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Studies on gene expression and promoter analyses
for protein production in *Aspergillus oryzae*

Hiromoto Hisada

2013
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Abbreviations

BSA  bovine serum albumin  
catA  catalase A-encoding gene  
catB  catalase B-encoding gene  
CBB  Coomassie Brilliant Blue  
ELISA  enzyme-linked immunosorbent assay  
EMSA  electrophoretic gel mobility shift assay  
Endo H  endoglycosidase H  
EST  expressed sequence tag  
glaB  glucoamylase-encoding gene  
GUS  β-glucuronidase  
hCG  human chorionic gonadotropin  
HSE  heat shock element  
ORF  open reading frame  
PAGE  polyacrylamide gel electrophoresis  
PCR  polymerase chain reaction  
SDS  sodium dodecyl sulfate  
SSC  solid-state culture  
sodM  superoxide dismutase-encoding gene  
TMB  3,3′,5,5′-tetramethylbenzidine  
uidA  β-glucuronidase-encoding gene  
VHH  heavy-chain antibody fragment  
WCE  whole-cell extracts
Introduction

The filamentous fungus *Aspergillus oryzae* is used widely in traditional Japanese fermentation industries, including sake, *mirin*, *miso* and soy sauce breweries. In sake brewing, *A. oryzae* has been used for making rice *koji*, malted rice, for over 1,000 years. Therefore, *A. oryzae* is called “koji mold” and is honorary titled as “national fungus (kokkin)” (Machida et al. 2008). In the process of *koji*-making in sake brewing, polished and steamed rice is traditionally used as a solid-state culture medium. As the advancement of fermentation technology, the process of sake brewing has been mechanized and regulated by a computer-assisted operation in a plant, but the process of making *koji* still remains as the traditional solid-state culture on steamed rice. It is extremely important to make a high quality *koji* for brewing good quality sake, but the process of making *koji* is still dependent largely on manual craftsmanship procedures of skilled artisans called *Toji* group. A chief sake brewer or *Toji* says that the most important process in sake brewing is in the process of *koji* making, sending words as “1st *koji*, 2nd yeast mash (*moto*) and 3rd alcohol fermentation (*tsukuri*)”.

A well-known “simultaneous saccharification and fermentation process” of sake brewing is quite unique. The saccharification process, a highly important step of starch hydrolysis before alcohol fermentation, depends on starch degradation enzymes such as glucoamylase and α-amylase, and it is *koji* that provides these starch degradation enzymes. Therefore, if the manufacturing process of *koji* fails, glucoamylase activity becomes so low as to inadequately degrade starch or maltooligosaccharide to glucose, thereby resulting in failure in subsequent alcohol fermentation to deteriorate the quality of the final product sake. For example, when the water content of steamed rice is too high, the manufacture of high quality *koji* is most likely to fail; this is so-called “submerged *koji* (*nure koji* or *hazeochi*)”. Although the
phenomenon of submerged koji is empirically known, this problem has been resolved only by intuitions and experiences of skilled craftsmanship of sake brewers with complicated process control.

The importance of koji is deeply concerned with the expression of saccharifying enzymes such as glucoamylase and α-amylase expressed in a solid-state culture of A. oryzae. The glucoamylase-encoding gene (glaB) has already been cloned from A. oryzae (Hata et al. 1998). The glaB is expressed excessively on solid-state culture such as koji, but is expressed neither in submerged koji nor in submerged (liquid) culture (Ishida et al. 1998). The difference between solid-state culture and submerged culture lies mainly in water activity, nutrient diffusion and the presence of insoluble material that serves as a scaffold of mold growth (Akao et al. 2007). Since these culture conditions and factors significantly affect the growth of A. oryzae and its gene expressions, it is highly important to elucidate the mechanism of regulation of gene expression of the glaB in a solid-state culture as compared to that in submerged koji or liquid culture.

Since the whole genome sequence of A. oryzae was determined in 2005 (Machida et al.), comprehensive analyses, such as DNA microarray analysis and proteomic analysis, have become available for various culture conditions (Akao et al. 2007; Oda et al. 2006). These comprehensive analyses have identified several proteins that are produced only in a solid-state culture, but the regulation mechanism of solid-state-specific gene expression, including the glaB, is yet to be elucidated. If this mechanism is revealed, it should significantly benefit not only sake brewing, but also mirin, miso and soy sauce brewing that includes a koji making process. Since the identification of the regulation mechanism would make koji manufacture much easier, the analysis of regulation of individual gene of culture-condition-specific expression has become increasingly important.
Whereas other *Aspergilli*, including *A. nidulans*, *A. fumigatus*, *A. niger* and *A. awamori*, produce a high level of glucoamylase in submerged cultures, *A. oryzae* produces a limited amount of glucoamylase in submerged culture (5 U/ml) compared with that on solid-state *koji* (200 U/g) (Narahara *et al*. 1982). It seems quite difficult to know the reason why *A. oryzae* produces glucoamylase in a culture-condition-specific manner, but it was proposed recently that *A. oryzae* was the specific man-made mold that has been bred for sake brewing over 500 years so that it produces a higher level of glucoamylase only in a solid-state culture (Kitamoto 2012).

In Chapter I of this study, the author focused catalases as an essential enzyme for detoxification of reactive oxygen species. Since catalase-encoding genes would be strongly expressed and tightly regulated under aerobic conditions, these genes are suitable for gene expression analysis. First, the author cloned two distinct, but apparently catalase-encoding genes (*catA* and *catB*). The overexpression of the *catA* and *catB* indicated that both genes encoded functional catalases. In the next step, the author revealed gene expression analysis that the *catB* gene was expressed under both submerged (liquid) and solid-state culture, and was induced with hydrogen peroxide addition. In contrast, the *catA* gene was found to be expressed only in solid-state culture and was not inducible with hydrogen peroxide.

Since a gene expression is regulated directly by its promoter, the analysis of promoters is a key to understand the regulation mechanism of culture-condition-specific gene expression. Hence, in Chapter II, the author focused on the analysis of the promoters of the genes that express in a culture-condition-specific manner to determine the relationship between culture condition and promoter regulation. Promoters of three types of genes were analyzed to see how the difference in gene regulation affects the difference in gene expression depending on culture conditions: a gene of submerged-specific expression, a gene of non-specific
expression, and a gene of solid-state-specific expression.

