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MINI-REVIEW

Engineering of protein secretion in yeast: strategies and impact on protein production

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Abstract Yeasts combine the ease of genetic manipulation and fermentation of a microorganism with the capability to secrete and modify foreign proteins according to a general eukaryotic scheme. Their rapid growth, microbiological safety, and high-density fermentation in simplified medium have a high impact particularly in the large-scale industrial production of foreign proteins, where secretory expression is important for simplifying the downstream protein purification process. However, secretory expression of heterologous proteins in yeast is often subject to several bottlenecks that limit yield. Thus, many studies on yeast secretion systems have focused on the engineering of the fermentation process, vector systems, and host strains. Recently, strain engineering by genetic modification has been the most useful and effective method for overcoming the drawbacks in yeast secretion pathways. Such an approach is now being promoted strongly by current post-genomic technology and system biology tools. However, engineering of the yeast secretion system is complicated by the involvement of many cross-reacting factors. Tight interdependence of each of these factors makes genetic modification difficult. This indicates the necessity of developing a novel systematic modification strategy for genetic engineering of the yeast secretion system. This mini-review focuses on recent strategies and their advantages for systematic engineering of yeast strains for effective protein secretion.

Keywords Yeast secretion system · Secretion pathway · Protein folding · Membrane trafficking · Protease · Glycosylation

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Introduction

Microorganisms are used widely as producers of heterologous proteins of medical or industrial interest. Bacteria are the most efficient producers, but they are not able to perform some post-translational processing (folding, glycosylation, phosphorylation, or removal of part of their initial sequence) of eukaryotic proteins. Eukaryotic yeasts are able to do the post-translational processing and secrete heterologous eukaryotic proteins in their native, biologically functional form. The rapid growth and high-density fermentation of yeast in chemically defined media have a high impact particularly in the large-scale industrial production of foreign proteins, where secretory expression is important for simplifying the downstream protein purification process (Porro et al. 2005). Therefore, the intrinsic commercial value of heterologous proteins has driven a wide range of studies of the optimization of yeast secretion systems as "cell factories." As a result, currently, many types of FDA-approved therapeutic proteins are being produced using yeasts (Rader 2007; Schmidt 2004).

However, secretory expression of heterologous proteins in yeast is often limited at comparatively low levels. Factors affecting titer of heterologous protein secretion include the properties of the target protein, the host strain and its

cultivation condition, vector system, promoter choice, codon usage, leader sequences, translation signals, processing and folding, and secretion (Niebaur and Robinson 2005). Thus, many studies on the yeast secretion systems have focused on engineering of the fermentation process, expression vector systems, and host strains in order to improve two different volumetric yields, cell titer and cell specific productivity, respectively (Palomares et al. 2004). If any one of above factors is suboptimal in protein secretion, it can create a bottleneck that leads to poor production yields (Mattanovich et al. 2004). Therefore, often it has been found that secretion titers of yeast are 100- to 1,000-fold lower than the theoretically estimated range (Schröder 2007). Improvements in the secretion titer of some heterologous proteins from milligrams to grams per liter have been achieved over the past decade. This is due mainly to optimization of the fermentation process, which includes increased cell density or reduced protein degradation by control of pH and temperature and optimization of the expression vector system. However, the wide range of cell specific secretion titers for different proteins in yeast still raises the important question as to whether host- or protein-based factors limit the cell specific protein secretion levels. Molecular engineering of the secretory target proteins to optimize their properties for effective secretion is difficult and crucial in most cases; thus, current studies on the improvement of cell specific secretion titers have shifted to focus on host selection and engineering.

Selection of the most suitable host based on host-protein compatibility is an initial important step, which helps to overcome drawbacks that may occur as a result of host-type-specific limitation in single-cell specific secretory productivity (Greene 2004). For such a purpose, distinct yeast secretion systems have been established (Böer et al. 2007; Gellissen et al. 2005a; Müller et al. 1998). The initial yeast system that was used for heterologous protein secretion was based on the baker's yeast *Saccharomyces cerevisiae*, which led to successful production of FDA-approved pharmaceuticals such as insulin (Melmer 2005) and hepatitis B surface antigen (Harford et al. 1987). More recently, defined systems have included fission yeast *Schizosaccharomyces pombe* (Giga-Hama 1997; Giga-Hama and Kumagai 1999; Giga-Hama et al. 1994, 2007; Takegawa et al. 2009), methylotrophic species *Candida boidinii* (Sakai et al. 1996), *Pichia methanolica* (Raymond et al. 1998), *Pichia pastoris* (Ilgen et al. 2005), and *Hansenula polymorpha* (Gellissen 2000; Kang and Gellissen 2005), *Klyveromyces lactis* (Gellissen and Hollenberg 1997), and the dimorphic species *Arxula adeninivorans* (Böer et al. 2005) and *Yarrowia lipolytica* (Madzack et al. 2005). These different yeast systems could be useful for confirming host-protein compatibility by simple screening, which then may result in savings in time and resource use. Recently, a wide-range vector system named CoMed™ has been established, which may be able to assess several yeast platforms in parallel to select the best host system (Gellissen et al. 2005b; Steinborn et al. 2006).

Protein-based host selection and promoter/signal-peptide optimization are the simplest initial approaches, and often yield 3–10-fold cell specific secretion improvements over wild-type strains, and are almost always protein specific. However, many proteins are still secreted only at comparatively low levels even though the transcription or translation level of the target protein is optimized sufficiently for overexpression in the most suitable host system (Macauley-Patrick et al. 2005; Porro et al. 2005; Punt et al. 2002; Schröder 2007). This implies that heterologous protein secretion is not only a simple homogeneous protein synthesis process but also involves many other co-factors such as co- or post-translational translocation of nascent proteins into the endoplasmic reticulum (ER), protein folding and quality control inside the ER, post-translational glycosylation in the ER and Golgi apparatus, intracellular protein trafficking and sorting, proteolytic degradation, and stress response for misfolding or overexpression. Therefore, strain engineering by genetic modification has become the most useful and effective method to overcome such drawbacks in the cell specific productivities. Such an approach is now being promoted strongly by current post-genomic technology and system biology tools, which has made it possible to modify hosts almost on theoretical grounds.

