Molecular cloning, expression and characterization of two potential antibacterial fusion hydrophobins in *Pichia pastoris*

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Biofilms constitute a successful protection mechanism for planktonic bacterial cells to survive in hostile environments. To date, biofilm-associated infections in medical devices represent a major cause of morbidity and mortality among patients. As a potential candidate for anti-biofilm therapy, fungal hydrophobins provide new solutions to manipulate the physical and chemical properties of surfaces, which in turn may give protection against bacterial colonization. However, in practice, native hydrophobin coatings generally have no impact on bacterial surface colonization, because of the lack of being antibacterial by these fungal proteins themselves. The aim of this study was to explore the feasibility of using recombinant fusion hydrophobins to control bacterial growth.

In this study, the class I hydrophobin *hgfI* gene isolated from the edible mushroom *Grifola frondosa* was in frame fused with two antimicrobial peptide genes (*bac8c* and *p11-5*), respectively, and subsequently cloned into the corresponding expression vectors with a view to obtain two recombinant fusion hydrophobins, *Bac8c-HGFI* and *P11-5-linker-HGFI*. These two chimeric genes were separately expressed in *Pichia pastoris* under the regulation of alcohol oxidase 1 promoter. SDS-PAGE and immunoblot analyses confirmed that these two fusion proteins were successfully expressed and secreted into the culture medium. Minimum inhibitory concentration (MIC) test demonstrated that the highly active antimicrobial peptide *Bac8c* became inactivated when it was fused with the hydrophobin HGFI. Interestingly, the hydrophobin HGFI gained an acquired antibacterial nature when it was fused with the antimicrobial peptide *P11-5* through a 10-mer flexible polypeptide linker, with the MIC of 100 μg/ml against *Escherichia coli*.

To the best of my knowledge, this study presents the first heterologous expression of an antibacterial fusion hydrophobin in *P. pastoris*. This finding in combination with surface modification mediated by hydrophobin may broaden the current approaches used for anti-biofilm therapies.

**Avainsanat – Nyckelord**

Hydrophobin; antimicrobial peptide; fusion expression; polypeptide linker; self-assembly; *Pichia pastoris*

**Additional information**

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PREFACE

This Master’s thesis project in molecular biology was carried out at the Department of Food and Environmental Sciences at the University of Helsinki from May 2014 to December 2014. This study was under the supervision of Prof. Per Saris in his laboratory.

Here I would like to express my sincere gratitude to my supervisor Prof. Per Saris for providing instructions and comments, and offering strong support during the entire thesis work. I also would like to thank Prof. Marina Heinonen and the program coordinator Tiina Naskali for their support during my study.

My special thanks are given to my beloved parents and friends for their consistent encouragement and support through all these years.
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>ABS</td>
<td>aqueous biphasic system</td>
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<tr>
<td>α-MF</td>
<td>alpha-mating factor of <em>Saccharomyces cerevisiae</em></td>
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<td>AMP</td>
<td>antimicrobial peptide</td>
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<td>Amp</td>
<td>ampicillin</td>
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<td>AOX</td>
<td>alcohol oxidase</td>
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<td>AP</td>
<td>alkaline phosphatase</td>
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<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
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<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>ATPS</td>
<td>aqueous two-phase system</td>
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<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3-indolyl phosphate</td>
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<td>BMG</td>
<td>buffered minimal glycerol</td>
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<td>bp</td>
<td>base pair</td>
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<td>Da</td>
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<td>ddH₂O</td>
<td>double-distilled water</td>
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<td>DGGE</td>
<td>denaturing gradient gel electrophoresis</td>
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<td>DNA</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>EG</td>
<td>endogluanase</td>
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<tr>
<td>Fc</td>
<td>fragment crystallization region</td>
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<td>FESEM</td>
<td>field emission scanning electron microscopy</td>
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<tr>
<td>H+L</td>
<td>heavy + light chains (antibody)</td>
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<tr>
<td>HAMBI</td>
<td>culture collection center, University of Helsinki</td>
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<td>HBOEC</td>
<td>human blood outgrowth endothelial cells</td>
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<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
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<tr>
<td>kbp</td>
<td>kilobase pair</td>
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<tr>
<td>LB</td>
<td>Luria Bertani</td>
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<td>Acronym</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>MIC</td>
<td>minimum inhibitory concentration</td>
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<tr>
<td>NAD⁺/NADH</td>
<td>nicotinamide adenine dinucleotide</td>
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<tr>
<td>NBT</td>
<td>nitro blue tetrazolium chloride</td>
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<tr>
<td>Ni-NTA</td>
<td>nickle-nitrilotriacetic acid</td>
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<td>OD</td>
<td>optical density</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PEG</td>
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<td>RE</td>
<td>restriction endonuclease</td>
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<tr>
<td>RNA</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<tr>
<td>ssDNA</td>
<td>single-stranded deoxyribonucleic acid</td>
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<td>PAGE</td>
<td>poly acrylamide gel electrophoresis</td>
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<tr>
<td>SOC</td>
<td>super optimal broth with catabolite repression</td>
</tr>
<tr>
<td>T4</td>
<td>T4 bacteriophage</td>
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<tr>
<td>TAE</td>
<td>Tris-acetic acid-EDTA</td>
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<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
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<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>Tris</td>
<td>tris-hydroxymethyl aminomethane</td>
</tr>
<tr>
<td>WCA</td>
<td>water contact angle</td>
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<tr>
<td>XPS</td>
<td>X-ray photoelectron spectroscopy</td>
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<tr>
<td>YPD</td>
<td>yeast extract-peptone-dextrose</td>
</tr>
<tr>
<td>YPDS</td>
<td>yeast extract-peptone-dextrose-sorbitol</td>
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1 INTRODUCTION

Bacterial biofilms are well-organized bacterial cell aggregates on various surfaces, which constitute a successful protection mechanism for planktonic bacterial cells to survive and proliferate in hostile environments (Donlan 2002; Simões et al. 2010). To date, biofilm-associated infections in medical devices represent a major cause of morbidity and mortality among patients (Shirtliff and Leid 2009; Simões et al. 2010; Chen et al. 2013a). As a result, various biofilm control approaches, generally focus on changing the surface characteristics, have been applied to practice (Champ et al. 1987), but often toxic substances are used (Evans et al. 1995; Chen et al. 2013a). The use of biocompatible bacteria-repellent agents such as hydrophobins, fluoropolymers and polydimethylsiloxanes (Rieder et al. 2011; Brady 2000; Krishnan et al. 2008) to prevent bacterial colonization on surfaces, thus negating the use of toxic substances is a new biofilm control strategy over the last fifteen years.

Hydrophobins are a family of small adhesive proteins exclusively produced by filamentous fungi and play a crucial role in fungal growth and development (Wösten and Scholtmeijer 2015; Wessels 1997). Apart from rich in hydrophobic amino acids, all of these fungal proteins possess eight cysteine residues at conserved positions that form four intramolecular disulfide bridges (Linder et al. 2005). Hydrophobins are extremely surface-active proteins; they are able to self-assemble into stable monolayers at hydrophilic-hydrophobic interfaces (Wösten 2001), and thereby altering the surface wettability of solids, from hydrophilic to hydrophobic and vice versa (Wösten and de Vocht 2000; Lugones et al. 1996). Due to these unique properties, many potential applications have been proposed for hydrophobins, including uses as emulsifiers in food products (Cox et al. 2008) and anti-biofilm agents (Rieder et al. 2011).

However, in practice, native hydrophobin coatings generally have no impact on bacterial surface colonization, because of the lack of being antibacterial by these fungal proteins themselves (Rieder et al. 2011). This finding has led to numerous proposals in developing fusion hydrophobins that tethered with antibiotics, specific enzymes or antimicrobial
peptides to avoid or retard bacterial colonization on surfaces (Rieder et al. 2011). As a potential candidate, antimicrobial peptides possess preferable characteristics in comparison with antibiotics, including broad-spectrum antimicrobial activity, rapid onset of cell killing (Hancock and Sahl 2006), and low propensity to cause drug resistance among pathogens (Steckbeck et al. 2014).

The aim of this study was to explore the feasibility of using recombinant fusion hydrophobins to control bacterial growth. In the first part of the thesis, bacterial biofilms and the corresponding control strategies, hydrophobins and the potential applications, antimicrobial peptides, especially antimicrobial peptides (Bac8c and P11-5) used in this study, and the *Pichia pastoris* protein expression system are reviewed. In the second part of the thesis, methods for molecular cloning, heterologous gene expression, protein identification, purification and characterization are described. In the final sections, the antibacterial potential of these recombinant fusion hydrophobins are evaluated and discussed.
2 LITERATURE REVIEW

2.1 Bacterial biofilms

Bacterial biofilms are well-organized bacterial cell aggregates on various surfaces, such as metals, plastics, biological tissues, implant materials and dental surfaces (Donlan 2002), which constitute a successful protection mechanism for planktonic bacterial cells to survive and proliferate in hostile environments (Simões et al. 2010). There is no denying that some bacterial biofilms (e.g. gut flora) are essential to human health, physiology and development, like *Eubacterium* spp., *Bifidobacterium* spp. and *Ruminococcus* spp. (Neufeld et al. 2009). However, under most circumstances, biofilms by human bacterial pathogens on indwelling medical devices, artificial implants and daily care products that are directly in contact with the human body, such as bile stents, urinary catheters, artificial skeletons and joints, and contact lens pose a critical medical problem (Simões et al. 2010; Costerton et al. 2005), which in turn may lead to acute infections and chronic illnesses (Bryers 2008; Bjarnsholt 2013). Many pathogenic bacteria can form biofilms on surfaces, including both gram-positive (e.g. *Listeria monocytogenes*, *Staphylococcus aureus* and *Enterococcus faecalis*) and gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*) bacteria (Chen et al. 2013a).

One well-accepted process of bacterial biofilm development is characterized by 5 stages (Hall-Stoodley et al. 2004) (Figure 1). (1) Reversible attachment of bacteria to surfaces. In this stage, planktonic bacterial cells use a variety of extracellular organelles and sticky membrane proteins for attaching to surfaces, including flagella (Daniels et al. 2004), pili (Sauer and Camper 2001) and stalks (Renner and Weibel 2011). (2) Irreversible attachment of bacteria to surfaces. Here bacteria are embedded within a ‘sticky scaffold’, a matrix of extracellular polymeric substances (EPS) that mainly contain nucleic acids, peptidoglycan, lipids/phospholipids and lipopolysaccharides (Sutherland 2001), and irreversibly cling to surfaces (Flemming and Wingender 2010). (3) Early development of biofilm architectures. Once bacterial cells are settled down on surfaces, they start to proliferate, accumulate and recruit free-floating bacterial cells from the surrounding environment (Stoodley et al. 2002), resulting in the formation of bacterial microcolonies with a diameter of tens or hundreds of
microns (Renner and Weibel 2011). Meanwhile, the EPS matrix acts as a ‘shield’ to protect the encapsulated bacterial cells from adverse factors in the surrounding environment, such as antibiotics, oxidizing agents, ultraviolet radiation (Flemming and Wingender 2010; Simões et al. 2005). (4) Maturation of biofilm architectures. Bacterial cells are further ‘glued’ together by EPS, leading to the formation of complex three-dimensional biofilm structures. For example, water channels that help to distribute nutrients and disposal of waste products within or outside of the biofilm matrix (Stoodley et al. 1994). (5) Detachment and dispersion of bacteria from mature biofilms. Bacterial cells detach from mature biofilms and subsequently disperse into the surrounding environment, where they may act as ‘germs’ for a new round of biofilm development (Costerton et al. 1987; Fey et al. 2010).

**Figure 1.** Schematic diagram of bacterial biofilm development on a surface. (1) Reversible attachment of bacteria to surfaces. (2) Irreversible attachment of bacteria to surfaces. (3) Early development of biofilm architectures (microcolonies). (4) Maturation of biofilm architectures. (5) Detachment and dispersion of bacteria from mature biofilms (Figure adapted from Stoodley et al. 2002).