In Section 1, the author focused on the promoter of a superoxide dismutase-encoding gene (sodM) as a liquid-culture-specific expression gene (Ishida et al. 2004). Although the sodM gene plays a central role in detoxification of reactive oxygen species in concert with catalase-encoding gene, the sodM gene is strongly expressed only in submerged or liquid culture. Deletion analysis and electrophoretic mobility shift assay (EMSA) revealed the region that contains cis-elements concerned with strong gene expression. Comparison with the sodM ortholog promoters in Aspergilli showed that the region was found only in A. oryzae. This genetic trait indicates that A. oryzae has specifically been bred as a safe fungus for brewing and that is totally different from other Aspergilli such as pathogenic A. fumigatus.

The catalase-encoding gene (catB) is expressed non-selectively depending on culture conditions. Therefore, the promoter of catB was studied as a second topic of promoter analysis (Section 2). Deletion analysis and EMSA identified the region containing cis-elements concerned with strong gene expression. The region contains a well-known heat shock element (HSE) that responds to various stresses. Comparison with the catB ortholog promoters showed that the region was conserved widely in Aspergilli, suggesting that non-specific expression gene promoter is conserved during breeding.

The glucoamylase-encoding gene (glaB) and acid protease-encoding gene (pepA) of A. oryzae are very important in koji and are expressed only in solid-state culture (Ishida et al. 1998; Kitano et al. 2002). Since common cis-elements concerned with solid-state-specific gene expression was not known, the glaB promoter was analyzed in Section 3. Deletion analysis and EMSA successfully found a region responding to low water activity, a unique property of solid-state culture. Comparison with other glaB ortholog promoters showed that the region was conserved in a limited member of Aspergilli, A. oryzae, A. clavatus and A.
These results suggested that *A. oryzae* have been selected and bred intentionally for sake brewing among *Aspergilli*.

*A. oryzae* is an attractive mold as a host for production of valuable proteins from different origins. In Chapter III, the author describes the production of a llama variable heavy-chain antibody fragments (VHH) fused to glucoamylase in *A. oryzae*. Because the VHH consisting of a single polypeptide is more easily manipulated through protein engineering, this class of antibodies has been the subject of significant research effort, e.g., for improved thermal stability (Hagihara *et al.* 2007; Hussack *et al.* 2011). The llama VHH was previously produced and secreted in submerged culture of *A. awamori* (Joosten *et al.* 2005a, b), but at very low expression levels (7.5 to 30 mg protein/l culture medium). By searching for a leader protein fused with llama VHH for optimal production, the author successfully expressed a functional VHH with antigen binding ability in *A. oryzae* by fusing with glucoamylase. The productivity was over 1.0 g/l culture medium in 5 day.

The results shown in this study reveals an interesting genetic trait of *A. oryzae* as the unique and safe microbe that has been bred specifically over years by skilled brewers for brewing traditional Japanese fermented foods where solid-state culture is a key. The author believes that the present study lays a “solid” platform for efficient production of saccharifying enzymes in submerged or liquid-culture *koji*, a long-cherished goal of brewing industries in Japan.
Chapter I

Expression analysis of two different catalase-encoding genes
under various culture conditions

Many organisms have functionally equivalent genes that are regulated differently. In *A. oryzae*, two genes encoding glucoamylase (*glaA* and *glaB*) were identified (Hata *et al.* 1998). Both genes encode the same class of enzyme, but *glaA* is expressed only in submerged cultures, while *glaB* is expressed only in solid-state cultures (*koji*). Recently, tyrosinase-encoding genes *melO* and *melB* were also found to act in a similar manner (Ishida *et al.* 2001; Obata *et al.* 2004).

The author screened for strong promoters for the overexpression of homo- and hetero-proteins. In the screening process, the author hypothesized that the genes required for detoxification of reactive oxygen species would be constitutively expressed under strong promoters and would be induced easily by increasing oxidative stress. Oxidative stress causes damage to various organs; it was recently reported that reactive oxygen species are involved in Alzheimer’s disease, Parkinson’s disease and amyotrophic lateral sclerosis (Barnham *et al.* 2004). Reactive oxygen is not only the cause of such diseases, but is also used to signaling processes such as apoptosis (Cantara *et al.* 2004), life span determination (Dröge 2003), cell differentiation (Carter *et al.* 2004; Hansberg and Aguirre 1990) and pathogen defense (Paris *et al.* 2003). Hydrogen peroxide, superoxides and hydroxyl radicals are formed inevitably during aerobic metabolism. All aerobic organisms have enzymatic and non-enzymatic detoxification systems to combat these reactive oxygen species. Catalases, superoxide dismutases and peroxidases play a central role in enzymatic detoxification (Muradian *et al.* 2002). Among the detoxification enzymes, catalases are the most important enzymes to degrade hydrogen
peroxide and are classified into three separate families: Mn-catalases, catalase-peroxidases and mono-functional catalases (Igarashi et al. 1996; Klotz et al. 1997). The mono-functional catalases are the best characterized, and they are homotetrameric and heme-containing enzymes. As catalases are found in organisms from eubacteria to eukaryotes (Fowler et al. 2003; Klotz et al. 1997; Michán et al. 2002; Paris et al. 2003; Varnado et al. 2004), they are essential, strongly expressed, and tightly regulated.

Here the author describes the cloning of two distinct catalase-encoding genes of *A. oryzae* and the characterization of their physiological roles in *A. oryzae* by promoter analysis.

**Materials and methods**

**Strains, plasmids and growth conditions**  The genomic DNA library was constructed from the wild-type *A. oryzae* OSI1013 (deposited with the International Patent Organism Depository [IPOD] as FERM P-16528) (Hata et al. 1998). The *A. oryzae* OSI1013 AON-2, a *niaD* mutant (deposited with IPOD as FERM P-17707) derived from *A. oryzae* OSI1013, was used for the gene expression and promoter experiments. Submerged cultures were incubated at 30˚C for 72 h in 100 ml of modified Czapek-Dox medium (2% glucose, 0.2% KCl, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O, 0.002% FeSO₄·7H₂O) or in 100 ml of DPY medium (2% dextrin, 1% peptone, 0.5% Yeast extract, 0.2% KCl, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O, 0.002% FeSO₄·7H₂O). Solid-state cultures were incubated at 30˚C for 48 h on DPY-agar (DPY medium, 1.5% agar) or on wheat bran (Sigma-Aldrich, St. Louis, MO; containing 80% H₂O[w/v]). The *Escherichia coli* JM109 strain (TaKaRa Bio, Otsu, Japan) was used for DNA manipulations.