The current strategy for strain engineering for protein secretion is focused mainly on four topics: (1) engineering of protein folding and quality control system in the ER, (2) engineering of the intracellular protein trafficking pathway, (3) minimization of post-secretory proteolytic degradation, and (4) engineering of post-translational glycosylation (which is particularly required for producing pharmaceutical glycoproteins). This mini-review will deal with such cell engineering strategies trying to improve cell specific productivities and not primarily vector/fermentation optimization, which are well-established (recently reviewed by Böer et al. 2007).

Engineering of protein folding and quality control in ER

Secretory proteins start their journey on the intracellular secretory pathway by co- or post-translational translocation through the Sec61 translocon into the crowded environment of the ER lumen, where they are folded into their native structure via the ER-resident protein-folding machinery, which is under strict quality control (QC) (Anelli and Sitia 2008; Dobson 2004; Ellgaard and Helenius 2003). After translocation into the ER, nascent polypeptides are bound

by the ER-resident chaperone protein binding protein (BiP; encoded by *Kar2*) for folding to native structures, whereas the nascent glycoproteins are bound by the ER chaperone calnexin (encoded by *CNE1*) to undergo their correct folding and *N*-glycan processing. The ER sustains a set of covalent modifications, which include signal sequence processing, disulfide bond formation, *N*-glycosylation, glycosyl-phosphatidyl-inositol addition, degradation, and sorting.

Following the substantial folding and modification process in the ER, only properly folded and assembled proteins can be exported from the ER to the Golgi apparatus, where they are further modified, before being transported to the extracellular space, vacuoles or other organelles (Klausner 1989). Meanwhile, misfolded or aggregated proteins in the ER are recognized by the QC system, which leads to binding of the proteins by the BiP complex and eventual redirection to the cytosol for degradation, namely ER-associated protein degradation (ERAD) (Yoshida 2007). Prolonged binding of BiP to partially misfolded proteins leads to the induction of unfolded protein response (UPR), which stimulates proteolysis by ERAD and inhibits the transcription and translocation of the target protein. Thus, ER-resident protein folding and the QC system mainly involves five components: (1) molecular chaperones (e.g., BiP, calnexin, and calreticulin) that assist protein folding, (2) enzymes such as protein disulfide isomerases (PDIs) and *cis–trans* peptidyl prolyl isomerases (PPIs), (3) degradation machinery linked with ERAD, (4) signal transduction pathways linked with UPR, and (5) post-translational modification enzymes related to glycosylation. As a result of such strict QC systems in the ER, protein folding often has a tendency to become the most rate-limiting bottleneck in heterologous protein secretion. Thus, genetic modification of the ER protein folding and QC system has become the most useful approach in the current strain engineering method.

The complexity and stringency of the protein folding and QC system in the ER are supported by numerous genes; therefore, their modification is not simple (Gasser et al. 2009; Travers et al. 2000). As a straightforward strategy, overexpression of multiple chaperones, PDIs, and other folding helpers seems to be an effective approach. Some studies have emphasized that overexpression of the chaperone BiP, a member of the Hsp70 family of ATPases, stimulates protein secretion in *S. cerevisiae*, for example, a 5-fold increase in secretion of human erythropoietin (Robinson et al. 1994) and a 26-fold increase in bovine prochymosin (Harmsen et al. 1996). Moreover, in some cases, reduction of BiP levels leads to decreased secretion of foreign proteins (Robinson et al. 1996). However, it has been found that the overexpression effect of BiP is protein or host specific and thus not beneficial in all cases, although promising expectations have emerged that increased BiP levels could result in increased folding capacity in the ER and improved secretion rates. Some studies even have suggested a negative impact of BiP overexpression, as extracellular levels of *Aspergillus niger* glucose oxidase decreased 10-fold upon BiP overexpression in *H. polymorpha* (van der Heide et al. 2002). Overexpression of BiP seems to stall the activity of other chaperone mechanisms, because of the hierarchy of the ER chaperone system, and attenuates UPR. BiP overexpression increases the potency of prolonged binding between BiP and its target proteins, which leads to ERAD rather than protein secretion. This may explain why the overexpression of BiP chaperone alone does not result in increased secretion, but can negatively influence it (Kauffman et al. 2002; van der Heide et al. 2002).

BiP plays a role in all known functions of the ER, including gating the translocon, folding nascent proteins, targeting misfolded proteins for degradation, regulating the unfolded protein response, and contributing to ER calcium stores. All except the last of these activities require BiP's ATPase activity, which is regulated by DnaJ co-chaperones and other cofactors like Sls1p/Sil1p (Kabani et al. 2000; Tyson and Stirling 2000) and Lhs1p (Steel et al. 2004). By single or multiple overexpression of the co-chaperones Jem1p (a DnaJ-homolog), Sil1p, Lhs1p, and Scj1p, secretion levels of recombinant human albumin (rHA), granulocyte–macrophage colony-stimulating factor (GM-CSF), and recombinant human transferrin (rTf) were significantly enhanced in *S. cerevisiae* (Payne et al. 2008). A synergistic increase in secretion of single-chain antibody fragments from *S. cerevisiae* has been observed with joint overexpression of BiP and PDI (Shusta et al. 1998). *Pyrococcus furiosus* β-glucosidase secretion in *S. cerevisiae* is diminished with increased BiP levels, but benefits from higher PDI levels, although the target protein does not contain any disulfide bonds (Smith et al. 2004). This indicates the chaperone effect of PDI and complex regulation effects between different chaperones.

Next to BiP/DnaJ and their cofactors, overexpression of PDI alone increases secretion of some, but not all, heterologous proteins (Butz et al. 2003; Damasceno et al. 2006; Inan et al. 2006; Robinson et al. 1994). Duplication of PDI1 with its partner Ero1p accelerates the secretion of recombinant human albumin in *K. lactis* (Gross et al. 2004; Lodi et al. 2005). Duplication of PDI and polyubiquitin genes has a strong stimulating effect on the production of the highly disulfide-bonded HSA, but not of interleukin 1β in *K. lactis* (Bao and Fukuhara 2001). By overexpressing polyubiquitin gene *UBI4* in *S. cerevisiae,* human leukocyte elastase inhibitor secretion was enhanced 7-fold, but no effect was found for production of α-factor and of certain natural yeast extracellular enzymes (Chen et al. 1994). This

result indicates that the modification of the ubiquitin–proteasome pathway in protein secretion is protein specific.