**Bacterial biofilm inhibitors as potential drugs**

Bacterial cells growing in biofilms are highly resistant to antibacterial agents (Smith 2005; Olson et al. 2002). This resistance is supposed to be due to a lower reproduction rate of bacterial cells within biofilms and the barrier effect of EPS shell (Davies 2003; Lewis 2010; Stewart and Costerton 2001). For example, Ito et al. (2009) found that bacteria *Escherichia coli* showed an increased ampicillin resistance when it was grown as biofilms on an abiotic surface. Usually these biofilm-associated bacterial cells can be between 100-1000 times
more resistant to antibiotics when compared with their planktonic counterparts (Gristina 1987), which makes them difficult to be eradicated with conventional antibiotic therapies (Chen et al. 2013a). In addition, overuses of antibiotics in bacterial biofilm therapies may give a rise to the antibiotic resistance among bacterial pathogens (Weinstein 2001). Antibiotic resistances in *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella pneumoniae* such as multiple beta-lactam antibiotic resistance is one of the most urgent public health problems nowadays (Muir and Weinbren 2010; Yong et al. 2009; Grundmann et al. 2006).

The initial stage of bacterial biofilm development is a surface-dependent reversible step; surface characteristics like wettability (Privett et al. 2011), roughness (Truong et al. 2010) and protein adsorption capacity (Stallard et al. 2012) may play an important role in facilitating bacterial colonization on surfaces. Based on these postulations, a research group at Harvard University developed a novel bacteria-repellent surface coating agent called SLIPS (Slippery-Liquid-Infused Porous Surfaces) (Wong et al. 2011). In the practical study, significant inhibition of *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus* biofilm development was observed on a SLIPS-coated surface, under both static and realistic flow cultivation conditions (Wyss Institute, Harvard University). Weickert et al. (2011) coated plastic bile stents with the recombinant fusion hydrophobin H*Protein A* (from BASF SE), decreased microbial adhesion and serum protein adsorption were observed on these hydrophobin-treated bile stents.

Other attempts have been focused on giving surfaces with an acquired antibacterial activity. This attempt is achieved primarily by incorporating antibacterial agents into/onto surface materials, such as antibiotics (Hickok and Shapiro 2012; Raad et al. 1997; Liu et al. 2012), chemicals (Jaramillo et al. 2012) and antimicrobial peptides (Costa et al. 2011; Tan et al. 2014).
2.2 Hydrophobins

Hydrophobins are a family of small (about 100 amino acids), amphipathic proteins, which are exclusively produced by filamentous fungi (Bayry et al. 2012; Wösten and Scholtmeijer 2015; Linder et al. 2005) and play a crucial role in fungal growth and development (Wösten and Willey 2000; Wessels 1997). These fungal proteins have been regarded as one of the most surface-active proteins known (Linder et al. 2005); they are able to self-assemble into amphipathic monolayers at hydrophobic-hydrophilic interfaces, for example, between air and water (Wösten et al. 1993), water and oil (Wang et al. 2004), hydrophobic surfaces and water, and hydrophilic surfaces and air (Wösten et al. 1994), and thereby altering the surface wettability of solids, from hydrophilic to hydrophobic (Lugones et al. 1996) and vice versa (Wösten et al. 1993) (Figure 2).

Figure 2. Self-assembly of hydrophobins on surfaces. (A) Hydrophobin coating makes the hydrophobic surface hydrophilic (water contact angle [WCA], ranging between 22 and 63°). (B) Hydrophobin coating makes the hydrophilic surface hydrophobic (WCA, 110°). (1) If the WCA is smaller than 90°, the surface is considered as hydrophilic. (2) If WCA is greater than 90°, the surface is considered as hydrophobic. (3) ○ hydrophilic region ● hydrophobic region (Figure adapted from Wösten and de Vocht 2000).

Hydrophobins show very low amino acid sequence homology in general, but all of them contain eight cysteine residues at conserved positions that form four intramolecular disulfide bridges with the conserved pattern (cys1-cys6, cys2-cys5, cys3-cys4, and cys7-cys8) (Kwan et al. 2006; Linder et al. 2005) (Figure 3). There are two categories of hydrophobins, class I and class II, which are firstly classified by Wessels (1994) based on biophysical properties, hydropathy patterns (Kyte and Doolittle 1982), and film-forming patterns of hydrophobins (Wessels 1997). Class I hydrophobins, such as SC3 of
Schizophyllum commune, EAS of Neurospora crassa and HGFI of Grifola frondosa can form rigid and highly insoluble aggregates on hydrophilic surfaces (e.g. mica and polystyrene plates) (Lugones et al. 1996), and can only be dissociated by strong acids (e.g. 100% formic acid or trifluoroacetic acid). In contrast, class II hydrophobins, including HFBI and HFBII of Trichoderma reesei can form less stable monolayers on hydrophilic surfaces, and can easily be dissociated by 60% ethanol or 2 % SDS (Linder et al. 2005; Wösten et al. 1993).

**Figure 3.** Ribbon diagram of the class I hydrophobin EAS of Neurospora crassa (Figure adapted from Bayry et al. 2012).

**Application prospects of hydrophobins**

Due to their amphipathic nature and self-assembly characteristics, many potential applications of hydrophobins have been proposed (Linder et al. 2005; Linder 2009; Wösten and Scholtmeijer 2015; Khalesi et al. 2012). Here applications in biotechnology and food industry are briefly summarized, including uses in protein/peptide immobilization or purification, food production and quality control, and anti-biofilm therapy.

**Immobilization of proteins and peptides as hydrophobin fusions**

The stable nanostructure of hydrophobin assembles on surfaces makes them suitable for immobilization of proteins/peptides (Linder et al. 2005). Niu et al. (2012a) have studied the surface modification characteristics of the class I hydrophobin HGFI of Grifola frondosa, and its fusion with a short human blood outgrowth endothelial cells (HBOECs) binding ligand, TPS (NH₂-TPSLEQRTVYAK). The TPS-linker-HGFI fusion could be efficiently and rigidly immobilized onto a hydrophobic surface, as analyzed by water contact angle (WCA) and X-ray photoelectron spectroscopy (XPS) measurements. More importantly, the HBOECs binding activity of TPS was highly retained upon the immobilization. Linder et
al. (2002) used two class II hydrophobins HFBI and HFBII of *Trichoderma reesei* to immobilize the cellulose endoglucanase I catalytic core (EGI-core) of *T. reesei*, onto both silanized glass and Teflon™ surfaces. The enzyme activity was remained unchanged after the immobilization.

**Purification of proteins as hydrophobin fusions in aqueous two-phase systems**

Aqueous two-phase system (ATPS), also called aqueous biphasic system (ABS) is frequently employed as a nondestructive method for purification of labile proteins (e.g. membrane proteins and enzymes) at large scales. The amphipathic nature of hydrophobins makes them suitable as a fusion partner for ATPS purification of proteins (Wösten et al. 1993).

Collén et al. (2002) used the class II hydrophobin HFBI of *Trichoderma reesei* for ATPS purification of the cellulose endoglucanase I (EGI) of *T. reesei*, with both non-ionic detergent and polymers. The ATPS purified EGI showed an unaffected catalytic activity with a good recovery rate of 90% at pilot scale, which appeared to be more efficient than EGI purified by other methods (Linder et al. 2005).

**Food production and quality control**

Gushing is an unpleasant quality index of carbonated beverages (e.g. beer, champagne and sparkling wine), a phenomenon where a carbonated beverage, without agitation, vigorously effervesces and jets out when the cap is removed (Linder et al. 2005; Sarlin et al. 2012). Haikara et al. (2006) concluded that hydrophobins (class II hydrophobins) of barley/malt microbiological contaminants, including pathogenic *Fusarium poae* and *Nigrospora* spp., and non-pathogenic *Trichoderma reesei* (HFBI and HFBII) are closely related with these gushing problems (Figure 4). This finding has led to the development of an immunological detection method for the evaluation of gushing potential of barley and malt materials (Haikara et al. 2006).
9

**Figure 4.** Class II hydrophobin HFBII of *Trichoderma reesei* causes gushing of beer. In this laboratory experiment, 50 μg purified hydrophobin HFBII was added to a 33 cl bottle of beer three days prior to opening (Figure adapted from Linder et al. 2005).

On the other hand, due to their superior foaming abilities and foam stability. Hydrophobins can thus be used in many aerated foods where foams are important, including chocolate, ice cream and buttercream (Cox et al. 2009). Recently, a research group from Unilever UK has successfully used hydrophobins from edible mushrooms to mimic the texture and mouthfeel of fats. As a result, up to 50% of the fat could be removed from the food products without affecting the mouthfeel (Green et al. 2013; Cox et al. 2008).

**Biofilm control**

Adsorption of serum proteins plays an important role for bacterial colonization on surfaces (Donlan 2002; Palmer et al. 2007), including dental plaque formation and the primary stage of urinary tract infections (Bernsmann et al. 2008; Wösten and Scholtmeijer 2015). Stallard et al. (2012) and Geoghegan et al. (2013) concluded that the fibrinogen loading of a surface was closely related to its colonization potential of bacteria. Based on this fact, von Vacano et al. (2011) successfully used the recombinant fusion hydrophobin protein H*Protein B (from BASF SE), to prevent the non-specific absorption of serum proteins, such as BSA, casein and collagen on a 1-octanethiol-coated gold surface. Sarparanta et al. (2012) employed the class II hydrophobin HFBII of *Trichoderma reesei* to wrap thermally hydro-carbonized porous silicon (THCPSi) nano-particles. Significant reduction in the non-specific adsorption of plasma proteins, such as serum albumin and fibrinogen was observed in these hydrophobin-treated nano-particles.
2.3 Antimicrobial peptides

Antimicrobial peptides (AMPs) are endogenous oligopeptide ‘antibiotics’, which are widely distributed in all classes of life (Lehrer and Ganz 1999; Brodgen et al. 2003) and generally execute a non-receptor mediated cell killing mechanism (Prenner et al. 1999) against the competing microorganisms (Boman 1995). Usually these small molecules are composed of less than 60 amino acid residues, with a net positive charge of +2 to +9 provided by lysine, arginine or, in acidic environments, histidine, and a dominant proportion (>50%) of hydrophobic resides (Hancock and Sahl 2006; Brodgen 2005; Peters et al. 2010).

Figure 5. Four major structural groups of antimicrobial peptides: (A) α-helical structure of magainin-2; (B) β-sheeted structure of β-defensin 1; (C) extended structure of indolicidin; (D) looped structure of gramicidin (Figure adapted from Peters et al. 2010).

The classification of AMP is difficult owing to the limited amino acid sequence homology between these peptides, thus the further classification of AMPs is made on the basis of their secondary structures (Epand and Vogel 1999; Hancock and Sahl 2006). Folded AMPs are classified into four major structural groups (Figure 5): α-helical peptides (for example, cecropins, magainins, pexiganans and the human cathelicidin LL-37); β-sheeted peptides predominantly stabilized by more than one disulfide bridge (for example, human α- and
β-defensins and protegrins); extended peptides enriched with specific amino acids like proline, tryptophan, arginine and histidine (for example, indolicidin, bactenecin-5 and bactenecin-7); looped peptides coiled by one disulfide bridge (for example, gramicidin) (Peters et al. 2010; Hancock and Sahl 2006).

Currently, more than 2,000 natural AMPs have been isolated from a wide range of organisms (more information can be found at http://aps.unmc.edu/AP/main.php). Some of them have been approved for clinical or food applications, including the cationic AMP (cAMP) gramicidin S for treatment of genital ulcers and the lantibiotic nisin for preservation of food products (Hancock and Sahl 2006). Of interest, AMPs exhibit a wide range of activities against various organisms, including gram-negative and gram-positive bacteria, fungi, enveloped viruses, parasites, and tumor cells with low propensity to cause drug resistance among pathogens (Baker et al. 1993; Hancock and Scott 2000; Hancock and Sahl 2006; Zasloff 2002; Chen et al. 2001; Hoskin and Ramamoorthy 2008). Due to these unique properties, AMPs are considered as attractive substitutes for conventional antibiotics to overcome the current drug resistance crisis (Hancock and Sahl. 2006; Lohner 2001).