The pNia2 plasmid used for the catalase gene expression experiments was generated by
inserting the nitrogen reductase (*niaD*) selectable gene marker into the pUC119 vector. The *melO* promoter (-999 to -1) was amplified by polymerase chain reaction (PCR) with the upper primer MO-1 (5’-AAAAACTGCAGGCGATGTCATTGCAGCGCAGGT-3’; the synthesized *Pst*I site underlined), and the lower primer MO-2 (5’-TATTGGCCATTGTGAACCA_AAGTAATCAGA-3’; the underlined sequence being capable of annealing with that in the CA-1 primer) (Ishida *et al.* 2001). The *catA* open reading frame (ORF) was amplified by PCR with the upper primer CA-1 (5’-TTGGTTCAACATGGCAATAATTGCTG-3’; the underlined sequence being capable of annealing with that in the MO-2 primer), and the lower primer CA-2 (5’-AAAACCTCAGTTAGAATGCAACTGTCGAGG-3’; the synthesized *Xho*I site underlined). Both PCR products were fused and subcloned into pNia2, and it was named pMCA (*catA*). Similarly, the *melO* promoter (-999 to -1) was amplified with the upper primer MO-1, and the lower primer MO-3 (5’-GGCGCAGCATTGTGAACCAAAGTAATCAGA-3’; the underlined sequence being capable of annealing with that in the CB-1 primer). The *catB* ORF was amplified with the upper primer CB-1 (5’-TTGGTTCAACATGGCGCGCCCTC TCCCTTGC-3’; the underlined sequence being capable of annealing with that in the MO-3 primer), and the lower primer CB-2 (5’-AAAAGTCGACTTAATGGTCAGCTTGAAAC-3’; the synthesized *Sal*I site underlined). Both PCR products were fused and subcloned into pNia2, and it was named pMCB (*catB*).

The *A. oryzae* integration vector pNGS1 used for promoter analysis was derived from pUC119 and harbors the *E. coli* *uidA* gene, the *A. oryzae* glaB terminator, and *A. oryzae niaD* as a reporter, terminator, and selectable marker, respectively (Ishida *et al.* 2000). The *catA* promoter region (-1000 to -1) was amplified by PCR with the forward primer CAp-1 (5’-AAAAACTGCAGTGATGGGATTCAAATAATTG-3’; the synthesized *Pst*I site underlined), and the reverse primer CAp-2 (5’-AAAAAGTCGACGATTGGGTTGATGATAT-3’; the
synthesized SalI site underlined). Similarly, The catB promoter region (-1000 to -1) was amplified with the forward primer CBp-1 (5′-AAAGTGCAGGCAATATTCTGCAACGTC-3′; the synthesized PstI site underlined), and the reverse primer CBp-2 (5′-AAAGTGCAGGCAATATTCTGCAACGTC-3′; the synthesized SalI site underlined).

Each promoter fragment was individually subcloned upstream of the reporter gene using these sites, and they were named pCAPG (catA) and pCBPG (catB).

Transformation of the plasmids into AON-2 was done by the method of Gomi et al. (1987). Transformants were selected on plates containing sodium nitrate as the sole source of nitrogen.

**Catalase assay** Catalase activity was measured by the method of Beers and Sizer (1952). One unit of catalase activity is defined as the amount of enzyme that decomposes 1 µmol of H₂O₂/min at 25°C (pH 7.0). Consumption of H₂O₂ was measured as the decrease in absorbance at 240 nm. The rate of H₂O₂ consumption was determined over the range of 10.3 mM to 9.2 mM of H₂O₂. Spectrophotometric assays were performed on a UV-2450 UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan).

**β-Glucuronidase (GUS) assay and determination of protein concentration** Cell-free extracts were prepared by the method of Ishida et al. (1998). β-Glucuronidase (GUS) activity was measured according to Jefferson et al. (1986). One unit of GUS is defined as the amount of enzyme that produces 1 nmol of p-nitrophenol/min at 37°C using a molar extinction coefficient of 14,000 at an absorbance of 430 nm. Protein concentration was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA) and bovine serum albumin (BSA) as the standard.
Results

Cloning of two developmentally distinct catalase genes  To identify genes expressed under strong and easily induced promoters, the author screened an *A. oryzae* expression sequence tag (EST) library consisting of approximately 80,000 clones. Accordingly, the author identified two catalase-encoding genes that were expressed in developmentally distinct ways. To clone the full-length genes from *A. oryzae*, the author constructed a phage genomic library from the *A. oryzae* OSI1013 strain. Approximately 15,000 plaques of the EMBL3 phage library were screened by hybridization with DNA probes made from the ESTs by PCR. Three positive clones were isolated, and sequence analysis confirmed that each EST sequence was represented by the cloned genes. According to standard nomenclature, the genes were named *catA* and *catB*. Sequence analysis of the *catA* gene revealed an ATG-initiated ORF interrupted by two putative introns that predicted a 747 amino acid polypeptide (Fig. 1-1). This polypeptide is homologous to the *A. fumigatus catA* protein (81%) and the *A. nidulans catA* protein (77%). The *catB* gene consists of an ATG-initiated ORF interrupted by five introns that predicts a putative polypeptide of 725 amino acids (Fig. 1-2). This polypeptide shares homology with the *A. fumigatus catB* protein (82%) and the *A. nidulans catB* protein (75%). However, the newly cloned *A. oryzae* catalase genes share much less homology (41%, Table 1-1).
The nucleotide and predicted polypeptide sequence of the catA gene. A potential TATA box and consensus splice sites are underlined. DDBJ/EMBL/GenBank accession no. is AB079611.
The nucleotide and predicted polypeptide sequence of the catb gene. A potential TATA box and consensus splice sites are underlined. DDBJ/EMBL/GenBank accession no. is AB078864.
Table 1-1  Homologies at amino acid level of catalase-encoding genes from *Aspergilli*

<table>
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<tr>
<th></th>
<th>AoCatA</th>
<th>AnCatA</th>
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**Overexpression of the *catA* and *catB* genes under the *melO* promoter**  To determine whether the *catA* and *catB* genes encode functional catalases, the author overexpressed each one in DPY medium. The coding regions of *catA* and *catB* were fused downstream of the *melO* promoter and were inserted into the pNia2 plasmid. The *melO* promoter was chosen because it is one of the strongest promoters available (Ishida et al. 2001). Both constructs, pMCA (*catA*) and pMCB (*catB*) (Fig. 1-3) were introduced into the host AON-2 strain, and transformants harboring single copies of the fused genes were analyzed. Figure 1-4 shows that the catalase activities of the overexpressed transformants were approximately 2-fold higher than the control transformant. These results demonstrate that the *catA* and *catB* genes encode functional catalases. In Czapek-Dox medium, the similar results were observed.
Fig. 1-3 Plasmids used in Chapter I. The constructed plasmids for overexpression of the catA and catB gene were pMCA and pMCB, respectively. The plasmids used for promoter analysis of the catA and catB promoters were pCAPG and pCBPG, respectively.