Another approach to stimulate the secretory pathway concertedly is manipulation of the UPR pathway regulator Hac1p. Accumulation of unfolded or misfolded proteins in the ER induces UPR by Hac1p, which then activates expression of chaperones (e.g., PDI, PPI, and ERAD genes) as well as suppresses transcription of the secretory target protein (Schröder and Kaufman 2005). Interestingly, heterologous overexpression of *Trichoderma reesei Hac1* in *S. cerevisiae* yielded a 2.4-fold improvement in *Bacillus* α-amylase secretion. Overexpression of *S. cerevisiae HAC1* has been shown to enhance secretion of the endogenous invertase (2-fold), and recombinant α-amylase (70% increase), but it did not affect secretion of an ER-accumulated *T. reesei* endoglucanase EGI (Valkonen et al. 2003). Overexpression of the spliced form of *HAC1* (namely, $HAC1^{i}$) in *S. cerevisiae* stimulates secretion (1.5-fold) of three types of recombinant proteins, probably due to induction of co-chaperones Sil1p, Lhs1p, and Jem1p (Payne et al. 2008). Unexpectedly, overexpression of co-chaperone genes *LHS1*, *JEM1*, and four co-chaperone genes (*SIL1*, *LHS1*, *JEM1*, and *SCJ1*) together reduced $HAC1^{i}$ transcript levels up to approximately 2-fold. Disruption of *HAC1* in *S. cerevisiae* led to reduced secretion of the two recombinant proteins (α-amylase, 75%; EGI, 50%), but not of the endogenous invertase (Valkonen et al. 2003). These results indicate that the effect of *HAC1* overexpression is protein or host specific and complex regulation effects between Hac1p and other chaperones.

Due to complex regulation between different chaperones or folding helpers and their connection with the ERAD and UPR pathways, modification of one step can lead to rate limitation of the following one, which can then become the bottleneck of the secretory expression system. Moreover, modification effects are often protein or host specific. Therefore, co-expression of multiple chaperones and folding helpers, or targeting cytosolic or heterologous chaperone to the ER lumen may yield more consistent improvements for different heterologous proteins as recommend by Schröder (2008). Single gene introduction of *S. cerevisiae* chaperones Kar2p, Ssa1p, or PDI to *P. pastoris* improved recombinant protein secretion four to seven times, while multiple introduction of YDJ1p/PDI, YDJ1p/Sec63, and Kar2p/PDI synergistically increases secretion levels 8.7, 7.6, and 6.5 times, respectively (Zhang et al. 2006). Ideally, the combination and co-expression levels of each chaperones and folding helpers should be tuned to provide a more comprehensive assessment. Such an approach has been used successfully in filamentous fungi to identify optimal levels of BiP and Pdi1p for the production of the plant sweet protein thaumatin (Lombraña et al. 2004; Moralejo et al. 2001).

Engineering of intracellular protein trafficking pathway

Intracellular trafficking of secretory proteins in the secretion pathway is initiated with their co- or post-translational translocation into the ER lumen and completed by stepwise vesicular transport, which involves ER-to-Golgi, intra-Golgi, and post-Golgi traffic, respectively (Fig. 1). After correct folding in the ER lumen, the selective trafficking of secretory proteins between organelles in the secretory pathways is accomplished predominantly by membrane-enclosed transport vesicles. Thus, intracellular protein trafficking is also named vesicular or membrane trafficking. In such trafficking systems, newly synthesized proteins are selected and concentrated into distinct vesicle populations, which are subsequently targeted to a specific acceptor compartment. The process of directing each newly expressed protein to the cell surface—referred to as protein targeting, or protein sorting—is crucial to the organization and functioning of yeast cells. The effects of each traffic step and correct vesicular destination at each traffic crossroads are controlled by numerous intracellular membrane proteins and significantly affect the overall secretory effects. Thus, genetic optimization of traffic pathway is required particularly in the case of inefficient traffic or mis-sorting, which often results in intracellular retention or accumulation of the target proteins for secretion.

As an initial approach to engineering, the early secretory traffic pathway from cytosol to ER must be considered. In yeast, two pathways for cytosol-to-ER targeting exist: co- and post-translational pathways. Polypeptide domains of secretory proteins, termed signal peptides, are necessary, and in most cases sufficient, for such protein targeting. These signal peptides are segments of 15–50 aa comprising a central hydrophobic core, which is flanked by an N-

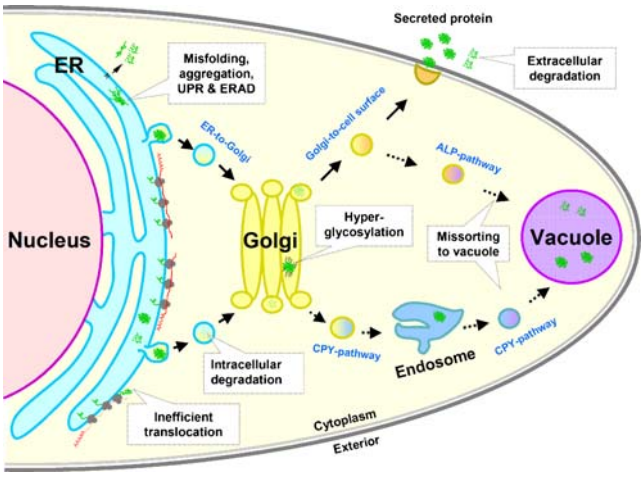

Fig. 1 A schematic diagram representing typical bottlenecks in the secretory pathway of heterologous proteins in yeast. The main membrane trafficking and vacuolar protein sorting pathways are indicated

terminal, positively charged and a C-terminal, hydrophilic region in the secretory proteins (Martoglio and Dobberstein 1998). In co-translational translocation pathway, the ribosome–nascent chain complex is transferred to the translocon channel in the ER membrane by signal recognition particle (SRP) and its receptor (SR) as soon as the N-terminal signal sequence emerges (Halic and Beckman 2005; Kida et al. 2007; Shan and Walter 2005). However, the post-translational translocation pathway seems to be used by a larger fraction of yeast secretory proteins, which possess only moderately hydrophobic signal sequences that cause them to escape recognition by the SRP during their synthesis in cytosol (Rapoport 2007). These post-translational secretory proteins need to remain unfolded or loosely folded in cytosol after their release from the ribosome by binding with cytosolic chaperones and their cofactors until post-translational translocation across the translocon channel. This may imply overexpression effect of some cytosolic chaperones and ribosome-binding factors in secretion improvement (Gasser et al. 2007).