**Mechanisms of antimicrobial-peptide-mediated bacterial cell killing**

Despite their vast structural varieties, most AMPs kill bacterial cells by disrupting the cytoplasmic membrane integrity, resulting in the effusion of intracellular ions and organic solutes (Prenner et al. 1999), which in turn may lead to cell death. Although the exact mechanism by which AMPs disengage the bacterial cytoplasmic membrane is still not fully understood. Indisputably, AMPs must be initially attracted to the bacterial surface, and this step is thought to be facilitated by electrostatic interactions between AMPs and lipopolysaccharides (LPS) on the surface of Gram-negative bacteria, and wall-associated teichoic acids on the surface of Gram-positive bacteria (Figure 6) (Hancock 2001; Brogden 2005).
Once non-covalently attached to the bacterial surface, AMPs must traverse the compact bacterial outer wall before they can interact with the cytoplasmic membrane, by a process described by Hancock and coworkers as ‘self-promoted uptake’ (Hancock and Sahl 2006; Hancock 1997). This process is very important but rarely mentioned in many earlier studies. Take gram-negative bacteria as an example, the bacterial outer wall stability is maintained by native divalent cations (e.g. Mg$^{2+}$ and Ca$^{2+}$) via salt bridges (Ledebo 1976). AMPs show higher affinity to LPS than these native divalent cations and thus competitively displacing them, resulting a disruption of the bacterial outer wall integrity. This local disturbance in the bacterial outer wall enables AMPs to pass through the bacterial outer wall and reach the negatively charged cytoplasmic membrane.

Once reached the cytoplasmic membrane, AMPs start to interact with the anionic phospholipid head groups of the cytoplasmic membrane (Figure 6). At low peptide/lipid (P/L) molar ratios, AMPs are bound parallel onto the cytoplasmic membrane through hydrophobic and electrostatic interactions, and slightly stretch the cytoplasmic membrane (Yang et al. 2001; Brogden 2005). Once the P/L ratio surpasses a critical threshold, AMPs start to aggregate into bundles and subsequently insert into the cytoplasmic membrane interior, leading to the formation of transmembrane pores or detergent-like micelles (Brogden 2005; Melo et al. 2009), which in turn may lead to cell death. Three modes of action have been proposed based on the model membrane study with AMPs, they are: (1)
barrel-stave model (Ehrenstein and Lecar 1977); (2) carpet model (Pouny et al. 1992; Jenssen et al. 2006) and (3) toroidal pore model (Yamaguchi et al. 2002) (Figure 7).

![Figure 7](image1.png)

**Figure 7.** Modes of action of antimicrobial peptides: (A) barrel-stave model; (B) toroidal pore model; (C) carpet model (Figure modified from Brogden 2005).

**Alternative mechanisms of antimicrobial-peptide-mediated bacterial cell killing**

Most of the existing AMPs (>90%) act directly on the bacterial cytoplasmic membrane, via a non-receptor dependent membrane permeabilization process, as described in detail above. However, some early studies revealed that there is a distinct group of AMPs, which can kill bacteria in a non-lytic process (Bahar and Ren 2013). One example is AMP Buforin II, which kills bacteria by inhibiting DNA and RNA synthesis (Park et al. 1998). Increasing evidence indicates that there are several intracellular sites of AMP activity, including DNA and RNA (Park et al. 1998), intracellular enzymes, and ribosomes (Boman et al. 1993) (Figure 8).

![Figure 8](image2.png)

**Figure 8.** Schematic diagram of intracellular sites of antimicrobial peptide activity in *Escherichia coli* (Figure adapted from Brogden 2005).
Antimicrobial peptides P11-5 and Bac8c

AMP P11-5 (NH2-GKLFFKILKIL) is a novel 11-amino-acid peptide derived from AMP BP76 (NH2-KKLFFKILKFL) with two amino acid substitutions (Qi et al. 2010). This peptide showed a broad-spectrum antimicrobial activity against a wide range of pathogenic microorganisms, including gram-negative bacteria (E. coli and P. aeruginosa), gram-positive bacteria (S. aureus) and fungi (C. albicans and F. solani) without significant cytotoxicity to mammalian cells (Table 1).

AMP Bac8c (NH2-RRWIVWIR) is a truncated 8-amino-acid peptide derived from AMP Bac2A (NH2-RAVRIVVIRALR), a C3A/C11A variant of bactenecin (also known as bovine dodecapeptide), with four amino acid substitutions (Hilpert et al. 2005). This peptide (Bac8c) is the smallest known antimicrobial peptide with broad-spectrum activity against a range of microorganisms, such as gram-positive bacteria (S. aureus and S. epidermidis), gram-negative bacteria (E. coli and P. aeruginosa) and a fungus (C. albicans) (Table 1).

Table 1. Peptide properties: MICs (μg/ml) for five test pathogenic microorganisms

<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>Peptide sequence (NH2-)</th>
<th>E. coli</th>
<th>P. aeruginosa</th>
<th>S. aureus</th>
<th>C. albicans</th>
<th>F. solani</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP76a</td>
<td>KKLFFKIILKFL</td>
<td>8</td>
<td>16</td>
<td>62.3</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>P11-5a</td>
<td>GKLFFKIILKIL</td>
<td>3.1</td>
<td>12.5</td>
<td>12.5</td>
<td>3.1</td>
<td>12.5</td>
</tr>
<tr>
<td>Bac2Ab</td>
<td>RAVRIIVIRAR</td>
<td>17</td>
<td>50</td>
<td>17</td>
<td>9</td>
<td>-</td>
</tr>
<tr>
<td>Bac8cb</td>
<td>RRWIVWIR</td>
<td>2</td>
<td>8</td>
<td>2</td>
<td>8</td>
<td>-</td>
</tr>
</tbody>
</table>

a values were obtained from Qi et al. 2010.
b values were obtained from Hilpert et al. 2005.

P11-5 is a cationic (+4) AMP with a substantial proportion (7/11) of hydrophobic amino acid residues. The structural study of P11-5 showed that this peptide is mainly folded up into an amphipathic α-helix structure, with the positively charged hydrophilic residues localized on one side of the helix and the hydrophobic residues on the other side (Figure 9).
Due to its amphipathic and cationic nature, P11-5 may spontaneously interact with the negatively charged bacterial outer wall and rapidly disintegrate the cytoplasmic membrane by the ‘carpet-like’ mode of action (Ferre et al. 2006; Melo et al. 2009). The field emission scanning electron microscopy (FESEM) images of *E. coli* cells challenged by P11-5 showed conspicuous membrane fraying and surface blistering, together with the effusion of intracellular materials (Figure 10).

Bac8c is a cationic (+3) AMP with an abnormal low amount (< 50%) of hydrophobic amino acid residues. The secondary structure of Bac8c is typically α-helix, with its specific structure being anion-, pH- and detergent-dependent. For example, it adopts α-helical
structure in a zwitterionic solution and β-turn structure in anionic solutions (Wieczorek et al. 2010).

Like the parent peptide Bac2A, Bac8c executes a two-stage mode of antimicrobial action, in a way not solely dependent on membrane depolarization as the cause of cell death (Spindler et al. 2011). In short, when Bac8c is present at concentrations that below the minimum inhibitory concentration (MIC), the bacterial cytoplasmic membrane integrity is well maintained (Figure 11B), while the cellular metabolism (e.g. ATP synthesis, NAD+/NADH balance and protein synthesis) is severely impaired. Upon the accumulation of a threshold concentration of Bac8c on microbial cytoplasmic membranes, the bacterial cytoplasmic membrane is rapidly (less than 5 min) depolarized via an unknown mechanism, resulting in the formation of transmembrane pores (between 2.2 and 3.3 nm) and effusion of intracellular ions and organic solutes (Figure 11C & D) (Lee and Lee 2015).

**Figure 11.** Transmission electron microscopy (TEM) images of *Escherichia coli* ATCC 9637 cells untreated and treated with the antimicrobial peptide Bac8c for 30 min. (A) Untreated control. (B) 3 μg/ml (IC₅₀). (C) 6 μg/ml (MBC). (D) 30 μg/ml (5x the MBC) (Figure adapted from Spindler et al. 2011). MBC: minimum bactericidal concentration. IC₅₀: the half maximal inhibitory concentration.

**2.4 The *Pichia pastoris* protein expression system**

Over the past few decades, scientists have learned how to identify, amplify and place genes into a variety of organisms that are different from the source organism (Macauley-Patrick et al. 2005). Meanwhile, in this proteomics era, the demand of proteins for scientific and
pharmaceutical research is steadily on the increase. Therefore, a major application of these transgenic organisms is to produce heterologous proteins (Macauley-Patrick et al. 2005; Adrio and Demain 2010).

To date, bacterial protein expression systems are still predominately used in most laboratories for heterologous protein production, due to their simplicity of genetic manipulation, low cultivation costs and generally high yields, when compared with other protein expression platforms (Chen 2012). However, insufficient and incorrect post-translational modifications of complex proteins are two obvious drawbacks of these bacterial systems. Take the class I hydrophobin HGFI of *Grifola frondosa* as an example, this fungal protein has been successfully expressed in both *Escherichia coli* (Wang et al. 2010a) and *Pichia pastoris* (Wang et al. 2010b) protein expression systems. In *E. coli*, HGFI is acquired as insoluble and miss-folded inclusion bodies, thus succeeding solubilization and re-folding operations are needed (Wang et al. 2010a), which are either technically difficult to achieve or time-consuming (Marston 1986; Daly and Hearn 2005; Tsujikawa et al. 1996). In addition, *E. coli* produced heterologous proteins usually tend to have an extra methionine residue (translation initiator) in their innate N-terminals, which may pose a negative effect on protein activity and stability (Chaudhuri et al. 1999). In contrast, in *P. pastoris*, HGFI is obtained in its biologically active form, and, more importantly, its innate N-terminal sequence is well retained (Wang et al. 2010b). Other fungal hydrophobins, initially produced as non-functional inclusion bodies through production in *E. coli*, but obtained in their active forms when produced in *P. pastoris*, including the class II hydrophobin HFBI of *Trichoderma reesei* (Nakari-Setälä et al. 1996; Niu et al. 2012b) and the class I hydrophobin EAS of *Neurospora crassa* (Kwan et al. 2006; Winefield 2004).

The *P. pastoris* protein expression system, except for allowing heterologous proteins to be produced in their innate active forms, the increased popularity of this system can be attributed to several additional factors: (1) the capability of performing eukaryotic post-translational modifications, especially for target proteins with multiple disulfide bridges or in need of glycosylation (Cereghino and Cregg 2000); (2) the ability of
producing heterologous proteins at high levels (Daly and Hearn 2005); (3) the availability of systematic genetic manipulation techniques and kits, thus the expression system is easy to set up and maintain in most laboratories.

**Pichia pastoris host strains**

*P. pastoris* is facultative methylotrophic yeast, which is capable of utilizing methanol as a sole source of carbon and energy. The methanol utilization pathway involves several enzymes, and the alcohol oxidase (AOX) is the most important one, catalyzing the oxidation of methanol to formaldehyde (HCHO) and hydrogen peroxide (H₂O₂) in peroxisomes.

The *P. pastoris* alcohol oxidase (AOX) is encoded by either the *AOX1* or the *AOX2* gene, and the majority of AOX activity arises from the *AOX1* gene (Tschopp et al. 1987; Cregg et al. 1989). A wild type *P. pastoris* strain (e.g. *P. pastoris* X-33) generally contains both AOX (*AOX1* and *AOX2*) genes and primarily uses the *AOX1* gene for its methanol metabolism. This phenotype is designated as methanol utilization fast (Mut⁺). Once the *AOX1* gene is disrupted or deleted on purpose, as a result, *P. pastoris* cells (e.g. *P. pastoris* KM71) are still capable of utilizing methanol via the transcriptionally weaker *AOX2* gene at a decreased rate, thus giving rise to the phenotype methanol utilization slow (Mut⁻). In addition, a rarely addressed phenotype exists for *P. pastoris* strains (e.g. *P. pastoris* MC 100-3) that have deletions at both AOX genes, which are unable to grow on methanol. This phenotype is denominated as methanol utilization minus (Mut⁻) (Macauley-Patrick et al. 2005).