Fig. 1-4 Catalase activities of the overexpressed transformants. The AON-2 was individually transformed with constructed plasmids. Control, transformant with pNia2; CatA, transformant with pMCA (catA); CatB, transformant with pMCB (catB). Each transformant was cultured in 100 ml DPY medium at 30°C for 8 d. Catalase activities were determined with cell-free extracts (Beers and Sizer 1952; Ishida et al. 1998).
Expression analysis of the *catA* and *catB* promoters by GUS reporter assay  To study the regulation of the two catalase genes, the author constructed pNGS1-based plasmids for the β-glucuronidase (GUS) reporter assay that contained the *catA* gene promoter (pCAPG) or the *catB* gene promoter (pCBPG) fused upstream of the β-glucuronidase-encoding gene (*uidA*). Both plasmids were introduced into AON-2, and transformants were cultivated under various conditions. GUS activity from the extracts of transformants was measured to analyze the regulation of the two catalases (Table 1-2). The *catB* gene was expressed under all culture conditions, but was highly induced in submerged (liquid) cultures containing DPY medium and Czapek-Dox medium. The *catA* gene was expressed only in wheat bran (solid-state) cultures in which many spores were formed.

<table>
<thead>
<tr>
<th>Culture</th>
<th><em>catA</em> promoter&lt;sup&gt;a&lt;/sup&gt;</th>
<th><em>catB</em> promoter&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sporulation</th>
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<tr>
<td>DPY</td>
<td>295±15</td>
<td>25,400±903</td>
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</tr>
<tr>
<td>Czapek-Dox</td>
<td>306±17</td>
<td>12,391±131</td>
<td>−</td>
</tr>
<tr>
<td>DPY-agar</td>
<td>500±21</td>
<td>8,479±321</td>
<td>+</td>
</tr>
<tr>
<td>Wheat Bran</td>
<td>2,532±21</td>
<td>2,154±189</td>
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</tr>
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</table>

<sup>a</sup>GUS activity (U/mg-protein)

The effect of hydrogen peroxide on *catA* and *catB* gene expression  In an *in vivo* study examining the gene expression of two *Saccharomyces cerevisiae* catalases, it was demonstrated that one catalase gene was induced upon hydrogen peroxide exposure (Wieser *et al*. 1991). To determine the effect of hydrogen peroxide on *A. oryzae* catalases, the author examined gene expression under two experimental conditions. Hydrogen peroxide was added either to a Czapek-Dox submerged culture or to a wheat bran solid-state culture (Fig. 1-5). Under both culture conditions, hydrogen peroxide induced the *catB* gene but not the *catA* gene.
Fig. 1-5  Time-course of catA and catB expression. GUS activity was measured from cultures grown in Czapek-Dox medium (a) and on wheat bran solid-state medium (b). H₂O₂ was added to the cultures (final concentration 0.1% v/v in Czapek-Dox medium, or 0.1% v/w on wheat bran solid-state medium mixed well with vigorously shaking) 24 h after the initial inoculation. Symbols: open circles, catA promoter; closed circles, catA promoter plus H₂O₂; open squares, catB promoter; closed squares, catB promoter plus H₂O₂.
Discussion

Here the author has cloned and characterized the *A. oryzae catA* and *catB* genes and shown that they encode different catalases. Based on sequence similarity, it is likely that *A. oryzae catA*, *A. fumigatus catA* and *A. nidulans catA* compose one family of genes, while *A. oryzae catB*, *A. fumigatus catB* and *A. nidulans catB* compose another (Table 1-1). The *catA* and *catB* genes show little similarity to one another (41%), further suggesting that they do not belong to the same gene family. Both gene families are conserved in other fungi. For example, the *Neurospora crassa cat1* gene shares 63% homology with the *catA* gene, and its *cat3* gene shares 61% homology with the *catB* gene (Michán et al. 2002).

Overexpression of the *catA* and *catB* genes under the strong *melO* promoter produced 2-fold higher activity than the control confirming that both genes encode a functional catalase. Because equal levels of activity were observed with equal amounts of protein from cell-free extracts, these experiments also suggest that there is no difference in *catA* and *catB* catalytic activities.

To elucidate the regulation of the *catA* and *catB* genes, the author employed the promoter-GUS gene fusion system. The transcriptional efficiency of the *catA* and *catB* promoters was measured under various culture conditions. These experiments revealed much about the regulatory mechanisms of both the *catA* and *catB* genes and allowed insight into their roles in vivo. The *catB* gene was constitutively expressed under all culture conditions and could be induced with hydrogen peroxide. In comparison to the *catB* promoter, the *melO* promoter was strong in submerged culture, but its overexpression needed more time (7-10 d) (Ishida et al. 2001). The *glaA* promoter was only expressed in submerged cultures, on the other hand the *glaB* promoter was specifically expressed on solid-state cultures. Furthermore,
polysaccharides were necessary for expression (Hata et al. 1998; Ishida et al. 1998). Thus, the catB promoter will be very useful for homo- or heterologous protein expression. On the other hand, the catA gene was expressed only in solid-cultures undergoing sporulation and was not induced with hydrogen peroxide. These developmental differences have also been noted in other fungi (Kawasaki et al. 1997; Navarro et al. 1996). The author hypothesizes that the main function of catB is to reduce the toxic effects of oxidative stress and that the catA gene product is required during the germination stage. Intriguingly, disruption of the catB gene but not the catA gene in A. nidulans rendered spores sensitive to hydrogen peroxide (Kawasaki et al. 1997). This piece of evidence suggests that the catA gene is required for a role other than detoxification of the spore.