In the both co- and post-translational translocation routes in the early secretory pathway, protein targeting specificity is mainly conferred by the hydrophobic core of the secretory signal sequences and its interaction compatibility with SRP in cytosol (Kida et al. 2009; Ng et al. 1996; Plath et al. 1998). Thus, many types of N-terminal secretory signal sequences have been developed for each host system and yeast pre-pro-sequences (e.g., mating pheromone α-factor signal MFα1) are used most commonly (Brake et al. 1984; Fuller et al. 1989). The pre- and pro-cleavage sites in the signal peptides are processed stepwise in the ER and Golgi apparatus, respectively, during trafficking, and make it easy to confirm traffic flow of the target protein from the cytosol to the Golgi apparatus via the ER. In the fission yeast *S. pombe*, heterologous proteins of *Escherichia coli* phytase, human lysosomal acid lipase, and interleukin-6 were secreted at high yields using a pre–pro-secretory signal peptide P4, which is derived from the fission yeast mating pheromone P-factor precursor (Giga-Hama 1997). This signal sequence (P3 or P4) includes a pre–pro cleavage region and a dibasic site, Lys-Arg, that is recognized by endoprotease in the Golgi apparatus. In contrast, cellulases of the fungus *T. reesei* are secreted at high levels when using their own signal sequences but not with the P4 signal (Okada et al. 1998a, b). By comparing different signal peptides for secretion of green fluorescent protein (GFP) and other heterologous proteins in fission yeast, the signal sequence of Cpy1p, a vacuolar carboxypeptidase Y (CPY), exhibited better secretion than the P-factor signal (Kjaerulff and Jensen 2005). Recently, using the viral K28 preprotoxin (*pptox*) signal peptide, which is a viral secretion signal peptide derived from the K28 preprotoxin precursor of the yeast K28 virus toxin, GFP was secreted from four yeast species, *Candida glabrata*, *P. pastoris*, *S. cerevisiae*, and *S. pombe*, which indicated the potential of the viral signal peptides as unique tools in recombinant protein production (Eiden-Plach et al. 2004). Thus, optimization of signal peptides is especially useful for secretion of some heterologous proteins, which are inefficiently led into the ER. In some cases, however, signal sequence optimization is almost inefficient for leading the target protein to the ER. As reported very recently, a major determinant of whether a protein follows the co- or post-translational pathway is not only the sequence of the N-terminal signal peptide but also the properties of the signal-anchor sequences (Berndt et al. 2009; Kida et al. 2009). Therefore, different approaches, such as fusion expression with other protein molecules or subdomains, tags, signal-anchor peptides may give improved results.

However, in some cases, secretory proteins are retained intracellularly without complete secretion, even though they have entered the ER lumen and folded into their native structure. This implies that other rate-limiting factors operate during the ER-to-Golgi or post-Golgi trafficking and the necessity for genetic optimization for membrane trafficking. Such trafficking pathways are known to be supported by numerous membrane proteins, and some of them are still not well characterized. Therefore, genetic optimization of intracellular vesicular traffic pathways is not simple, and identification of the rate-limiting step in the trafficking flow is the most important step. In our recent study on the secretory production of recombinant human growth hormone (hGH) from engineered fission yeast strains, intracellular retention of partial hGH was found, which was known to have resulted mainly from Golgi-to-vacuole mis-sorting (Idiris et al. 2010). Such vacuolar mis-sorting is known to be caused mainly by the vacuolar protein sorting (*vps*) receptor Vps10p, which is responsible for recognition and targeting of vacuolar carboxypeptidase Y (CPY, which is encoded with genes *Cpy1*$^+$ and *PRC1*$^+$, respectively, in *S. pombe* and *S. cerevisiae*) to the vacuole (Iwaki et al. 2006). Thus, Vps10p is referred to as a CPY receptor, and its vacuolar sorting pathway has been named as a CPY pathway. In the late-Golgi compartment, other enzymes that are destined for the vacuole are known to be separated from secretory proteins by binding to the receptor Vps10p (Vida et al. 1993; Marcusson et al. 1994). This suggests the presence of a Vps10p-mediated Golgi-to-vacuole protein mis-sorting pathway that may result in vacuolar accumulation of secretory proteins in yeast. In *S. cerevisiae*, there have been several similar reports about the positive effects of the *vps10* deletion on heterologous protein secretion (Holkeri and Makarow 1998; Hong et al. 1996; Zhang et al. 2001).

As a result of the important physiological roles of the yeast vacuoles, the vacuolar traffic pathway has become

more complex, and its modification is not simple (Banta et al. 1988; Raymond et al. 1992; Bonangelino et al. 2002; Takegawa et al. 2003). In contrast to the above result, *vps10* deletion has no effect on vacuolar retention of an insulin-containing fusion protein that is secreted from *S. cerevisiae*, whereas deletion of another five *vps* genes (*vps4*, *vps8*, *vps13*, *vps35*, and *vps36*) enhances heterologous protein secretion (Zhang et al. 2001). It is difficult to block completely the vacuolar mis-sorting pathway by a single *vps10* deletion because of the presence of another vacuolar sorting pathway, the alkaline phosphatase (ALP) pathway (Burd et al. 1998; Conibear and Stevens 1998). Transport of proteins through the ALP pathway to the vacuole requires the function of the adaptor protein (AP) complex AP-3 and Vps41p, which is also known as the AP-3 pathway (Darsow et al. 2001). Therefore, like other intracellular vesicular traffic pathways, genetic modification of the vacuolar mis-sorting pathway is not simple and is probably protein specific, which indicates the necessity of further systematic analysis of gene functions.