It is not entirely clear that which phenotype (Mut⁺, Mut⁻ and Mut⁻) is the most suitable one for expression of a given protein (Hellwig et al. 2001). However, for extracellular production of heterologous proteins, Mut⁺ strains are more often used than Mut⁻ strains, due to their higher growth rates and better environmental adaptabilities (Hohenblum et al. 2004; Slibinskas et al. 2004). This phenotype (Mut⁺) can easily be identified either by growing *P. pastoris* cells on minimal dextrose (MD) and minimal methanol (MM) plates.
sequentially (Vassileva et al. 2001), or by colony PCR with specific primers 5’ AOX1 and 3’ AOX1 (Linder et al. 1996).

Protein degradation has long been a problem associated with *P. pastoris* protein production practices, due to the action of endogenous proteases (e.g. vacuole peptidase A). The use of protease defect strains (e.g. SMD1168) has proven to reduce protein degradation in many heterologous protein production studies with this system (Cereghino and Cregg 2000; Macauley-Patrick et al. 2005).

**Table 2. Pichia pastoris expression host strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-33</td>
<td>wild-type</td>
<td>Mut⁺, His⁹</td>
<td>Li et al. 2001</td>
</tr>
<tr>
<td>GS115</td>
<td>his⁴</td>
<td>Mut⁺, His⁹</td>
<td>Cregg et al. 1985</td>
</tr>
<tr>
<td>KM71</td>
<td>△aox1::SARG4 his4 arg4</td>
<td>Mut⁺, His⁹</td>
<td>Cregg and Madden 1987</td>
</tr>
<tr>
<td>SMD1168</td>
<td>△pep4::URA3 his4</td>
<td>Mut⁺, His⁹</td>
<td>White et al. 1995</td>
</tr>
<tr>
<td>MC 100-3</td>
<td>△aox1::SARG4△aox2::Phis4 his4 arg4</td>
<td>Mut⁺, His⁹</td>
<td>Cregg et al. 1989</td>
</tr>
</tbody>
</table>


**Pichia pastoris expression vectors**

There is a wide range of commercially available vectors that can be used to express heterologous proteins in *P. pastoris* (Table 3). All *P. pastoris* expression vectors have been designed as an *E. coli/P. pastoris* shuttle vector. Therefore, an origin of replication for vector maintenance and replication in *E. coli*, AOX1 promoter or alternative promoters, one or more restriction sites for insertion of foreign genes, and selection markers functional in one or both host organisms are needed (Cereghino and Cregg 2000).

pPIC9 (Figure 12) is a commercially available *P. pastoris* expression vector that has been intensively used for the production of hydrophobins (Niu et al. 2012a; Wang et al. 2010b; Niu et al. 2012b) and antimicrobial peptides (Zhang et al. 2005; Zhang et al. 2000). Except for the basic features that cited above, this vector contains the functional histidine dehydrogenase gene (*HIS4*), which can be used as a selection marker for the transformation of histidine dehydrogenase defective (*his4*) strains (e.g. GS115, KM71,
In addition, to facilitate the extracellular production of heterologous proteins, an extracellular secretion signal sequence (usually, alpha-mating factor of *Saccharomyces cerevisiae*) is incorporated upstream of the multiple cloning site (MCS).

![pPIC9 vector diagram](Image)

**Figure 12.** Vector diagram of pPIC9 (Figure adapted from *Pichia* expression kit user guide, MAN0000012, Lifetechnologies)

**Table 3.** Commercially available *P. pastoris* expression vectors

<table>
<thead>
<tr>
<th>Name</th>
<th>Secretion signal</th>
<th>Selection maker</th>
<th>Promoter</th>
</tr>
</thead>
<tbody>
<tr>
<td>pPIC9</td>
<td>α-MF</td>
<td>HIS4, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>AOX1</td>
</tr>
<tr>
<td>pPIC9K</td>
<td>α-MF</td>
<td>Amp&lt;sup&gt;R&lt;/sup&gt;, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>AOX1</td>
</tr>
<tr>
<td>pPICZαA</td>
<td>α-MF</td>
<td>Zeo&lt;sup&gt;R&lt;/sup&gt;</td>
<td>AOX1</td>
</tr>
<tr>
<td>pGAPZαA</td>
<td>α-MF</td>
<td>Zeo&lt;sup&gt;R&lt;/sup&gt;</td>
<td>GAP</td>
</tr>
<tr>
<td>pHIL-D2</td>
<td>no</td>
<td>HIS4, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>AOX1</td>
</tr>
<tr>
<td>pHIL-S1</td>
<td>PHO1</td>
<td>HIS4, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>AOX1</td>
</tr>
<tr>
<td>pP-αSUMOstar</td>
<td>α-MF</td>
<td>Zeo&lt;sup&gt;R&lt;/sup&gt;</td>
<td>AOX1</td>
</tr>
</tbody>
</table>

Secretion signals: α-MF (*S. cerevisiae* α-mating factor); PHO1 (*P. pastoris* acid phosphatase).

Selection markers: Amp<sup>R</sup> (ampicillin resistance); Kan<sup>R</sup> (kanamycin resistance); Zeo<sup>R</sup> (zeocin resistance); HIS4 (histidine dehydrogenase).

Promoters: GAP (glyceraldehyde-3-phosphate dehydrogenase); AOX1 (alcohol oxidase 1).

More information can be found at www.lifetechnologies.com.

To date, a wide range of fungal origin hydrophobins have been successfully expressed in *P. pastoris*, including HFBI (Niu et al. 2012), HFBII (Kottmeier et al. 2011) and Hfb2 (Lutterschmid et al. 2011) of *Trichoderma reesei*, FcHyd5p and FcHyd3p of *Fusarium culmonorum* (Lutterschmid et al. 2011; Stübner et al. 2010), HGFI of *Grifola frondosa* (Wang et al. 2010b), RodA and RodB of *Aspergillus fumigatus* (Pedersen et al. 2011) and EAS of *Neurospora crassa* (Winefield 2004).
EXPERIMENTAL RESEARCH

3 AIMS OF THE STUDY

The aim of this study was to explore the feasibility of using recombinant fusion hydrophobins to control bacterial growth. The specific aims were:

1. To construct expression vectors that can thus be used to transform \( P.\) \emph{pastoris} with a view to obtain two recombinant fusion hydrophobins, Bac8c-HGFI and P11-5-linker-HGFI.
2. To express these two recombinant fusion hydrophobins in \( P.\) \emph{pastoris} and purify them from the culture medium.
3. To determine the minimum inhibition concentrations (MICs) of these two recombinant fusion hydrophobins against bacteria.

4 MATERIALS AND METHODS

4.1 Materials

4.1.1 Bacteria and yeast strains, plasmids, oligonucleotides and primers

\emph{Pichia pastoris} GS115 (Genotype: \(\text{his}^4\); Phenotype: His\(^+\), Mut\(^+\)) and X-33 (Wild type; Phenotype: Mut\(^+\)) strains were kindly provided by Dr. Kristiina Hildén (University of Helsinki, Finland). Library Efficiency® DH5\(\alpha\)™ Competent Cells (Genotype: F- \(\Phi80\text{lacZ}\Delta\text{M15} \Delta (\text{lacZYA-argF})\) U169 \text{recA1 endA1 hsdR17 (r}\(_{K^-}\), \text{m}\(_{K^+}\)\)) \text{phoA supE44 }\lambda\text{-thi-1 gyrA96 relA1} \) were purchased from Lifetechnologies (Amsterdam, Netherlands). Reference strains \emph{Escherichia coli} ATCC 8739, \emph{Staphylococcus aureus} subsp. \emph{aureus} ATCC 12600, \emph{Pseudomonas aeruginosa} ATCC 10145 and \emph{Micrococcus luteus} ATCC 4698 were obtained from HAMBI culture collection center (University of Helsinki, Finland).

Reconstructed \emph{P. pastoris} expression vectors pPIC9-linker-hgfI preserved in \( E.\) \emph{coli} TG1 [Genotype: \( F^+(\text{traD36 proAB}^+ \text{ lacI}^q \text{ lacZ}\Delta\text{M15})\text{ supE thi-1 }\Delta(\text{lac-proAB})\Delta(\text{mcrB-hsdSM})5, (\text{r}\(_{K^-}\), \text{m}\(_{K^+}\)\)] and pP-secSUMOpro3-hgfI preserved in \( E.\) \emph{coli} DH5\(\alpha\) (Genotype: \( F^-\Phi80\text{lacZ}\Delta\text{M15} \Delta (\text{lacZYA-argF})\) U169 \text{recA1 endA1 hsdR17 (r}\(_{K^-}\), \text{m}\(_{K^+}\)\])
*phoA supE44 λ- thi-1 gyrA96 relA1* were kindly provided by Prof. Mingqiang Qiao (Nankai University, China). All primers and oligonucleotides (Table 4) were custom synthesized by Oligomer Oy (Helsinki, Finland).

**Table 4. Overview of primers and oligonucleotides used in this study**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>5′ AOX</td>
<td>GACTGGTTCCAATTGACAAGC</td>
</tr>
<tr>
<td>3′ AOX</td>
<td>GCMAATGGCATTCTGACATCC</td>
</tr>
<tr>
<td>α-factor</td>
<td>TACTATTGCCAGCATTGCTGC</td>
</tr>
<tr>
<td>p11-5 forward</td>
<td>TCGAGAAAAGAGTAAAGTTGTGTTCAAGAAGATCTTGAAGATCTTGG</td>
</tr>
<tr>
<td>p11-5 reverse</td>
<td>AATTCAGGGATCTTCAAGAAGATCTTGAAGATCTTGG</td>
</tr>
<tr>
<td>AMP forward</td>
<td>CAAATGAAAGCTGACA</td>
</tr>
<tr>
<td>hgfI reverse</td>
<td>TGGTGCCTGGCTTGG</td>
</tr>
<tr>
<td>hgfI*</td>
<td>CAACAGTGACCCACTGGCCA</td>
</tr>
<tr>
<td>bac8c*</td>
<td>TCTTTCTCCAAAATACCCAAATTCTACCTCCCGTGCTGCTGCTGGA</td>
</tr>
</tbody>
</table>

*5′-phosphorylated

### 4.1.2 Antimicrobial peptides, chemical reagents and culture media components

Antimicrobial peptides Bac8c and P11-5 were custom synthesized by Thermoscientific (Germany) using the solid phase method and standard 9-fluorenyl-methoxy-carbonyl (FMOC) chemistry. These peptides were purified to > 95% purity using reversed-phase high performance liquid chromatography (RP-HPLC). Mass spectrometry was used to confirm the peptide identity.

All chemical regents and culture media components were purchased from Sigma-Aldrich (Germany), Merck (Germany) or Lab M (Heywood, UK) unless otherwise stated.

### 4.2 Methods

All techniques used for the molecular biology experimental work were based upon these standard protocols found in Molecular Cloning: A Laboratory Manual (Green and Sambrook, 2012) and Cold Spring Harbor Online Protocols (http://cshprotocols.cshlp.org/) unless otherwise stated.
4.2.1 Isolation and purification of plasmid DNA from *Escherichia coli*

Glycerol stock of *E. coli* TG1 cells containing the reconstructed *P. pastoris* expression vector pPIC9-linker-hgfI was streaked onto a freshly prepared LB agar plate (1% [w/v] trypton, 0.5% [w/v] yeast extract, 1% [w/v] sodium chloride, 1.5% [w/v] agar) containing 100 μg/ml ampicillin. In the same way, glycerol stock of *E. coli* DH5α cells containing the reconstructed *P. pastoris* expression vector pP-secSUMOpro3-hgfI was streaked onto a freshly prepared low salt LB agar plate (1% [w/v] trypton, 0.5% [w/v] yeast extract, 0.5% [w/v] sodium chloride, 1.5% [w/v] agar) containing 50 μg/ml zeocin (Invitrogen, Carlsbad, CA, USA). These plates were incubated overnight at 37°C until single colonies appeared.

A single colony from the LB agar plate was inoculated into 5 ml LB broth (1% [w/v] trypton, 0.5% [w/v] yeast extract, 1% [w/v] sodium chloride) containing 100 μg/ml ampicillin (for *E. coli* DH5α, 5 ml low salt LB broth [1% [w/v] trypton, 0.5% [w/v] yeast extract, 0.5% [w/v] sodium chloride] containing 50 μg/ml zeocin was used), and incubated at 37°C with shaking at 200 rpm until OD₆₀₀ reached 0.4 (log-phase growth, approximately 8 hours). Bacterial cells were collected by centrifugation (Sigma 1-14 Microfuge) for 5 min at 12000g. Plasmid DNA was extracted from bacterial pellets and purified with GeneJET Plasmid Miniprep Kit (Thermoscientific) according to the manufacturer’s instructions, and stored in a sterile nuclease-free Eppendorf tube at -20°C. The concentrations of DNA samples were measured by NanoDrop-1000 Spectrophotometer (Thermoscientific).