Both catalase genes subcloned in Chapter I belong to large-subunit catalase families (Klotz et al. 1997). A motif search of the proteins (GenomeNet, http://www.genome.jp/) showed that catB contains a catalytic domain (44-434 amino acid residues) and a catalase-related domain (514-567 amino acid residues). However, catA not only contains a catalytic domain (81-469 amino acid residues) and a catalase-related domain (475-529 amino acid residues), but also harbors a DJ-1/PfpI domain (598-745 amino acid residues). The DJ-1/PfpI domain is found in members of the DJ-1/ThiJ/PfpI superfamily, which have both chaperone and protease activities (Bandyopadhyay and Cookson 2004; Wilson et al. 2004). This suggests that the function of the catA gene is required for proper folding and degradation of proteins in the spore.
Chapter II

Promoter analysis of culture-condition-specific expression genes and comparison of homologous promoters in Aspergilli

Section 1

Analysis of superoxide dismutase-encoding gene (sodM) promoter driving strong expression only in submerged cultures

*A. oryzae* is one of the most important fungi used in the fermentation of traditional Japanese foods such as sake, *miso* and soy sauce. Because *A. oryzae* produces a variety of useful enzymes without concurrent production of harmful secondary metabolites such as aflatoxins, it is considered to be an excellent host for the production of homologous and heterologous proteins (Barbesgaard et al. 1992). Economical production of such proteins in *A. oryzae* requires a better understanding of how expression of each gene is regulated.

Previous promoter analysis in *A. oryzae* focused on two gene families. One comprises the saccharolytic enzyme encoding-genes such as *amyB* (Tsuchiya et al. 1992; Tsukagoshi et al. 1989), *glaA* (Hata et al. 1992), *agdA* (Minetoki et al. 1996), and *xynF1* (Kitamoto et al. 1998). Regulatory regions of these promoters such as Region III (Minetoki et al. 1998) and the GGCTAAA-box (Van Peij et al. 1998), and regulatory factors such as AmyR (Gomi et al. 2000) and AoXlnR (Marui et al. 2002) have been described. The other family comprises genes that encode glycolytic enzymes such as *gpdA, pgkA*, and *enoA* (Nakajima et al. 2000). The consensus regulatory region of these glycolytic promoters has been analyzed using an electrophoretic gel mobility shift assay (EMSA) (Toda et al. 2001).

The author previously reported that genes required for detoxification of active oxygen in
A. oryzae were highly expressed. The manganese superoxide dismutase (sodM) (DDBJ/EMBL/GenBank accession no. AB078724) promoter is one of the strongest promoters in A. oryzae, and has been exploited for protein production (Ishida et al. 2004). The sodM gene was highly expressed in submerged cultures but less expressed in solid-state cultures. Thus, the present promoter analysis of the sodM gene was undertaken to learn the regulatory basis for its high-level expression and to allow rational construction of an even stronger promoter. Although Aspergillus fumigatus manganese superoxide dismutase is known to be one of the factors responsible for aspergillosis (Latge 1999), A. oryzae is not a causative agent, in spite of having the sodM gene. Therefore, the sodM promoter from A. fumigatus and from A. oryzae was compared to determine if sequence divergence could account for this difference in pathogenicity.

Materials and methods

Strains, growth conditions, expression plasmid, and primers The A. oryzae OSI1013 AON-2 was used throughout the transformation experiments for GUS reporter assay. GPY medium (2% glucose, 1% peptone, 0.5% yeast extract, 0.2% KCl, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O, and 0.002% FeSO₄·7H₂O) was used to grow the A. oryzae OSI1013 AON-2 and transformants. E. coli JM109 was used for DNA manipulations. The A. oryzae integration vector pNGS1 was used for promoter deletion analysis. Primers are listed in Table 2-1.

Transformation experiments A. oryzae AON-2 mycelia were grown in GPY at 30°C for 24 h and transformed as described (Gomi et al. 1987). Transformants were selected on plates containing sodium nitrate as sole nitrogen source.
**Construction of sodM promoter deletion mutants**  
DNA fragments containing the sodM promoter region of various lengths were amplified by PCR using A. oryzae genomic DNA and the appropriate primers listed in Table 2-1. To generate the PstI and SalI sites at both ends of all PCR products, additional sequences, (5′-AAAACTGCAG-3′) and (5′-ACGCGTCGAC-3′), respectively, were added to the 5′-ends of each upstream and the common downstream (CDS) primer was shown in Table 2-1. The CDS primer contained the antisense nucleotide sequence between -20 to -1 of the sodM promoter. As an example of how the deleted promoters were constructed, the mutant promoter with a site-specific deletion of 30-bp between -209 to -178 was prepared by overlap extension PCR method (Higuchi 1989). The upper fragment (-1,038 to -209) was amplified by PCR using the Del. 1 and Del. 9-u primers, and the lower fragment (-178 to -1) was amplified using the Del. 9-d and CDS primers. To obtain the final site-specific deletion promoter, the two PCRs were combined and subjected to a second PCR using Del. 1 and CDS as primers. All synthesized promoters were digested with PstI and SalI, and individually subcloned upstream of the uidA gene using these same sites in pNGS1.

**Construction of enhanced sodM promoters**  
A partial sodM promoter region (-209 to -1) was amplified by PCR using TD-u and CDS primers, and was ligated to pNGS1 to yield pTD1. The region from -209 to -178 of the sodM promoter was then amplified by PCR using TD-u and TD-d primers and was then digested with PstI and SalI, and subcloned into the PstI and XhoI sites of pTD1. This method was repeated multiple times to construct pTD5 and pTD9, containing five and nine copies of this region, respectively.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>(5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Del. 1</td>
<td>AAAACTGCAGTTATGTACTCCGTACTCGGT</td>
<td></td>
</tr>
<tr>
<td>Del. 2</td>
<td>AAAACTGCAGTTCTTGAACTATATCTTCTT</td>
<td></td>
</tr>
<tr>
<td>Del. 3</td>
<td>AAAACTGCAGATATCAGAGTTCTACAGAGT</td>
<td></td>
</tr>
<tr>
<td>Del. 4</td>
<td>AAAACTGCAGAGGCTGTAGTAGGCGGCCTT</td>
<td></td>
</tr>
<tr>
<td>Del. 5</td>
<td>AAAACTGCAGTCCCGCCAACATGATTGGTA</td>
<td></td>
</tr>
<tr>
<td>Del. 6</td>
<td>AAAACTGCAGTATATTCTCCCTCGCCATAA</td>
<td></td>
</tr>
<tr>
<td>Del. 7</td>
<td>AAAACTGCAGAGTCACATCCCGCCAACATG</td>
<td></td>
</tr>
<tr>
<td>Del. 8</td>
<td>AAAACTGCAGATGAAGGTACCTTGGGGGTC</td>
<td></td>
</tr>
<tr>
<td>Del. 9-u</td>
<td>GTACCTTCTATGAGCGAGTGGAGGGAAT</td>
<td></td>
</tr>
<tr>
<td>Del. 9-d</td>
<td>ACTCGCTCATGAGGTTTGGTA</td>
<td></td>
</tr>
<tr>
<td>TD-u</td>
<td>AAAACTGCAGTCCCGCCAACATG</td>
<td></td>
</tr>
<tr>
<td>TD-d</td>
<td>ACGCGTACCTTTACCAATCATGGGGTG</td>
<td></td>
</tr>
<tr>
<td>CDS</td>
<td>ACGCGTACCTTTACCAATCATGGGGT</td>
<td></td>
</tr>
</tbody>
</table>

**GUS assay**  *A. oryzae* transformants were cultured in 100 ml GPY at 30°C for 72 h. Cell-free extracts were prepared by the method of Ishida *et al.* (1998). GUS activity was measured as described (Jefferson *et al.* 1986). Protein was determined (Bradford 1976) using a commercial kit (Bio-Rad Laboratories) with BSA as a standard.