By screening an *S. cerevisiae* mutant library, Kanjou et al. (2007) have identified that deletion of the gene *MON2*, which encodes a scaffold protein for vesicle formation located in the late-Golgi, enhances secretion of recombinant luciferase. Overexpression of BiP and disruption of the Golgi-resident calcium-ATPase-encoding gene, *PMR1*, synergistically stimulate secretion of bovine prochymosin but not plant thaumatin in yeast (Harmsen et al. 1996). Furthermore, Ruohonen et al. (1997) have reported that overexpression of the yeast syntaxins, Sso1p or Sso2p, which act at the targeting/fusion of the Golgi-derived secretory vesicles to the plasma membrane, resulted in 4- or 6-fold enhanced secretion of *Bacillus* α-amylase or invertase, respectively, in *S. cerevisiae*. These results indicate the importance of genetic modification of the late-Golgi to plasma membrane traffic, besides blocking the vacuolar mis-sorting that is directed from the late-Golgi to the vacuole.

Although some studies have demonstrated the possible application of traffic modification in strain engineering, it seems to be difficult to block completely or change a flow direction or rate by modifying one or a few genes that are related to the trafficking, because of the complexity of membrane trafficking mechanisms. Moreover, traffic modification often affects cell viability or gives rise to protein-specific effects. Thus, as mentioned above, transformational fusion expression of the secretory target proteins, which are hardly trafficked in the secretory pathway, with other tags or secretion enhancer proteins may be another useful approach to improve secretion. Recently, Ahn et al. (2004) have reported that secretion of *Bacillus stearothermophilus* L1 lipase was enhanced 7-fold in *S. cerevisiae* by N-terminal molecular fusion with a cellulose-binding domain (CBD) from *Trichoderma harzianum* endoglucanase II. By such an approach, the fusion protein was secreted into the culture medium, and reached a concentration of ~1.3 g/L in high-cell-density, fed-batch cultures. They identified that insertion of a Kex2p cleavage site, which is cleaved in the Golgi apparatus, into the junction between the two fused proteins, CBD-linker and the target L1 lipase, resulted in the same level of enhanced secretion. This indicates that the CBD-linker fusion probably plays an important role in trafficking from the ER to the Golgi apparatus. Furthermore, secretion efficiency of *Bacillus subtilis* lipase A in *S. cerevisiae* is also significantly enhanced by fusing with cell wall protein Pir4p (Mormeneo et al. 2008). Thus, molecular fusion is also an effective approach for traffic improvement, particularly when strain optimization or molecular engineering of the target protein itself is difficult.

Engineering proteases related to proteolytic degradation

One of the major problems that hinders effective secretion and purification of secretory heterologous proteins from yeast is post-secretory degradation of the recombinant gene products by host-specific proteases, which are present in relatively high levels in yeast, and are induced readily by environmental stresses, especially during high-density fermentation processes. There are some reports that indicate contribution of cell lysis is to the presence of proteases in the culture medium in yeast fermentations of *P. pastoris* (Sinha et al. 2005; Xiao et al. 2006). The significance of this problem increases during secretory production of proteolytically sensitive heterologous proteins. Thus, a number of approaches have been attempted as a solution, such as control of cultivation conditions (e.g., culture pH and temperature), changing medium composition (e.g., nitrogen and carbon sources), and addition of protease inhibitors, peptone, casamino acids or specific amino acids (Enfors 1992; Gonzalez-Lopez et al. 2002; Jones 1991; Kang et al. 2000; Siegel and Brierley 1990; Turner et al. 1991). However, this approach is mostly host or protein specific and limited in its effects. Consequently, genetic manipulation of the host proteases can reduce host-specific degradation; therefore, it has been used to develop many protease-deficient yeast strains (Chung and Park 1998; Copley et al. 1998; Kang et al. 1998; Komeda et al. 2002; Jønson et al. 2004).

In the budding yeast, the major vacuolar proteases PrA and PrB, encoded by *PEP4* and *PRB1*, respectively, are essential for maturation and activation of several vacuolar proteases; therefore, the double-mutant Δ*PEP4*–Δ*PRB1* generally is protease deficient (Jones 1991). A similar result was obtained for *P. pastoris* (Gleeson et al. 1998) and *C.*

boidinii (Komeda et al. 2002). The fission yeast proteases Isp6p and Psp3p are known to be similar to the major vacuolar serine protease B (Prb1p) in the budding yeast *S. cerevisiae*. Expectedly, double deletion of the vacuole protease genes *psp3* and *isp6* in fission yeast has shown a significant reduction in extra- and intracellular protease activity in our study (Idiris et al. 2006b). These main vacuolar proteases are supposed to relate to the activation of other vacuolar proteases such as Cpy1p, which is transported from the late-Golgi to the vacuole via the endosome, by its receptor Vps10p, and then matured and activated in the vacuole by vacuolar proteases (Takegawa et al. 2003). In *S. cerevisiae*, *C. boidinii*, and *P. pastoris*, deletion of the vacuolar protease genes *PEP4* and *PRB1* has been attempted, together with other protease genes such as *CPY1*, *YPS1*, and *KEX2* (Kang et al. 2000; Komeda et al. 2002; Werten and de Wolf 2005). It has been reported recently that deletion of the mitochondrial metalloendoprotease gene *CYM1* in *S. cerevisiae* not only reduced intracellular proteolysis but also enhances the secretion of heterologously expressed peptides such as growth hormone, pro-B-type natriuretic peptide, and pro-cholecystokinin (Jønson et al. 2004). This implies that some vacuolar or other intracellular proteases also share the early secretory pathway for their trafficking, and thus, their deletion is important for control of protein degradation in the secretion pathway.

However, the approaches described above were based mainly on deletion of a few proteases and may have a limited effect on the control of protein degradation. Thus, systematic screening of proteases and their multiple modifications are expected to be more useful for minimization of proteolytic degradation during production of secretory proteins. As a novel approach for systematic screening and multiple deletion of yeast protease genes, we attempted recently to construct a set of single protease-deficient mutants in the fission yeast *S. pombe* by respective disruption of 52 dispensable protease genes (Idiris et al. 2006a). These putative protease genes were selected depending on the fission yeast genomic database (in which a total of 91 proteases were listed). Genes for proteases that are involved in the proteasome system or required for cell viability were avoided. By screening the resultant protease deletants for secretion of recombinant hGH, a proteolytically sensitive model protein, we succeeded in selecting 13 deletant strains, which were effective for hGH secretion (Idiris et al. 2006a). Deleted protease genes in such selected strains included *atg4*, *cdb4*, *fma2*, *isp6*, *pgp1*, *psp3*, *sxa2*, *oma*1, *ppp16*/SPBC1711.12, *ppp20*/SPAC4F10.02, *ppp51*/SPAC22G7.01C, *ppp52*/SPBC18A7.01, and *ppp60*/SPCC1919.12C. However, only two genes, *sxa2* and *ppp16*, are predicted to encode secretory proteases, whereas the others code for intracellular proteases. Furthermore, we have attempted multiple deletion of the above-selected protease genes and have succeeded in creating a multiprotease-deficient strain, namely A8, in which eight protease genes were multiply deleted (Fig. 2) (Idiris et al. 2010). The resultant multiprotease-deficient strain A8 gave approximately 30-fold enhanced hGH secretion, which indicates the importance of systematic screening and multiply deletion of proteases.