4.2.2 DNA agarose gel electrophoresis

Separation and visualization of DNA fragments was performed by agarose gel electrophoresis. The percentage (w/v) of agarose (Lonza, Basel, Switzerland) used was varied between 1% and 3% depending on the size of the DNA fragments. Gel solutions were prepared with 1x TAE buffer (40 mM tris-HCl, 40 mM glacial acetic acid, 1 mM EDTA, pH 8.0) and ethidium bromide was added to a final concentration of 0.5μg/ml. DNA samples were pre-mixed with 6x DNA Loading Dye (Thermoscientific) prior to gel loading. 1 kbp GeneRuler™ DNA Ladder (Thermoscientific) or 100 bp plus GeneRuler™ DNA ladder (Thermoscientific) was used as a marker of molecular mass and run parallel to
the DNA samples. Electrophoresis was carried out in 1x TAE buffer at 80 V. Complete gels were observed and image captured with Gel Doc XR+ imaging system (Bio-Rad Laboratories, Hercules, CA, USA).

4.2.3 Construction of pP-secSUMOpro3-bac8c-hgfI

The inverse fusion PCR (IF-PCR) (Figure 13) was conducted with Phusion® Hot Start II High-Fidelity DNA polymerase (Thermoscientific) (Table 5). 5’ phosphorylated primers (bac8c and hgfI) were designed using a protocol described by Spiliotis (2012). Yield optimization was performed with respect to the annealing temperature via gradient PCR in Bio-Rad C1000 Thermal Cycler under the following conditions: 98°C for 1 min, 30 cycles of 98°C for 10 s, (63°C-75°C) for 30 sec, 72°C for 2 min, and a final extension step at 72°C for 10 min. The PCR products were analyzed on 1% agarose gel.

Table 5. Pipetting instructions for the inverse fusion PCR reaction

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (μl)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X Phusion HF Buffer</td>
<td>10</td>
<td>1x</td>
</tr>
<tr>
<td>Phusion Hot Start II DNA polymerase (2 U/μl)</td>
<td>0.5</td>
<td>0.02 U/μl</td>
</tr>
<tr>
<td>hgfI (10 μM)</td>
<td>2.5</td>
<td>0.5 μM</td>
</tr>
<tr>
<td>bac8c (10 μM)</td>
<td>2.5</td>
<td>0.5 μM</td>
</tr>
<tr>
<td>pP-secSUMOpro3-hgfI (template, 1 ng/μl)</td>
<td>1</td>
<td>0.02 ng/μl</td>
</tr>
<tr>
<td>dNTPs (10 mM)</td>
<td>1</td>
<td>200 μM each</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>32.5</td>
<td></td>
</tr>
</tbody>
</table>

The required DNA band was excised from the agarose gel with x-tracta™ Gel Extractor (Promega, Madison, WI, USA) on an UV transilluminator (Bio-Rad Laboratories) and subsequently purified with GeneJET Gel Extraction Kit (Thermoscientific) following the manufacturer’s instructions.

For self-circularization of blunt-ended PCR products, the reaction mixture was prepared as follows: 50 ng blunt-ended linear DNA, 2 μl 10x T4 DNA ligase buffer, 1 μl T4 DNA ligase (5 Weiss/μl, Thermoscientific), 2 μl PEG 4000 (50% [w/v]) and ddH₂O to a total volume of 50 μl. The reaction mixture was incubated at 22°C for 2 h, and the ligase was heat inactivated at 65°C for 10 min.
Figure 13. Outline of inverse fusion PCR (IF-PCR). For IF-PCR, a PCR master mix containing 5’ phosphorylated primer bac8c with the insert, circular plasmid template and 5’ phosphorylated primer hgfI was prepared. The bac8c primer was annealed with the complementary sequence (black) within the plasmid forward strand (2.1 & 2.2), the insert (white) was elongated by overlap extension (2.3), thus generating the single-stranded DNA (ssDNA) template with the insert (2.4). The complementary strand of the ssDNA template was generated by primer extension of primer hgfI, finalizing the double-stranded DNA (dsDNA) template (2.5), which was now exponentially amplified via primers bac8c and hgfI. The linear insert-plasmid fusions were self-circularized by T4-ligation (2.6) (Figure adapted from Spiliotis 2012).

4.2.4 Construction of pPIC9-p11-5-linker-hgfI

Restriction endonuclease (RE) digestion of pPIC9-linker-hgfI

The freshly prepared vector pPIC9-linker-hgfI from the plasmid isolation step (section 4.2.1) was double-restricted with FastDigest® EcoRI and XhoI restriction endonucleases (Thermoscientific). The double REs digestion was performed under the optimal reaction condition described by the manufacturer.

One step phosphorylation and annealing of oligonucleotides

As oligonucleotides (p11-5 forward and p11-5 reverse) used in this study are supplied 5’ non-phosphorylated. Therefore, to improve the ligation efficiency, T4 polynucleotide kinase (T4 PNK, Thermoscientific) was used to phosphorylate oligonucleotides. The reaction mix was prepared and the one-step phosphorylation and annealing reaction was carried out in Bio-Rad C1000 Thermal Cycler (Table 6).


Table 6. Pipetting instructions and thermocycler program for the one-step phosphorylation and annealing reaction of oligonucleotides

<table>
<thead>
<tr>
<th>Component</th>
<th>Reaction mix composition</th>
<th>Thermocycler program</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X T4 PNK buffer A</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>T4 PNK (10 U/μl)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>p11-5 forward (10 μM)</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>p11-5 reverse (10 μM)</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>ATP (10 mM)</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>ddH2O</td>
<td>10</td>
<td>6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (℃)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>37</td>
<td>30 min</td>
</tr>
<tr>
<td>2</td>
<td>95</td>
<td>5 min</td>
</tr>
<tr>
<td>3</td>
<td>95 (-1/cycle)</td>
<td>1 min/cycle</td>
</tr>
<tr>
<td>4</td>
<td>74</td>
<td>30 min</td>
</tr>
<tr>
<td>5</td>
<td>74(-1/cycle)</td>
<td>1 min/cycle</td>
</tr>
</tbody>
</table>

Ligation of DNA fragments

The 5’ phosphorylated double-stranded oligo (ds oligo) fragment synthesized from the previous step was ligated into the prepared pPIC9-linker-hgfI vector. For DNA insert ligation into vector DNA, the reaction mixture was prepared as follows: 100 ng linear vector DNA, 10:1 molar ratio of insert DNA over vector, 2 μl 10x T4 DNA ligase buffer, 0.2 μl T4 DNA ligase (5 Weiss/μl, Thermoscientific), and ddH2O to a total volume of 20 μl. The reaction mixture was incubated overnight at room temperature (20℃).

![Outline of annealed oligo cloning](image)

**Figure 14.** Outline of annealed oligo cloning. A short double-stranded oligo (ds oligo) fragment was synthesized by annealing oligonucleotides p11-5 forward and p11-5 reverse, with sticky ends specific to XhoI and EcoRI restriction endonucleases in 5’- and 3’-terminus, respectively. The double-stranded oligo fragment was ligated into the prepared pPIC9-linker-hgfI vector by T4-ligation.

4.2.5 Transformation of electrocompetent *Escherichia coli* DH5α cells

Electrocompetent *E. coli* DH5α cells from -80℃ storage were thawed on ice. 1 μl ligation mix (from section 4.2.3 or section 4.2.4) was added to 50μl cells, gently mixed and transferred to a pre-chilled 2 cm disposable electroporation cuvette (VWR International, Germany). Electroporation was conducted with a Gene Pulser™ apparatus (Bio-Rad Laboratories) set at 2.5 kV, 200 Ω and 25 μF. Immediately after the electroporation, 950 μl SOC broth (Lifetechnologies) was added to the cuvette and a total volume of 1 ml bacterial
suspension was transferred to a sterile Eppendorf tube and placed on a shaking incubator at 37°C for 1 hour. The bacterial cells were then plated out on selection agar plates with the corresponding antibiotics and incubated overnight at 37°C until single colonies appeared.

4.2.6 Colony PCR

Colony PCR was used as a rapid method to screen ligation reactions for positive clones. Visible bacterial colonies were picked from selection agar plates with pipette tips, suspended in 10 μl nuclease-free ddH₂O and thoroughly mixed by vortex. 1 μl bacterial liquid was directly added to the PCR master mix as the DNA template (Table 7).

The colony PCR was conducted with vector specific primers in Bio-Rad C1000 Thermal Cycler under the following conditions: 94°C for 5 min, 30 cycles of 94°C for 1 min, 53°C for 1 min, 72°C for 8 sec, and a final extension step at 72°C for 10 min. The PCR products were analyzed on 3% agarose gel. Two colony PCR verified positive transformants were sent for sequencing (Institute of Biotechnology, University of Helsinki, Finland).

| Table 7. Pipetting instructions for E. coli colony PCR |
|---------------------------------|-----------------|----------------|
| Component                        | Volume (μl)     | Final concentration |
| 10X optimized DyNAzyme buffer    | 2               | 1x               |
| 10 mM dNTPs                      | 0.4             | 200 μM each      |
| α-factor/AMP forward (10 μM)     | 1               | 0.5 μM          |
| hgfI reverse (10 μM)             | 1               | 0.5 μM          |
| Bacterial liquid (DNA template)  | 1               |                  |
| DyNAzyme II polymerase (2 U/μl)  | 0.2             | 0.02 U/μl       |
| H₂O                              | 14.4            |                  |

a primers AMP forward and hgfI reverse were used for E. coli transformants that transformed with the pP-secSUMOpro3-bac8c-hgfI ligation mix, and primers α-factor and hgfI were used for E. coli transformants that transformed with the pPIC9-p11-5-linker-hgfI ligation mix.

4.2.7 Transformation of electrocompetent Pichia pastoris cells

The sequencing-confirmed P. pastoris expression vector pPIC9-p11-5-linker-hgfI was linearized with StuI (Thermoscientific) (for pP-secSUMOpro3-bac8c-hgfI, PmeI was used). The linearized vector DNA was concentrated by isopropanol precipitation, suspended in a small amount of nuclease-free Milli-Q water and subsequently used to transform electrocompetent P. pastoris GS115 cells (for pP-secSUMOpro3-bac8c-hgfI,
Electrocompetent *P. pastoris* X-33 cells were used. Electroporation was conducted with a Gene Pulser™ apparatus set at 2.0 kV, 200 Ω and 25 μF. Immediately after the electroporation, 1 ml 1M ice cold sterile sorbitol was added to the cuvette and the cell suspension was transferred to a 15 ml Falcon™ tube and left to incubate for 2 hours at 28°C without shaking. Electrocompetent *P. pastoris* GS115 and X-33 cells were prepared using a protocol described by Cereghino et al. (2005).

For *P. pastoris* GS115 transformants, cells were initially plated on MD (minimal dextrose) agar plates (1.34% [w/v] YNB, 4 × 10⁻⁵ % [w/v] biotin, 2% [w/v] dextrose, 1.5% [w/v] agar) and incubated at 30°C for 3-4 days until single colonies appeared. Subsequently, thirty colonies (His⁺) were picked from MD agar plates and pasted on MM (minimal methanol) agar plates (1.34% [w/v] YNB, 4 × 10⁻⁵ % [w/v] biotin, 0.5% [v/v] methanol, 1.5% [w/v] agar) to identify the methanol utilization type. For *P. pastoris* X-33 transformants, cells were initially plated on YPDS agar plates (1% [w/v] yeast extract, 2% [w/v] peptone, 2% [w/v] dextrose, 1 M sorbitol, 2% [w/v] agar) containing 100 μg/ml zeocin and incubated at 30°C for 3-4 days until single colonies appeared. Subsequently, twenty zeocin-resistant colonies were picked from YPDS agar plates and further polished on YPD agar plates (1% [w/v] yeast extract, 2% [w/v] peptone, 2% [w/v] dextrose, 2% [w/v] agar) containing 100 μg/ml zeocin.