**Southern blot analysis**  Genomic DNA from *A. oryzae* transformants prepared according to Tsuchiya *et al.* (1992) was digested with *SalI* before Southern blot analysis. The entire 1.8-kbp *uidA* gene fragment was used as a probe. The DIG-DNA labeling and detection kit (Roche, Basel, Switzerland) was used for signal detection.
Preparation of whole-cell extracts (WCE) and EMSA  Whole-cell extracts (WCE) were prepared from *A. oryzae* OSI1013 mycelia that were cultured in 100 ml GPY at 30°C for 24 h (Machida and Jigami 1994). Oligonucleotides and those labeled with IRDye800 were chemically synthesized and annealed to prepare double-stranded probes. The coding region of the *A. oryzae pgmA* gene (+191 to +240) (DDBJ/EMBL/GenBank accession no. AB032275) was used as a non-specific competitor. DNA fragments that had the same sequences as the probes were used as specific competitors. WCE and probes were incubated for 20 min at room temperature as described by Toda *et al.* (2001), and were then subjected to EMSA performed using a LI-COR 4200L DNA sequencer (LI-COR, Lincoln, NE) (Sano *et al.* 2001).
Results

Deletion analysis of the sodM promoter  Plasmids for deletion analysis were constructed by insertion of truncated sodM promoter fragments into pNGS1 for fusion with the uidA gene. A series of sequential deletions at the 5′-end of the 1,038-bp sodM promoter was constructed (Fig. 2-1). These plasmids were introduced into the AON-2 strain, and transformants were selected on the basis of nitrate utilization as sole nitrogen source. Transformants harboring a single copy of the uidA gene were confirmed by genomic Southern blot analysis. Functional analysis of the sodM promoter was performed on submerged cultures by measuring GUS activity. Deletions, spanning the region from -1,038 to -200 (Del. 1-5 in Fig. 2-1), were found not to affect GUS activity. In contrast, a deletion from -1,038 to -138 (Del. 6), resulting in loss of the sequence upstream of the TATA-box resulted in almost complete loss of activity.

Fig. 2-1  GUS activity of the sodM promoters. The numbers noted in each mutant denote the distance from the start codon ATG. GUS activities are the means (filled bars) and standard deviations (error bars) for cell-free extracts from three independent transformants.
Determination of protein-binding regions  EMSA was employed to detect specific binding of intracellular factors to the regulatory regions identified by the deletion analysis. The probe for EMSA was designed to include a part of the sodM promoter sequence (-209 to -138). The competitors were synthesized so as to contain an entire (C0, -209 to -138) or a part (C1, -169 to -138; C2, -189 to -158; C3, -209 to -178) of the promoter sequence. A strongly shifted band (arrow adjacent to lane 2, Fig. 2-2) was detected in the sample incubated with a WCE. The shifted band was detected in the presence of the non-specific competitor, but disappeared upon the addition of the specific competitor (C0). While the short competitor, C3, inhibited formation of the shifted band as did C0, the competitors C1 and C2 did not. These results clearly indicate that the 30-bp region from -209 to -178 is the target of the sequence-specific binding factor(s) present in the WCE.

Fig. 2-2  EMSA of the sodM promoter. WCE (5 µg) and 200 fmol of DNA probe derived from the sodM promoter region (-209 to -178) were mixed and incubated. The specific competitor (C0), partial competitor 1 (C1), partial competitor 2 (C2), and partial competitor 3 (C3) were derived from regions -209 to -178, -169 to -138, and -189 to -158 of the sodM promoter, respectively. NS indicates non-specific competitor. Either 10 pmol (lanes 3, 6, 9, 12, and 15), 5 pmol (lanes 4, 7, 10, 13, and 16), or 1 pmol (lanes 5, 8, 11, 14, and 17) of each competitor were added. SB and FP indicate the shifted band and the free probe, respectively.
**Detailed promoter deletion analysis of sodM promoter**  Detailed deletion analysis was undertaken to confirm the EMSA results. Deletion of the sodM promoter from -209 to -178 (Del. 8, Fig. 2-3) and the site-specific deletion (Del. 9) led to almost complete loss of GUS activity. Weak activity remained after deletion of the region from -209 to -178 and was not affected by further deletions up to position -138 (Del. 6, 8). These results together with the EMSA strongly suggest that the 30-bp region from -209 to -178 is the cis-regulatory element essential for strong expression of the sodM promoter.

![Diagram of sodM promoter deletion analysis](image)

**Fig. 2-3**  GUS activity of the sodM promoter with detailed deletion. The numbers, filled bars, and error bars are as described in the Fig. 2-1 legend.

**Construction of an enhanced sodM promoter**  To confirm the deletion analysis and EMSA results, sodM promoters containing multiple insertions of the putative cis-acting element (-209 to -178) upstream of the minimum promoter (-209 to -1) were constructed. Five and nine tandem copies of the putative cis-element are present upstream of the minimum
promoter (Del. 7 in Fig. 2-3) in pTD5 and pTD9, respectively. These promoters containing the multiple repeats of the putative *cis*-element had about 2-fold higher activity than the minimum *sodM* promoter (Fig. 2-4).

