS.

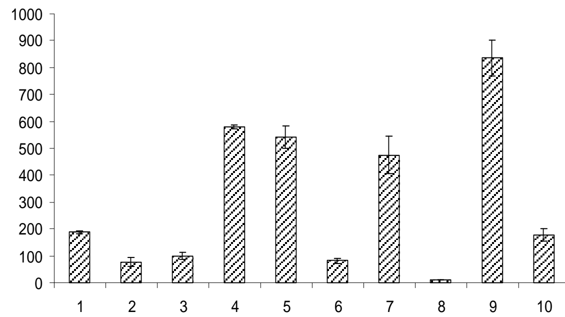

FIG. 6B. Whole cell *in vitro* acid phosphatase activity results of *B. halodurans* BhFC04 24 h cultures harbouring the different promoter constructs. 1, pNW33N1; 2, pNW33N2; 3, pNW33N3; 4, pNW33N4; 5, pNW33N5; 6, pNW33N6; 7, pNW33N7; 8, pNW33N8; 9, pNW33N9.

B. halodurans BhFC04 harbouring pNW33N showed no acid phosphatase activity with the plate screen assay (Fig. 6A). The host strain harbouring pNW-SapS stained black indicating high expression levels of the *SapS* gene from its own promoter and signal sequence. *E. coli* CU1867 harbouring the $P\sigma^{D}$-reporter gene construct (pNW33N8) stained light brown with the plate screen assay. No colour was detected for the host strain harbouring any of the other promoter-reporter gene constructs (Fig. 6A).

Of the heterologous promoter-reporter gene constructs the best activity (18mU/mg 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.

1 **DISCUSSION**

2 In this study we evaluated the *S. aureus* acid phosphatase SapS enzyme as a reporter for
3 promoter and signal sequence characterization in *E. coli* CU1867, *B. subtilis* 154 and *B.*
4 *halodurans* BhFC04. Initial studies focused on determining whether the *SapS* gene was
5 expressed in the three host strains from its own promoter and signal sequence. The SapS
6 enzyme was found to be active after acetone precipitation which made it possible to
7 concentrate the enzyme from protein extracts. A zymogram of active acid phosphatase
8 led to the determination of the molecular masses of the *sapS* gene expressed in the three
9 different host strains and showed that the enzyme was processed differently in *E. coli* as
10 compared to *B. subtilis* and *B. halodurans*. The precursors of secreted proteins from
11 Gram-positive bacteria generally have longer and more hydrophobic signal peptides than
12 those of gram-negative bacteria (von Heijne, 1990). In *S. aureus* alanine is at the -1
13 position and lysine at the +1 position of the signal peptide of the *sapS* gene as determined
14 with N-terminal sequencing. When applied to *B. halodurans* BhFC04 alanine was found
15 to be at the -1 position and serine at the +1 position in the signal peptide of the *sapS* gene.
16 In *B. subtilis* alanine is the predominant residue (>90%) at the -1 and -3 position of the
17 *Bacillus* signal peptides (Nagarayan, 1993). Therefore it can be concluded that the *sapS*
18 gene was cleaved at the same position as in *B. subtilis.*

19 The suitability of the *SapS* gene as an expression reporter system was evaluated by the
20 ligation of a range of heterologous promoters which included boh well known as well as
21 newly isolated promoters and signal sequences. Expression/secretion ability of the
22 transcriptional and translational fusion products were estimated by acid phosphatase
23 activity determination. Since Vasantha et al., (1992) reported the successful use of the *B.*

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/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 its ability to express/secrete the reporter gene. It has been reported that the *Bacillus* temperature 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 origin in lactococci, *B. subtilis* and *E. coli* (Van de Guchte et al., 1989). *SapS* gene expression from this promoter was therefore 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 the host strains. 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 when compared to non-fused controls (Strathdee et al., 2000). Piruzian et al., (2002) reported a decrease in thermostable lichinase (LicB) and Gus activity for cells expressing the fusion constructs when 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 *sapsS* gene sequence in all three the host strains, showing the versatility of the *sapS* gene as an enzymatic reporter gene. The fact that very low levels of extracellular SapS activity was detected for the constructs in the three host strains and the activity was cell wall-associated makes it unsuitable for the isolation or evaluation of signal peptides for the extracellular production of heterologous proteins.

From the range of promoters evaluated using this system the strongest promoter for the expression of heterologous proteins were 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* temperature 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), we found in our study that the *B. subtilis* temperature 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 the P32 promoter.

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