**Table 8.** Pipetting instructions and thermocycler program for *P. pastoris* colony PCR

<table>
<thead>
<tr>
<th>PCR master mix composition</th>
<th>Thermocycler program</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Component</strong></td>
<td><strong>Volume (μl)</strong></td>
</tr>
<tr>
<td>10X standard Taq buffer</td>
<td>2</td>
</tr>
<tr>
<td>10 mM dNTPs</td>
<td>0.4</td>
</tr>
<tr>
<td>5’ AOX1 (10 μM)</td>
<td>1</td>
</tr>
<tr>
<td>3’ AOX1 (10 μM)</td>
<td>1</td>
</tr>
<tr>
<td>Hot start Taq DNA polymerase&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.2</td>
</tr>
<tr>
<td>Template DNA</td>
<td>1</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>to 20</td>
</tr>
</tbody>
</table>

<sup>a</sup>New England Biolabs (NEB)

The fast-growing/zeocin-resistant transformants were further analyzed by colony PCR with primers 5’ AOX and 3’ AOX (Table 8). To obtain the genomic DNA template for PCR,
single colonies were picked and lysed with 0.2% hot SDS treatment (microwave oven set at full power) for 2 min. Cell debris was then pelleted by a pulse spin in a bench top centrifuge and the supernatant was directly used for PCR analysis.

4.2.8 Inducible expression of recombinant fusion hydrophobins

The colony PCR-validated *P. pastoris* transformants together with the corresponding control strains (*P. pastoris* GS115 transformed with pPIC9, *P. pastoris* X-33 transformed with pP-secSUMOpro3) were used for a small-scale trial expression of recombinant fusion proteins.

For optimal expression, *P. pastoris* transformants and control strains were initially inoculated into 25 mL buffered minimal glycerol (BMG) broth (100 mM potassium phosphate, 1.34% [w/v] yeast nitrogen base, 4 x 10^{-5} % [w/v] biotin, 1% [v/v] glycerol, pH 6.0) and grown at 30°C with shaking at 280 rpm until OD_{600} reached 2-6 (log-phase growth, approximately 16-18 hours). These cultures were centrifuged for 5 min at 3000g and pelleted cells were suspended in 50 mL buffered minimal methanol (BMM) broth (100 mM potassium phosphate, 1.34% [w/v] yeast nitrogen base, 4 x 10^{-5} % [w/v] biotin, 0.5% [v/v] methanol, pH 6.0). Cultures were grown for 96/120 h at 30°C with shaking at 280 rpm, filter-sterilized methanol was added to a final concentration of 0.5% (v/v) every 24 h. Meanwhile, 1 ml culture samples were taken at 24 h intervals for protein expression analysis. After 96 h/120 h of cultivation, culture supernatants were collected by centrifugation (8000g, 10 min, JA-14, Beckman Avanti™ J-251) and stored in sterile SCHOTT® glass bottles (Duran, Germany) at -80°C.

4.2.9 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of proteins

Tricine-SDS-PAGE with the discontinuous pH system described by Schägger (2006) was used to separate and visualize small proteins/peptides. Culture supernatant samples from the previous step were concentrated by trichloroacetic acid (TCA) precipitation, mixed with Tricine Sample Buffer (Bio-Rad) containing 125 mM dithiothreitol (DTT) and heated immediately for 10 minutes at 95°C. Spectra Multicolor Low Range Protein Ladder
Spectra Multicolor Low Range Protein Ladder (Thermoscientific) was loaded as a marker of size. Gels were run at 30 V through the stacking gel and 200 V through the separating gel. When the electrophoresis had finished, gels were stained with Silver Stain Plus Kit (Bio-Rad).

Glycine-SDS-PAGE with the discontinuous pH system originally described by Laemmli (1970) was used to separate and visualize proteins. Culture supernatant samples were concentrated by acetone precipitation, mixed with 2x Laemmli Sample Buffer (Bio-Rad) containing 355 mM 2-mercaptoethanol and heated immediately for 10 minutes at 95°C. Spectra Multicolor Low Range Protein Ladder (Thermoscientific) was loaded as a marker of size (for Western blot analysis, 7-175 kDa prestained protein marker [New England Biolabs] was used). Gels were run at 100 V through the stacking gel and 200 V through the separating gel. When the electrophoresis had finished, complete gels were stained for three hours and de-stained, using laboratory stocks of filtered Coomassie staining solution (0.04% [w/v] Coomassie Brilliant Blue R-250, 40% [v/v] methanol, 10% [v/v] glacial acetic acid) and de-staining solution (10% [v/v] glacial acetic acid), respectively.

4.2.10 Western blot analysis of the recombinant fusion hydrophobin SUMO3-Bac8c-HGFI

Western blot originally reported by Towbin et al. (1979) was used as a more sensitive and specific approach for visualizing proteins. Culture supernatant samples were initially separated with Glycine-SDS-PAGE as described in section 4.2.9. Instead of staining, proteins on SDS-PAGE gel were electro-blotted to a polyvinylidene fluoride (PVDF) membrane (Pall, Port Washington, NY, USA) with a wet-transfer apparatus (Bio-Rad) set at 30 V for 2 h. After transfer, the PVDF membrane was blocked in the blocking solution (WesternBreeze®, Invitrogen) at room temperature for 30 minutes.

Transferred proteins were probed with the primary antibody anti-Penta-His IgG1 (Mouse, 1:1000 dilution) (Qiagen, Netherlands) for 1 h at room temperature. After rinsed three times with the antibody wash solution (WesternBreeze®, Invitrogen), alkaline phosphatase (AP) conjugated Goat anti-Mouse IgG (H+L) secondary antibody (1:5000 dilution) (Pierce,
Rockford, IL, USA) was added and left to bind for 1 h and visualized with BCIP/NBT Color Development Substrate (Promega).

4.2.11 Purification of the recombinant fusion hydrophobin SUMO3-Bac8c-HGFI

The recombinant fusion hydrophobin SUMO3 [6x His-tag]-Bac8c-HGFI was purified with Ni-NTA slurry (Invitrogen) following the manufacturer’s instructions. The purified protein solution was transferred into an Amicon Ultra-15 ultrafiltration unit (Millipore) and buffer exchanged against phosphate buffered saline (PBS) solution (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) to remove imidazole and chemical impurities.

SUMO protease 2 (Lifesensors, Malvern, PA, USA) was added to the substrate (1 unit enzyme to 10-100 μg target fusion protein) to remove the SUMO3 fusion tag. The reaction was performed overnight at 4°C. In the same way, the SUMO3 fusion tag and SUMO protease 2 in the digestion mixture was removed with Ni-NTA slurry and buffer exchanged against ultrapure Milli-Q water. The purified protein solution was then lyophilized and stored at -80°C.

4.2.12 Purification of the recombinant fusion hydrophobin P11-5-linker-HGFI

Culture supernatant samples were separated with 16% Tricine-SDS-PAGE as described in section 4.2.9, instead, gel was stained with Coomassie staining solution (10% acetic acid, 40% methanol and 0.1% Coomassie Brilliant Blue-R250). The required protein band was excised from the gel and eluted with Model 422 Electro-Eluter apparatus (Bio-Rad) following the manufacturer’s instructions. In short, the excised protein gel slice was placed into an Electro-Eluter capsule and the electroelution was performed at 50 V for 12 h at 4°C in Tris-glycine buffer (25 mM Tris-HCl, 250 mM glycine, 0.1% [v/w] SDS). Eluent in the membrane cap was collected and transferred into an Amicon Ultra-4 ultrafiltration unit (Millipore) and buffer exchanged against ultrapure Milli-Q water to remove chemical impurities. The protein solution was then lyophilized and stored at -80°C.
4.2.13 Dot blot analysis of the recombinant fusion hydrophobin P11-5-linker-HGFI

The purified recombinant fusion hydrophobin P11-5-linker-HGFI was analyzed by dot blot, 2 μl protein solution was directly applied to a PVDF membrane (Pall) via a micropipette and air-dried at room temperature for at least 30 min. The PVDF membrane was then blocked in the blocking solution (WesternBreeze®, Invitrogen) at room temperature for 30 minutes.

Proteins on the PVDF membrane were probed with the primary antibody anti-rHGFI (1:1000 dilution) (from a male New Zealand white rabbit, Nankai University, China) for 1 h. After rinsed three times with the antibody wash solution (WesternBreeze®, Invitrogen), alkaline phosphatase (AP) conjugated secondary antibody anti-Rabbit IgG (Fc) (1:3000 dilution) (Promega) was left to bind for 1 h and visualized with BCIP/NBT Color Development Substrate (Promega).

4.2.14 Protein quantification

Protein quantification was performed with Modified Lowry Protein Assay Kit (Pierce, Waltham, MA, USA) following the manufacturer’s instructions.

4.2.15 Minimum inhibitory concentration test

Minimum inhibitory concentrations (MICs) were determined using a modified microplate dilution method described by Wiegand et al. (2008). Reference bacteria were grown to a mid-log phase (OD$_{600}$ ≈ 0.4) in Mueller-Hinton broth (BD Difco™) and diluted to $2-7 \times 10^5$ CFU/ml inoculum sizes.

Antimicrobial peptides and purified fusion proteins were serial diluted to obtain final concentrations of 200, 100, 50, 25, 12.5, 6.25 and $3.1 \mu$g/ml, respectively (Qi et al. 2010; Hilpert et al. 2005). Meanwhile, bacterial growth control with no test peptides/proteins and environmental sterility control with no bacterial cells were prepared. Plates were incubated at 37°C for 24 h and bacterial growth was determined with Infinite 200 microplate reader (Tecan, Switzerland) set at 600 nm. The experiment was independently repeated two
times. MIC was defined as the lowest concentration that needed to reduce bacterial growth by more than 50% after 24 hours (Wiegand et al. 2008).

### 4.2.16 Tools for data analyses

1. Thermoscientific Tm calculator was used to predict the annealing temperature for PCR or sequencing.
2. NEBcutter V2.0 was used to determine the potential endonuclease cleavage sites of vectors.
3. NCBI Nucleotide BLAST was used to generate sequence alignments for DNA sequence comparison.
5 RESULTS

5.1 Construction of pP-secSUMOpro3-bac8c-hgfI

Yield optimization of inverse fusion PCR (IF-PCR) was performed with respect to the annealing temperature via gradient PCR. In lanes 1-5, a band was apparent above 4 kbp. The no-template control in lane C did not display any bands (Figure 15).

![Figure 15. 1% agarose gel displaying inverse fusion PCR products. Lane L: Generuler™ 1 kbp DNA ladder. Lanes 1-5: gradient PCR products (from left to right, 65°C, 67°C, 69°C, 71°C, 73°C). Lane C: No-template control.](image)

The >4 kbp DNA fragment was purified from the agarose gel, self-circularized and transformed into E. coli DH5α cells. Eight zeocin-resistant transformants growing on selection plates were picked and analyzed by colony PCR with vector specific primers. In lanes 5, 6 and 9, a band was apparent below 200 bp. In lanes 2-4, 7 and 8, three bands were apparent below 1000 bp, between 1000 bp and 1200 bp, and above 1200 bp, respectively. The positive control (E. coli DH5α transformed with pP-secSUMOpro3-hgfI) in lane 1 displayed a band around 150 bp. The negative control (E. coli DH5α cells) in lane C did not show any bands (Figure 16C).
Figure 16. (A) Schematic representation of the reconstructed *P. pastoris* expression vector pP-secSUMOpro3-bac8c-hgfI. (B) The amino sequence of the recombinant fusion hydrophobin Bac8c-HGFI. (C) 3% high-resolution agarose gel (Sigma-Aldrich, Germany) displaying *E. coli* colony PCR products. Lane L: Generuler™ 100 bp Plus DNA ladder. Lane 1: positive control (*E. coli* DH5α transformed with pP-secSUMOpro3-hgfI). Lane C: negative control (*E. coli* DH5α cells). Lanes 2-9: eight zeocin-resistant *E. coli* transformants.