**Fig. 2-4**  GUS activity of the enhanced *sodM* promoters. The numbers, filled bars, and error bars are as described in the Fig. 2-1 legend. The open box indicates Region M (-209 to -178). TD1 contains only the *sodM* promoter region (-209 to -1) and original single copy of Region M. TD5 and TD9 contain five and nine tandem copies of region M, respectively.
Discussion

The initial deletion analysis of the sodM promoter revealed that a 63-bp region (-200 to -138) upstream of the TATA-box was essential for high-level expression of sodM. By way of comparison, the cis-element(s) needed for effective expression in other promoters such as amyB and glaA are more distant from the TATA-box, although the basic enhancers such as the CCAAT-box are located close to the TATA-box (Minetoki et al. 1996). These basic enhancers, in general, do not have a full promoter activity. However, the short region (-209 to -138) of the sodM promoter appeared to have a full promoter activity, judging from similarity to that of the enoA enhancer (Toda et al. 2001), the strongest promoter reported to date.

The EMSA identified the region from -209 to -178 which contained cis-acting element(s), and these results were consistent with the detailed deletion analysis. This region required for high-level expression was named Region M. In Region M, two cis-acting elements were found, the CCAAT-box (Tanaka et al. 2000) and the GC-box (Ishida et al. 2000) (Fig. 2-5a). These motifs have been reported in various promoters and have enhanced activity by changing local chromatin structure (Tsukagoshi et al. 2001). The author speculated that the strong enhancing activity of Region M was due to nearby arrangements of both the CCAAT-box and the GC-box. These arrangements are usually found in the promoters of vertebrates (Bernadt and Rizzino 2003), but have not been reported in other promoters of A. oryzae that have been analyzed to date.

To confirm that Region M has a strong enhancing activity, the enhanced sodM promoters were constructed by connecting five or nine tandem copies of this region. These promoters exhibited 2-3 times higher expression than wild-type (Fig. 2-4), indicating that Region M is a bona fide cis-acting enhancer element. The observation that GUS activity was almost the
same, irrespective of the presence of five or nine consecutive elements, suggests exhaustion of the regulatory protein(s) interacting with Region M, which is known as titration previously reported by Minetoki et al. (1998). A slight decrease in the expression level when the copy number was increased from five to nine is likely to be within the experimental error, but possibly is due to other uncharacterized effects (e.g., conformational change of DNA unfavorable to the binding of the regulatory factor).

In Section 1, the author showed that Region M was responsible for high-level expression of the sodM gene in submerged (liquid) cultures. The A. fumigatus manganese superoxide dismutase (MnSOD) gene (DDBJ/EMBL/GenBank accession no. U53561, Crameri et al. 1996) is a sodM homologue. The product of this homolog is known to be one of the factors responsible for aspergillosis (Latge 1999). The A. fumigatus MnSOD gene was reported to be expressed strongly on the surface of human lung under growth conditions resembling solid-state cultures (Crameri et al. 1996). In Section 1, the A. oryzae sodM gene was found to be repressed in solid-state cultures such as growth on the surface of an agar plate, wheat bran, or rice koji (GUS activities< 100 unit/mg-protein). The sodM homologs from the A. nidulans and A. fumigatus genomes were searched, and their promoter regions were compared. A highly conserved region (HCR) was found upstream of Region M (Fig. 2-5b), but this region did not affect promoter activity under various culture conditions in A. oryzae. Alignment of the regions spanning from the HCR to the translation initiation codon revealed that Region M was found only in the A. oryzae sodM promoter, but not in the other two Aspergilli, although approximately 50% similarity was observed throughout the promoter regions of the three genes, except in Region M (Fig. 2-5b). This would suggest that Region M evolved in the sodM promoter after A. oryzae branched off from A. fumigatus. Modification of sodM expression by insertion of Region M might be one of the mechanisms that prevent human
pathogenicity. The very weak expression of the modified sodM promoter lacking Region M (Del. 9 in Fig. 2-3) in solid-state cultures suggests extensive mutations in HCR of the A. oryzae promoter. This feature of the promoter could contribute to the safety of A. oryzae, as truncation of the transcriptional regulator, AflR, is already known to work as one of the multiple safety locks that prevents aflatoxin production (Kusumoto et al. 1998).

**Fig. 2-5a, b** Nucleotide sequence of *cis*-acting regions. a Nucleotide sequence of Region M. The open boxes indicate the GC-box and the CCAAT-box. b Interspecific comparison of regulatory regions in the sodM promoter. The solid box, open box, and hatched box indicate the TATA-box, Region M, and the highly conserved region (HCR), respectively.
Section 2

Analysis of catalase-encoding gene (catB) promoter expressed constitutively and induced with hydrogen peroxide

Aspergilli are one of the most important fungi for useful enzyme production. In particular, *A. oryzae* is used not only in the manufacture of traditional Japanese foods such as sake and soy sauce, but is also in the production of homo- and heterologous proteins. Recently, the *A. oryzae* genome sequence was identified (Machida et al. 2005), and post-genomic analysis such as transcriptome was done (Masai et al. 2006). Gene expression analysis is very important in the analysis of transcriptome, but promoter analysis and determination of *cis*-elements have not well been examined. These analyses are laborious and require much time, but the results are necessary not only for basic research but also for applications such as constructing strong promoters (Ishida et al. 2006a).

Recently, the authors isolated the catalase-encoding gene (catB) from *A. oryzae* and found strong expression in various cultures. In other *Aspergilli*, catB homolog genes are well expressed, but it is difficult to determine their regulatory *cis*-elements *in silico*; a catalase is commonly involved in detoxification of active oxygen, but it has been reported that regulatory *cis*-elements are different in species, such as *S. cerevisiae*, *Schizosaccharomyces pombe*, and *Homo sapiens* (Nakagawa et al. 1999; Nenoi et al. 2001; O’Rourke et al. 2002).

Furthermore, catB homolog genes such as the *A. fumigatus* cat1 gene are involved in aspergillosis (Shibuya et al. 2006), and the elucidation of the *cis*-elements of catB promoter should be useful for understanding this disease. Hence in Section 2, the author chose the *A. oryzae* catB promoter for its analysis.
Materials and methods

Strains and growth conditions  The A. oryzae OSI1013 AON-2 was used in all the transformation experiments by GUS reporter assay. GPY medium was used to grow the A. oryzae AON-2 and transformants. Czapek-Dox medium was used to select transformants. E. coli JM109 was used in DNA manipulations.

Transformation experiments  A. oryzae AON-2 mycelia were grown in GPY at 30°C for 24 h and transformed as described previously (Gomi et al. 1987). Transformants were selected using Czapek-Dox medium containing sodium nitrate as sole nitrogen source.