The resultant multiprotease-deficient strain A8 is not only effective for heterologous secretion but also useful for improvement of intracellular heterologous expression (unpublished observations), probably because of deletion of multiple intracellular proteases. Among eight proteases multiply deleted in the A8 strain, Psp3p and Isp6p are two major vacuolar serine proteases, Atg4p is a cytosolic cysteine protease required for autophagy (Klionsky et al. 2003), Fma2p is a cytosolic aminopeptidase specific to N-terminal methionine, Oma1p is a mitochondrial membrane protease, and Ppp20p is a vacuolar/cytosolic aminopeptidase specific to N-terminal aspartate or glutamate. The starvation-specific vacuolar protease Isp6p is also known to be involved in autophagy and sexual development and is released into the culture medium especially in the late stage of cell cultivation (Nakashima et al. 2006). These results also imply the importance of screening protease deletion targets by practical secretion of ideal protease-sensitive model proteins, which will go through the whole course of the trafficking pathway and thus make it possible to screen completely for intra- and extracellular dispensable proteases that probably are related to proteolytic degradation.

Finally, as a general strategy for minimizing host-specific total protease activity in heterologous protein secretion, optimization of cell cultivation conditions must be also considered besides using the multiprotease-deficient strains. This is because of the difficulty of complete deletion of all dispensable proteases and their complicated regulation mechanism, which is controlled by cell growth and cultivation conditions (e.g., temperature, pH, C/N-source level, cell density, viability, and stresses).

Engineering of post-translational glycosylation

Protein glycosylation is a major post-translational modification process in the yeast secretory pathway and confers an advantage in the secretory production of heterologous proteins that require glycosylation for proper folding and biological activity. However, for the production of therapeutic glycoproteins intended for use in human and animals, yeasts have been less useful because of their inability to modify proteins with human or animal glycosylation structures. In contrast to human-type glyco-

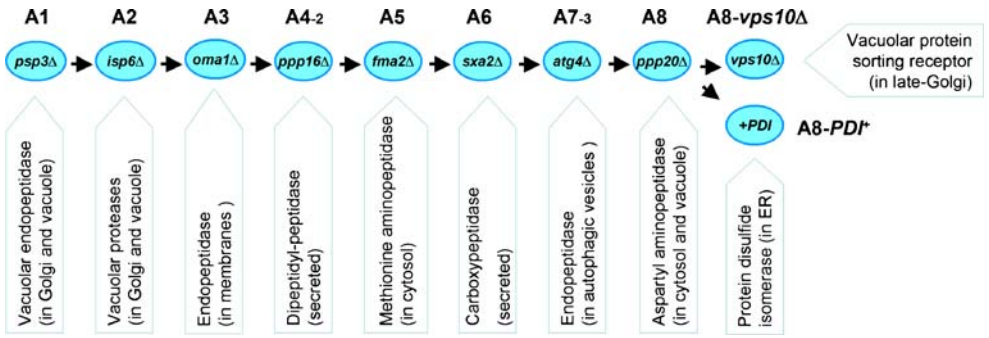

Fig. 2 Schematic representation of an example of engineering of the fission yeast *S. pombe* strains by multiple gene modification. A multiprotease-deficient strain A8 was constructed by deleting eight intra- and extracellular proteases to minimize proteolytic degradation in heterologous protein secretion (Idiris et al. 2010). All resultant multiprotease-deletant strains were named with their group name A plus the total number of deleted protease genes, such as A1–A8. Using the resultant strain A8, A8-*vps10*Δ was created by deleting the vacuolar protein sorting receptor gene *vps10*, whereas three A8-*PDI*$^{+}$ strains were constructed by, respectively, homogenously introducing PDI genes (Mukaiyama et al. 2010)

sylation, yeast *N*-glycosylation is of the high-mannose type, which confers a short in vivo half-life to the protein and may render it less efficacious or even immunogenic (Gerngross 2004). Therefore, the properties of post-translational glycosylation in each yeast host system and its optimization must be considered. This is especially important in the case of producing human therapeutic glycoproteins, which currently occupy about 70% of all approved therapeutic recombinant proteins, with their requirement for human-like *N*-glycosylation.

To overcome the disadvantage in the yeast glycosylation (mainly *N*-type), "humanizing" of yeast glycosylation systems has become the current main strategy for glyco-engineering of yeast species. However, success in this direction has been limited over the past decade, although several approaches have been attempted. This is because *N*-glycosylation is a complicated multi-step process that is localized to the secretory pathway, and the complex metabolic engineering of replicating the mammalian glycosylation machinery in yeast requires the cloning and functional expression of a large number of foreign glycosylation pathway enzymes in the yeast host strains (Bretthauer 2003; Gerngross 2004; Wildt and Gerngross 2005). Each enzyme in the glycosylation process catalyzes a reaction that yields the substrate for the subsequent enzyme, and must be targeted properly and function at high efficiency in its location in the secretory pathway. Recently, however, advances in the glycoengineering of yeast and the expression of therapeutic glycoproteins with humanized *N*-glycosylation structures have shown significant promise using current post-genomic information.