5.2 Expression of the recombinant fusion hydrophobin SUMO3-Bac8c-HGFI

The sequencing-confirmed expression vector pP-secSUMOpro3-bac8c-hgfI was linearized with *Pme*I and subsequently transformed into *P. pastoris* X-33 cells. Six zeocin-resistant *P. pastoris* transformants growing on selection plates were picked and analyzed by colony PCR with primers 5’AOX1 and 3’AOX1. In lanes 1-6, two clear bands were apparent above 2 kbp and above 1 kbp, respectively (Figure 17).

Figure 17. 1% agarose gel displaying *P. pastoris* colony PCR products. Lane L: Generuler™ 1 kb DNA ladder. Lanes 1-6: six zeocin-resistant *P. pastoris* X-33 transformants.
Six *P. pastoris* X-33 clones identified as positive transformants by colony PCR together with the control strain (*P. pastoris* X-33 transformed with pP-secSUMOpro3) were subjected to a small-scale trial expression. After the expression was induced with 0.5% methanol for 96 h, supernatant samples from each induced culture were collected, concentrated and analyzed by SDS-PAGE. Two protein bands with apparent molecular weights of 40 kDa and > 40 kDa were observed in all six *P. pastoris* X-33 transformants (lanes 1-6, Figure 18A), but not in the control strain at the corresponding positions (lane C, Figure 18A). Western blot showed a protein with apparent molecular weight of 80 kDa could react positively (purple band) with the protein specific primary antibody, which was observed in all six *P. pastoris* X-33 transformants (lanes 1-6, Figure 18B), but not in the control strain at the corresponding position (lane C, Figure 18B).

**Figure 18.** (A) Coomassie stained 12% SDS-PAGE analysis of the recombinant fusion hydrophobin SUMO3-Bac8c-HGFI expressed in *P. pastoris* X-33. Lane L: Spectra™ multicolor low range protein ladder. Lanes 1-6: six *P. pastoris* X-33 transformants. Line C: control strain (*P. pastoris* X-33 transformed with pP-secSUMOpro3). (B) Western blot analysis of the recombinant fusion hydrophobin SUMO3-Bac8c-HGFI expressed in *P. pastoris*. Lane L: Prestained protein marker (7-175 kDa, NEB). Lane C: control strain (*P. pastoris* X-33 transformed with pP-secSUMOpro3). Lanes 1-6: six *P. pastoris* X-33 transformants. Primary antibody: 1:1000 anti-Penta·His IgG1 (Mouse). Secondary antibody: 1:3000 Goat anti-Mouse IgG (H+L), AP conjugate. Color development substrate: NBT/BCIP.

### 5.3 Minimum inhibitory concentrations of the recombinant fusion hydrophobin Bac8c-HGFI

The SUMO3 fusion tag was removed from the fusion protein SUMO3-Bac8c-HGFI by protease digestion followed by an affinity chromatography cleaning step. Minimum inhibitory concentrations (MICs) of the antimicrobial peptide Bac8c and the recombinant
fusion hydrophobin Bac8c-HGFI against *E. coli*, *S. aureus*, *P. aeruginosa* and *M. luteus* were measured. A summary of results can be found in Table 9. The MIC of Bac8c was 3.1 μg/ml against *E. coli*, *S. aureus* and *M. luteus*, respectively, and 12.5 μg/ml against *P. aeruginosa*. There was no detectable growth inhibition effect of the recombinant fusion hydrophobin Bac8c-HGFI in all tested bacteria strains.

**Table 9.** Minimum inhibitory concentrations (μg/ml) of the recombinant fusion hydrophobin Bac8c-HGFI and the antimicrobial peptide Bac8c

<table>
<thead>
<tr>
<th>Name</th>
<th><em>E. coli</em></th>
<th><em>P. aeruginosa</em></th>
<th><em>S. aureus</em></th>
<th><em>M. luteus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Bac8c</td>
<td>3.1</td>
<td>12.5</td>
<td>3.1</td>
<td>3.1</td>
</tr>
<tr>
<td>Bac8c&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2</td>
<td>8</td>
<td>2</td>
<td>N.A.</td>
</tr>
<tr>
<td>Bac8c-HGFI</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values were obtained from Hilpert et al. 2005.


**5.4 Construction of pPIC9-p11-5-linker-hgfl**

The double-stranded oligo (ds oligo) fragment containing the encoding sequence of the antimicrobial peptide P11-5 was ligated into the expression vector pPIC9-linker-hgfI and transformed into *E. coli* DH5α cells. Seven ampicillin-resistant colonies growing on selection plates were picked and analyzed by colony PCR with vector specific primers. In lanes 2-8, a band was apparent below 200 bp. The positive control (*E. coli* DH5α transformed with pPIC9-linker-hgfI) in lane 1 displayed a band around 150 bp. The negative control (*E. coli* DH5α cells) in lane C did not show any bands (Figure 19C).

![Figure 19.](image)

(A) Schematic diagram of the reconstructed *P. pastoris* expression vector pPIC9-p11-5-linker-hgfI. (B) The amino acid sequence of the recombinant fusion hydrophobin P11-5-linker-HGFI. (C) 3% high-resolution agarose gel displaying the colony PCR products. Lane L: Generuler™ 100 bp Plus DNA...
ladder. Lane 1: positive control (E. coli DH5α transformed with pPIC9-linker-hgfl). Lanes 2-8: seven ampicillin-resistant E. coli transformants. Lane C: Negative control (E. coli DH5α cells).

5.5 Expression of the recombinant fusion hydrophobin P11-5-linker-HGFI

The sequencing-confirmed expression vector pPIC-p11-5-linker-hgfl was linearized with StuI and transformed into P. pastoris GS115 cells. Six fast-growing P. pastoris GS115 transformants on MM agar plates were picked and analyzed by colony PCR with primers 5’AOX1 and 3’AOX1. In lanes 1-6, two clear bands were apparent above 2 kbp and above 750 bp, respectively (Figure 20).

![Figure 20](image)

Figure 20. 1% agarose gel displaying the colony PCR amplification products. Lane L: Generuler™ 1kb DNA ladder. Lanes 1-6: Six P. pastoris GS115 transformants.

Six P. pastoris GS115 clones identified as positive transformants by colony PCR together with the control strain (P. pastoris GS115 transformed with pPIC9) were subjected to a small-scale trial expression. After the expression was induced with 0.5% methanol for 96 h, supernatant samples from each induced culture were collected, concentrated and analyzed by Tricine-SDS-PAGE. A band with apparent molecular weight of <15 kDa was observed in all six P. pastoris GS115 transformants (lanes 1-6, Figure 21A), but not in the control strain at the corresponding position (lane C, Figure 21A). Dot blot analysis showed that the eluent from the <15 kDa protein band could react positively (purple) with the protein specific antibody (Figure 22).
Figure 21. (A) Silver-stained 16% Tricine-SDS-PAGE analysis of the recombinant fusion hydrophobin P11-5-linker-HGFI expressed in *P. pastoris* GS115. Lane L: Spectra™ multicolor low range protein ladder. Lanes 1-6: six *P. pastoris* GS115 transformants. Line C: control strain (*P. pastoris* GS115 transformed with pPIC9). (B) Silver-stained 4-15% SDS-PAGE (Mini-PROTEAN® TGX™ Precast Protein Gel, Bio-Rad) analysis of the recombinant fusion hydrophobin P11-5-linker-HGFI expressed in *P. pastoris* GS115 transformant one. Lane L: Spectra™ multicolor low range protein ladder. Lanes 1-5: culture supernatant samples collected after 24, 48, 72, 96, 120 hours of induction, respectively. Lane C: control strain (*P. pastoris* GS115 transformed with pPIC9).

Figure 22. Dot blot analysis of the eluent from the <15 kDa band. 1: *P. pastoris* GS115 transformant one. Dot 2: *P. pastoris* transformant two. Dot C: control strain (*P. pastoris* GS115 transformed with pPIC9). Primary antibody: anti-rHGFI antibody (rabbit source). Secondary antibody: Anti-Rabbit IgG (Fc), AP conjugate. Color development substrate: NBT/BCIP.

Meanwhile, time course analysis to determine the optimal induction time for the production of P11-5-linker-HGFI was performed, daily supernatant samples of transformant one were collected and analyzed by SDS-PAGE. A protein band with apparent molecular weight of <15 kDa was observable after 24 h, increasing in intensity up to 96 h or 120h (Figure 21B).

5.6 Minimum inhibitory concentration of the recombinant fusion hydrophobin P11-5-linker-HGFI

Minimum inhibitory concentration (MICs) of the antimicrobial peptide P11-5 and the recombinant fusion hydrophobin P11-5-linker-HGFI against *E. coli* were measured. A
summary of results can be found in Table 10. The MIC of P11-5 was 3.1 μg/ml against *E. coli*. The MIC of the fusion protein P11-5-linker-HGFI was 100 μg/ml against *E. coli*.

**Table 10.** Minimum inhibitory concentrations (μg/ml) of the recombinant fusion hydrophobin P11-5-linker-HGFI and the antimicrobial peptide P11-5

<table>
<thead>
<tr>
<th>Name</th>
<th>E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td>P11-5</td>
<td>3.1 (3.1μM)</td>
</tr>
<tr>
<td>P11-5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.1</td>
</tr>
<tr>
<td>P11-5-linker-HGFI</td>
<td>100 (9.1μM)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Value was obtained from Qi et al., 2010. N.D.: no detectable.

As shown in Figure 23, abnormal cell clumps were observed, when *E. coli* cells were treated with the fusion protein P11-5-linker-HGFI at concentrations (25 and 50 μg/ml) below the MIC (100 μg/ml). The treated *E. coli* culture had a cloudy and yellowish appearance.

![Figure 23](image)

**Figure 23.** Light microscopic images of *E. coli* ATCC 9027 untreated, and treated with 50 μg/ml and 25 μg/ml purified recombinant fusion hydrophobin P11-5-linker-HGFI for 24 h. Macroscopic appearances of *E. coli* culture untreated and treated with 50 μg/ml purified recombinant fusion hydrophobin P11-5-linker-HGFI for 24 h.
6 DISCUSSION

6.1 Molecular cloning, expression and characterization of the recombinant fusion hydrophobin Bac8c-HGFI

In this study, *Pichia pastoris* X-33 strain was used to express the recombinant fusion hydrophobin Bac8c-HGFI, as it secretes extremely low levels of endogenous proteins, which in turn could facilitate purification of the expressed fusion protein from the culture medium (Cereghino and Cregg 2000). More importantly, being a fungus, *P. pastoris* shares the similar post-translation modification (PTM) strategies and extended glycosylation modes of hydrophobins as *Grifola frondosa* (Wang et al. 2010b), and thus may ensure that the hydrophobin HGFI domain of the fusion protein Bac8c-HGFI could be processed to its optimally active form (Niu et al. 2012a; Huang et al. 2013).

Heterologous expression of antimicrobial peptides is facing two challenges. Firstly, the peptide’s antimicrobial nature makes it potentially lethal to the expression host (Parachin et al. 2012; Li 2009). Secondly, the peptide’s linear structure and high base amino acid content makes it highly vulnerable to proteolytic degradation in all steps of the expression (Raimondo et al. 2005). Thus, in order to achieve successful heterologous expression, the antimicrobial peptide is usually fused to a chaperone protein to mask its cytotoxicity against the host cells (Lee et al. 2000; Parachin et al. 2012). To date, many fusion chaperone proteins have been used successfully to express a wide range of host-unfavorable antimicrobial peptides, including thioredoxin (Xie et al. 2013), small ubiquitin-like modifiers (SUMO) (Li et al. 2013; Wang et al. 2014) and phosphoribosyltransferase (PurF) (Lee et al. 2000). Following this approach, the reconstructed *P. pastoris* expression vector pP-secSUMOpro3-hgfI that carries the human SUMO3 gene and the hgfI gene isolated from *Grifola frondosa* (GenBank Accession NO.EF486307) was used to achieve secretory expression of the recombinant fusion hydrophobin Bac8c-HGFI in *P. pastoris*.