Glycoengineering in yeast was started by deleting some yeast-specific glycosyltransferases and introducing many other genes responsible for human-like sugar-nucleotide synthesis, their transport from the cytosol to the Golgi lumen, as well as hydrolysis. It has been identified that mammals and yeast share the initial biosynthetic pathway for the synthesis of *N*-glycans in the ER (Hamilton and Gerngross 2007; Chiba and Akeboshi 2009). Early steps in *N*-glycan processing, which involve the assembly of the core oligosaccharide, its site-specific transfer onto the protein, and its trimming by several glycosidases in the ER, are all highly conserved from yeast to humans. However, a series of glycosyltransferase reactions in the Golgi apparatus are distinctly different in humans compared with yeast. In yeast, several mannosyltransferases act on the *N*-glycan intermediate, and more than 50 mannose residues are attached to yield high-mannose type *N*-glycoproteins (Gemmill and Trimble 1999). Such large outer chains are attached to the oligosaccharide cores of glycoproteins during their export via the Golgi apparatus. A key gene for humanized *N*-glycan production in yeast was established in 1992, when a mutant strain (*och1*) of *S. cerevisiae* was isolated (Nagasu et al. 1992). It was found that formation of the yeast-specific, large outer chain is initiated by α-1,6-mannosylatransferase that is encoded by the *OCH1* gene, deletion of which blocks outer chain elongation. Och1p is highly conserved in all yeast species with the identical activity of transferring an initial mannose residue to an *N*-glycan intermediate ($Man_8GlcNAc_2$) in the Golgi apparatus, and its deletion causes a loss of hyper-mannosylated structure in the secreted glycoproteins. It was further identified that deletion of another two genes *MNN1* (encoding α-1,3-mannosyltransferase) and *MNN4* (encoding a positive regulator for phosphomannosyltransferase; Mnn6p), together with *OCH1* leads to the production of an intermediate *N*-glycan structure identical to the human structure (Chiba et al. 1998; Nakanishi-Shindo et al. 1993). This has provided the basis for the humanization of yeast glycans. The recent production of humanized sugar chain was achieved in the methylotrophic yeast *P. pastoris* by deleting glycotransferase genes *alg3* and *och1* and

introducing several glycosylation-related genes such as *MnsI*, *GnTI*, *GnTII*, *GalT*, and *SiaT* (Hamilton et al. 2003; Hamilton and Gerngross 2007). This elegant technique used combinatorial libraries that consisted of transmembrane domains of known Golgi- and ER-localized proteins, and catalytic domains of several glycosyltransferases and glycosidases from many species. Hamilton et al. (2006) also succeeded in the final stage of humanization of transfer of sialic acid onto terminal β-1,4-galactose sugars of complex glycoproteins, by introducing key enzymes that are required for CMP–sialic acid biosynthesis and sialyltransferase. Using such a fully humanized yeast strain, secretory production of sialylated human-type recombinant erythropoietin was demonstrated (Hamilton et al. 2006).

Besides the above *N*-glycosylation engineering, the glycoengineering of yeasts also includes *O*-glycosylation, which is characterized by shorter glycan structures. However, unlike *N*-glycosylation pathways, *O*-glycosylated modifications (other than *O*-Man) have not been attempted in yeast because of the lethality of the deletion of the *O*-mannosyltransferases (Gemmill and Trimble 1999). Recently, two modified production systems (a mucin-type *O*-glycosylation and *O*-fucosylation) for mammalian-type *O*-glycoproteins have been created in *S. cerevisiae* by introducing several genes (Oka and Jigami 2006; Chigira et al. 2008). Furthermore, our group recently identified six α-1,2-mannosyltransferase gene homologs, namely *omh1–omh6*, which are related to *O*-glycosylation of the fission yeast *S. pombe*, and has found that deletion of *omh1* blocks elongation of *O*-linked oligosaccharides (Ikeda et al. 2009). The resultant *omh1*Δ strain will be useful for controlling *O*-glycosylation or further glycoengineering in fission yeast. Recently, we have shown that *N*- and *O*-linked oligosaccharides completely lack galactose residues in the *gms1*Δ-*och1*Δ double-mutant strains of *S. pombe* (Ohashi and Takegawa 2009). The *N*-linked oligosaccharides of *gms1*Δ-*och1*Δ cells consist of α-1,2-linked Man-extended core oligosaccharides ($Man_{8–12}GlcNAc_2$), from which the fission-yeast-specific α-linked Gal residues are completely absent. These α-1,2-linked Man-extended core structures might not cause an immune response and could therefore be better tolerated in the human body. Still, such compounds could be quickly turned over in the human body (Wildt and Gerngross 2005). Next steps for humanization of the *S. pombe N*-linked oligosaccharides are screening and introduction of the active α-1,2-mannosidase into *S. pombe* of the screened α-1,2-mannosidase genes. These studies are now under way.

Success in the full humanization of *N*-glycosylation in *P. pastoris* implies a similar possibility in other yeast species such as the fission yeast *S. pombe* and the budding yeast *S. cerevisiae*. The industrial production of recombinant proteins in yeast is a well-established technology; therefore, it is reasonable to expect the impact of glycoengineered yeast strains to make a significant breakthrough in the biopharmaceutical production of human glycoproteins.

Perspective

Novel post-genomic information that involves genomic, proteomic, transcriptomic, or metabolic databases offers a general solution for yeast strain engineering. One approach is to screen yeast cDNA-overexpression libraries for improved secretion of heterologous proteins. Using such an approach, Shusta et al. (1999) have reported that the levels of surface-displayed single-chain T-cell receptors correlate strongly with the soluble expression of the respective proteins. Recently, high-throughput screening for potential secretion enhancers by flow cytometry and cell sorting have been used to attempt to isolate overproducing clones (Mattanovich and Borth 2006). Transcriptome-based screening of a yeast cDNA library in *S. cerevisiae* surface display strains has identified cell wall proteins (Ccw12p, Cwp2p, and Sed1p), ribosomal subunit protein Rpp0p (Wentz and Shusta 2008), and the PDI assistant Ero1p (Gross et al. 2004) as beneficial for the secretion of various antibody fragments (Wentz and Shusta 2007). A similar approach has been used to improve membrane protein production in *S. cerevisiae*, based on engineered expression of Bms1p, which is involved in ribosomal subunit assembly (Bonander et al. 2009; Rakestraw et al. 2006). Previously, they had identified by transcriptome analysis of *S. cerevisiae* that deletion of three transcription factors, Gcn5p, Spt3p, and Spt5p is effective for secretion of glycerol facilitator Fps1 (Bonander et al. 2005).

Furthermore, genome-wide analytical tools such as DNA microarrays are regarded as data mining sources for physiological effects, stress regulation, and host engineering. Sauer et al. (2004) have analyzed the differential transcriptome of a *P. pastoris* strain that overexpresses human trypsinogen compared with a non-expressing strain. Thirteen of the 524 significantly regulated genes were selected, and their *S. cerevisiae* homologs were overexpressed in a *P. pastoris* strain that produced a human antibody Fab fragment (Gasser et al. 2007). In that study, five previously known secretion helpers (Pdi1p, Ero1p, Sso2p, Kar2p/BiP, and Hac1p), as well as six novel, hitherto unidentified factors, Bfr2p and Bmh2p, the chaperones Ssa4p and Sse1p, and the vacuolar ATPase subunits Cup5p and Kin2p, proved their benefits for secretion of antibody in *P. pastoris* (Gasser et al. 2007).

Using several classic and novel genome-wide screening approaches, many yeast genes have been found that are related to secretion engineering. Examples of some genes for which co-expression or deletion is beneficial for

secretion are listed in Table 1. However, it is clear from the list that the identified genes vary in function, and their single modification may have a limited effect on protein secretion. This indicates the necessity of multiple modifications, such as knockout, knockdown, or co- or overexpression of several genes. Recently, we have investigated multiple modification of the fission yeast *S. pombe* by combining several genetic manipulations (Fig. 2). In our study, introduction of PDI genes or deletion of the vacuolar sorting receptor gene *vps10* was attempted after multiply deleting eight proteases in a single strain. The resultant strains A8-*PDI*$^{+}$ (Mukaiyama et al. 2010) and A8-*vps10*Δ showed 50–100% enhanced heterologous protein secretion compared with the A8 parental strain, which already had about a 30-fold increase in hGH secretion (Idiris et al. 2010; Takegawa et al. 2009). Such results clearly demonstrate the synergistic effect of multiple genetic modifications in strain engineering, although further combination of other modifications is still required. Moreover, we are also trying to combine such multiple modifications of the secretion pathway with large-scale chromosome deletions (known as the minimum genome factory or MGF project)

Table 1 Some genes modified in strain engineering for secretion improvement

Class	Gene	Product localization	Function	References
Folding and QC system	*CNE1*	ER membrane	Glycoprotein chaperone (calnexin) (*Sc*)	Klabunde et al. 2007
	ERO1	ER membrane	PDI assistant	Gross et al. 2004; Lodi et al. 2005
	HAC1	Nucleus	Transcriptional activator (*Sc*)	Schröder and Kaufman 2005; Valkonen et al. 2003
	JEM1	ER membrane, MMP	DnaJ-like co-chaperone (*Sc*)	Payne et al. 2008
	KAR2	ER lumen	BiP, a major Hsp70 chaperone in the ER (*Sc*)	Robinson et al. 1994; Harmsen et al. 1996
	PDI	ER lumen	Protein disulfide bond formation	Shusta et al. 1998; Smith et al. 2004
	SSA4	Cytoplasm	Cytosolic chaperone (Hsp70 family) (*Sc*)	Gasser et al. 2007
	SSE1	Cytoplasm	Cytosolic co-chaperone (Hsp70 family) (*Sc*)	Gasser et al. 2007
	UBI4	Cytoplasm, nucleus	Ubiquitin, required for poly-ubiquitination	Bao and Fukuhara 2001; Chen et al. 1994
Transcription, translation, and signaling	*GCN5*[a]	Nucleus	Histone acetyltransferase (*Sc*)	Bonander et al. 2005
	RPP0	Cytoplasm	Component of the large ribosomal subunit	Wentz and Shusta 2008
	SPT3[a]	Nucleus	Histone acetyltransferase complex subunit	Bonander et al. 2005
	SPT5[a]	Nucleus, *mit.*	Transcriptional elongation factor	Bonander et al. 2005
	KIN2	Plasma membrane	Protein kinase regulating exocytosis (*Sc*)	Gasser et al. 2007
Trafficking	*BFR2*	Nucleus, nucleolus	ER-to-Golgi transport, rDNA processing (*Sc*)	Gasser et al. 2007
	BMH2	Cytoplasm, nucleus	Vesicle transport, multi-process regulation (14-3-3 protein) (*Sc*)	Gasser et al. 2007
	CUP5	Vacuole membrane, MMP	Vacuolar ATP synthase subunit (*Sc*)	Gasser et al. 2007
	MON2[a]	Golgi membrane	Golgi-to-endosome traffic, endocytosis and vacuole integrity (*Sc*)	Kanjou et al. 2007
	PMR1[a]	ER membrane, MMP	Ca^{2+} and Mg^{2+} transporting ATPase	Harmsen et al. 1996
	PSE1	Cytoplasm, nucleus	Nuclear import of ribosomal proteins (*Sc*)	Chow et al. 1992
	SSO1	Plasma membrane	Secretory vesicle fusion with the plasma membrane (*Sc*)	Aalto et al. 1993; Ruohonen et al. 1997
	SSO2	Plasma membrane	Secretory vesicle fusion with the plasma membrane (*Sc*)	Aalto et al. 1993; Ruohonen et al. 1997
	VPS10[a]	Golgi membrane, late-Golgi	Vacuolar protein sorting	Hong et al. 1996; Idiris et al. 2010; Zhang et al. 2001

Sc S. cerevisiae, *Sp S. pombe*, *mit.* mitochondrion, *MMP* multi-pass membrane protein

[a] Deletion is beneficial for protein secretion

in *S. pombe*, to simplify the genome-wide regulation system that may directly or indirectly affect protein secretion (Giga-Hama et al. 2007; Takegawa et al. 2009). Further optimization of the cultivation process for the multiply engineered *S. pombe* strains also has been studied and found that changes in medium components and addition of dextran sodium sulfate, a poly-anion surfactant, to the culture medium of the engineered A8 strain significantly (7-fold) enhanced secretion efficiency of recombinant human transferrin (Mukaiyama et al. 2009).

In conclusion, on the basis of the well-established vector/fermentation optimizations, systematic modification of secretion pathways is a general strategy for yeast secretion engineering. Rapidly progressed genome-wide novel approaches will provide new systematic information and solutions to overcome unknown bottlenecks in the yeast secretion system.

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