Inverse fusion PCR cloning (IFPC) is a seamless in-frame cloning strategy, requires simple starting materials, one step routine PCR, and generally results in the insertion of the
foreign gene into a wide range of plasmids with high success rates (Spiliotis 2012). To perform a successful IFPC, high-fidelity DNA polymerases (with proofreading activity) that generate blunt-ended PCR products should be used, since blunt ends are mandatory for the final self-circularization step (Garrity and Wold 1992; Spiliotis 2012). In this study, the encoding sequence of the antimicrobial peptide Bac8c (bac8c) was successfully cloned into the vector (pP-secSUMOpro3-hgfI) backbone by IFPC, which was confirmed by colony PCR as the corresponding band (174 bp fragment) was observed by gel electrophoresis (lanes 5, 6 and 9, Figure 16C) and sequencing. Although the primer pair (hgfI and bac8c) used in this study was calculated (Tm Calculator, Thermoscientific) to have an average annealing temperature of 72°C, PCR with either lower or higher annealing temperatures did also provide very good amplification performances (Figure 15), demonstrating that IFPC could be successfully carried out even under some non-ideal conditions.

On the other hand, two drawbacks of IFPC emerged in this study: (1) non-specific PCR amplification products formed (data not shown) probably due to primer mismatching (Arnheim and Erlich 2000; Spiliotis 2012). This problem was successfully solved with a hot start PCR protocol to allow for more specific primer binding during the annealing step (Zhang and Gurr 2000); (2) high background of false positive clones (5/8, lanes 2-4 and 7-8, Figure 16C), which was likely due to poly-circularization of the blunt-ended PCR products.

To perform a reliable colony PCR screening of P. pastoris positive transformants, the proposed protocols usually involve a lyticase pretreatment followed by extraction and purification of genomic DNA template for PCR analysis (Linder et al. 1996; Lin et al. 2012). However, in practice, preparation of genomic DNA template is either tedious or time-consuming (Haaning et al. 1997). Instead of this, I developed a hot SDS method for rapid preparation of crude genomic DNA template for PCR analysis (section 4.2.7). This method was reliably used in the whole study (Figure 17 and 20). The colony PCR results confirmed the integration of pP-secSUMO3pro-bac8c-hgfI into all six P. pastoris X-33
transformants tested (1093 bp fragment, Figure 17). Meanwhile, all six transformants were also confirmed with the phenotype Mut\(^+\) (2.2 kbp fragment, Figure 17).

Dimerization and tetramerization of hydrophobins in an SDS-PAGE gel due to the self-assembly of hydrophobins into stable polymers in aqueous solutions (de Vries et al. 1993) was reported in many studies with hydrophobins (Hakanpää et al. 2006; Kisko et al. 2008; Wang et al. 2004; Szilvay et al. 2007). In the expression study, two unique proteins with apparent molecular masses of 40 kDa and >40 kDa were identified in all P. pastoris X-33 transformants (Figure 18A). These two values were inconsistent with the theoretical value (21 kDa) of the fusion protein SUMO3-Bac8c-HGFI, which would probably be a dimer (40 kDa \(\approx\) 2 x 21 kDa) and a tetramer of the fusion protein SUMO3-Bac8c-HGFI. The >40 kDa protein could react positively with the fusion protein specific primary antibody (Figure 18B). These results indicate that the fusion protein SUMO3-Bac8c-HGFI was expressed and secreted into the culture medium, but subsequently self-assembled into polymers.

Protease cleavage of the SUMO3 fusion tag followed by an affinity chromatography cleaning step was not successful as expected, only 1.04 mg fusion protein Bac8c-HGFI was recovered from 100 mL culture broth (data not shown), probably because the purification protocol (more details can be seen in the Ni-NTA purification system user manual [25-0496], Invitrogen) that I used has not been optimized for hydrophobins yet. In the following minimum inhibitory concentration test, the results showed that the highly active antimicrobial peptide Bac8c became inactivated when it was fused with the hydrophobin HGFI (Table 9). Similarly, in an earlier study, Rieder et al. (2014) found that a highly active antimicrobial peptide became inactivated when it was fused with the class I hydrophobin DewA of Aspergillus nidulans. To the best of my knowledge, steric hindrance between the adjacent domains (Bac8c and HGFI) might have been responsible for this inactivation (Linder et al. 2002; Iwanaga et al. 2011).
6.2 Molecular cloning, expression and characterization of the recombinant fusion hydrophobin P11-5-linker-HGFI

Flexible polypeptide linkers, generally composed of small, non-polar amino acids (e.g. glycine, serine and threonine) are often used in fusion protein studies when the adjacent domains require a certain degree of spacing to reduce unfavorable inter-domain interferences (Chen et al. 2013b). Following this approach, a 10-mer flexible polypeptide linker (NH₂-GGGGSGGGGS), which has been used successfully in an earlier fusion hydrophobin study (Niu et al. 2012a) was employed to construct the recombinant fusion hydrophobin P11-5-linker-HGFI.

The double-stranded oligo (ds oligo) fragment (p11-5) encoding the antimicrobial peptide P11-5 was successfully subcloned into the expression vector pPIC9-linker-hgfI, which was confirmed by colony PCR as the corresponding band (188 bp fragment) was observed by gel electrophoresis (lanes 2-9, Figure 19C) and sequencing. When the p11-5 fragment and the hgfI gene were fused, the linker sequence was laid between them, in order to reduce the steric hindrance between the adjacent peptide (P11-5) and protein (HGFI), when the fused gene (p11-5-linker-hgfI) was translated into protein (Figure 19B). In addition, the innate nucleotide sequence encoding the STE13 protease cleavage site (Glu-Ala-Glu-Ala) was deleted when the p11-5 fragment was inserted into the XhoI site of pPIC9-linker-hgfI. Although Glu-Ala repeats has been recommended in many studies, in order to ensure that the protein secretory signal (in this study, α-MF of saccharomyces cerevisiae) could be processed correctly in the endoplasmic reticulum of P. pastoris (Sreekrishna and Kropp 1996). However, there are numerous cases where STE13 cleavage of Glu-Ala repeats is not sufficient enough as expected, and thus Glu-Ala repeats are left on the N-terminus of the expressed protein (Kim et al. 1997; Emberson et al. 2005), which in turn may pose a negative effect on the activity and stability of the expressed protein (Wu and Hancock 1999). For example, Cabral et al. (2013) found that the P. pastoris produced Pisum sativum defensin 1 (Psd1), which contained Glu-Ala repeats on its innate N-terminal region presented at least 10-fold decrease in antifungal activity, as compared with the native counterpart. In this study, the KEX2 protease cleavage site (Lys-Arg) was designed to be
located after the XhoI site, in order to ensure that the fusion protein P11-5-linker-HGFI could be secreted into the culture medium with an intact N-terminus.

The colony PCR results confirmed the integration of pPIC9-p11-5-linker-hgfl into all six P. pastoris GS115 transformants tested (784 bp fragment, Figure 20). All tested transformants were also confirmed with the phenotype Mut⁺ (2.2 kbp fragment, Figure 20). In the expression study, all six P. pastoris GS115 transformants could produce a unique protein with apparent molecular mass of <15 kDa (Figure 21A). This value was inconsistent with the theoretical value (10.3 kD) of the fusion protein P11-5-linker-HGFI. This phenomenon was similar with those found in earlier studies (Tagu et al. 2001; Wang et al. 2010a). For example, Wang et al. (2010a) found that the apparent molecular weight (14 kDa) of the recombinant hydrophobin HGFI was slightly higher than its theoretical size (10.9 kDa). This change in gel mobility is proposed to be due to an insufficient reduction of the intramolecular disulfide bonds within hydrophobins during the sample pretreatment step, thus resulting in a retarded gel mobility (Cumming et al. 2004; Tagu et al. 2001). The eluent from the <15 kDa protein band could react positively with the primary antibody raised against the hydrophobin HGFI (Figure 22), which indicates that the fusion protein P11-5-linker-HGFI was successfully expressed and secreted into the culture medium.

The antibacterial activity of the antimicrobial peptide P11-5 against E. coli as determined in this study was well conformed to the literature value (Table 10). In contrast, the peptide represented 3-time reduced antibacterial activity when it was fused with the hydrophobin HGFI, in terms of the molecular concentration (Table 10). It is well acknowledged that the length of polypeptide linker may affect the cytoplasmic membrane penetration efficiency of tethered antimicrobial peptides (Qi et al. 2011). Many of the existing studies have demonstrated that a tethered antimicrobial peptide would exhibit better activities, if a long and flexible polypeptide linker is used (Bagheri et al. 2009). More specifically, Robinson et al. (1998) concluded that polypeptide linkers of more than 10-mer in length are excellent candidates for fusion proteins. In this fusion protein construct, a relatively short
polypeptide linker (10-mer) was used, which may still not sufficient long enough to prevent the reciprocal interference between the adjacent domains.

There is currently no clear explanation for this abnormal aggregation behavior of *E. coli* cells, when they were treated with the fusion protein P11-5-linker-HGFI at concentrations that below the MIC (Figure 23). It is well known that the cell-wall-associated hydrophobins play an important role in facilitating the aggregation of filamentous hyphal cells into large clumps (Talbot 1997). Based on this fact, Nakari-Setälä et al. (2002) engineered the cell wall of *Saccharomyces cerevisiae* with the class II hydrophobin HFBI of *Trichoderma reesei*, increased cell-cell adhesion (clumping) ability was observed among these hydrophobin-modified yeast cells, as compared with the unmodified counterpart. The proposed cell-killing mechanism of the antimicrobial peptide P11-5 is the ‘carpet-like’ mode of action. Therefore, if the concentration of peptides is not sufficient enough to disintegrate the bacterial cytoplasmic membrane, they are bound parallel onto the bacterial cytoplasmic membrane (Brogden 2005), thus leading to the immobilization of the attached hydrophobins on the bacterial surface. In the aqueous environment, these bacterial surface immobilized hydrophobins would self-assembly into polymers, and thereby resulting in the aggregation of bacterial cells.

### 6.3 Future work: specific-protease-enhanced antibacterial fusion hydrophobins

Many pathogenic microorganisms possess unique proteases to invade host organisms (Aoki and Ueda 2013). For example, *Listeria monocytogenes*, the bacterium that causes the invasive infection listeriosis, secretes a stress-induced serine protease that plays a crucial role in intracellular survival of this pathogen (Gaillot et al. 2000). On the basis of this, designing fusion antimicrobial peptides that can be activated by species-specific proteases may provide high selectivity toward each target pathogenic microorganism. Following this postulation, Aoki et al. (2012) designed a fungicidal fusion protein (Figure 24) that could be exclusively activated by a unique aspartic protease of *Candida albicans*. This peptide showed a highly selective *in vitro* activity against *C. albicans*, but not against other tested microorganisms, which do not possess the corresponding aspartic protease.
Figure 24. Schematic diagram of the aspartic protease-activated fungicidal fusion protein. This peptide is composed of three functional domains: an antimicrobial peptide, a protective peptide and a protease-cleavable linker. Generally, the fungicidal activity of the tethered antimicrobial peptide is inhibited by the protective peptide. Species-specific protease cleaves the linker and subsequently releases the antimicrobial peptide, causing its activation (Figure adapted from Aoki and Ueda, 2013).

The potential cleavage site analysis of the fusion protein P11-5-linker-HGFI demonstrated that this protein contains an endoprotease AspN cleavage site, which is located in the C-terminal region of the antimicrobial peptide. Therefore, upon the enzymatic digestion with AspN, the antimicrobial peptide P11-5 could be neatly released from the fusion protein. In the future work, once we confirmed that the enzymatic digestion could improve the antibacterial activity of the fusion protein. By using the design approach described by Aoki et al. (2012), a series of specific-protease-enhanced antimicrobial hydrophobins could thus be designed.
7 CONCLUSIONS

In summary, two recombinant fusion hydrophobins, Bac8c-HGFI and P11-5-linker-HGFI were successfully expressed and secreted into the culture medium. Similar to hydrophobins, the fusion protein Bac8c-HGFI could form stable polymers in the aqueous environment. The highly active antimicrobial peptide Bac8c became inactivated when it was fused with the hydrophobin HGFI. Interestingly, the hydrophobin HGFI gained an acquired antibacterial nature when it was fused with the antimicrobial peptide P11-5 through a 10-mer flexible polypeptide linker, with the minimum inhibitory concentration of 100 μg/ml against *Escherichia coli*. However, there is currently no clear explanation for the abnormal aggregation behavior of *E. coli* cells in this study. Further experiments are needed to clarify this issue.
8 REFERENCES