Construction of catB promoter deletion mutants  The A. oryzae integration vector pNGS1 used for promoter deletion analysis. Various lengths of DNA fragments of the catB promoter region were amplified by PCR using A. oryzae genomic DNA as a template. Each upstream primer (pd2-pd10) was tailed with 10-mer oligonucleotide (5′-AAAACCTGCAG-3′) to generate a PstI site at the 5′ end (pd2: 5′-AAAACCTGCAGTAGCTATCAACGCAAGTAGT-3′, pd3: 5′-AAAACCTGCAGCCAATATTCTCGAAACGTC-3′, pd4: 5′-AA AACTGCAGTCAACATTCTCATTCTTAT-3′, pd5: 5′-AAAACCTGCAGCTCTCATACC AATTATTGGG-3′, pd6: 5′-AAAACCTGCAGTAGAGGAGCAAGACCCAGTAG-3′, pd7: 5′-AAAACCTGCAGGCAATATTCTCGAACGTC-3′, pd8: 5′-AAAACCTGCAGCATGAGAGCAAGACCCAGTAG-3′, pd9: 5′-AAAACCTGCAGTCGCCGACCCACGCAAGGGG-3′, pd10: 5′-AAAACCTGCAGGGAATATTCTGAATAAG-3′, pd11: 5′-AAAACCTGCAGGAGGTATTGATCATCGAAGC-3′). One upstream primer and the common downstream primer (cdp) were tailed with 10-mer oligonucleotide (5′-ACGCGTCGAC-3′) to generate a SalI site at the 5′ end (pd1: 5′-ACGCGTCGACTTAGGTATATCTCGATC-3′, cdp: 5′-ACGCGTCGACCCTGTGTGCTAAGGT-3′). The cdp primer contained an antisense nucleotide sequence between -20 to -1 of the catB promoter. The mutant promoter with site-specific deletion of 25-bp between -1,000 to -975 was prepared by overlap extension
PCR (Higuchi, 1989). The upper fragment (-1,400 to -1,000) was amplified by PCR using the pd1 and the pd12u primers (5′-CGATCTGATGGAACACCTTTCTCTGAAT-3′), and the lower fragment (-975 to -1) was amplified using the pd12d (5′-AAGTGTCCACATCAGA TCGGGATGAGGCC-3′) and cdp primers. To obtain the site-specific deletion promoter, the two PCR products were combined and subjected to a second PCR using the pd1 and cdp primers. Each promoter fragments were digested with PstI and/or SalI, and were individually introduced upstream of the uidA gene using these same sites in pNGS1.

**GUS assay**  
A. oryzae transformants were cultured in 200 ml GPY at 30°C for 72 h with shaking. Preparation of cell-free extracts was done as described by Ishida et al. (2004). The GUS activity of cell-free extract was measured according to Jefferson et al. (1986). Protein concentration was determined using a protein assay kit (Bio-Rad Laboratories) with BSA as a standard.

**Southern blot analysis**  
Preparation of genomic DNA from A. oryzae transformants was carried out as described Tsuchiya et al. (1992). The entire 1.8-kbp uidA gene fragment was used as a probe. A DIG-DNA labeling and detection kit (Roche) was used for signal detection.

**Preparation of WCE and EMSA**  
WCE were prepared from A. oryzae OSI1013 mycelia that were cultured in 100 ml GPY at 30°C for 24 h (Machida and Jigami 1994). A double-stranded probe was prepared by PCR with a chemically labeled IRDye800 primer (5′-AATGTTGACTGTTTCTTGGCAGCATCCCC-3′) and a non-labeled primer (5′-GCCAATATTTCTCGAAACGTCTCTAGCATCA-3′). A specific competitor was prepared by the same method as both non-labeled primers. The coding region of the A. oryzae pgmA gene (+191 to +240, DDBJ/EMBL/GenBank no. AB032275) was used as a non-specific competitor. WCE and probes were incubated for 20 min at room temperature, as described by Toda et al. (2001), and were then subjected to EMSA performed using a LI-COR 4200L DNA sequencer (LI-COR) (Sano et al. 2001).
Results

**Deletion analysis of the catB promoter** Plasmids for promoter deletion analysis were obtained by insertions of the truncated catB promoter fragments into the pNGSI to fuse the uidA gene. A series of sequential deletions at the 5′-end of the 1400-bp catB promoter was constructed. These plasmids were introduced into the AON-2 strain, and the transformants were selected according to their ability to utilize sodium nitrate as sole nitrogen source. Transformants harboring a single copy of uidA gene were selected by the genomic Southern blot analysis. Functional analysis of the catB promoter was performed on submerged cultures by measuring GUS activity. In the culture condition without hydrogen peroxide, deletion of the region from -1,200 to -1,000 caused a dramatic increase in GUS activity (Del. 2, 3 in Fig. 3-1). Oppositely, deletion of the region from -1,000 to -800 caused marked decreases (Del. 3, 4). A deletion from -800 to -600 (Del. 4, 5) resulted in almost complete loss of activity. Deletions spanning the region from -600 to -200 (Del. 5-7) were found not to affect GUS activity. In the culture condition containing hydrogen peroxide, deletions spanning the region from -1,400 to -1,000 had higher GUS activities than the first condition (Del. 1-3), but deletions spanning the region from -1,000 to -200 (Del. 4-7) were not different in GUS activity.
Determination of protein-binding regions with EMSA

EMSA was employed in order to determine the specific binding of intracellular factors to the transcription regulatory regions identified by promoter deletion analysis. The probe and a specific competitor for EMSA were designed to include part of the catB promoter sequence (-1,000 to -791). The partial competitors were synthesized so as to contain part (C1, -1,000 to -956; C2, -965 to -921; C3, -930 to -886; C4, -895 to -851; C5, -860 to -816; and C6, -825 to -791) of the promoter sequence. A strongly shifted band (Fig. 3-2, arrow adjacent to lane 2) was detected in the sample incubated with WCE. The shifted band was detected in the presence of the non-specific competitor, but disappeared upon addition of the specific competitor. While the short competitor, C1, inhibited formation of the shifted band, as did specific competitor,
competitors C2 to C6 did not. These results clearly indicate that the 45-bp region from -1,000 to -956 was the target of the sequence-specific binding factors present in the WCE. Other probes including the catB promoter sequences (-1,200 to -1,000 and -800 to -600) were designed. But no shifted bands were detected when these probes were used in EMSA.

Fig. 3-2  EMSA of the catB promoter. WCE (10 µg) and 100 fmol of DNA probe derived from the catB promoter region (-1,000 to -791) were mixed and incubated. The specific competitor (SP) and partial competitors (C1-6) were derived from catB promoter regions (SP, -1,000 to -791; C1, -1,000 to -956; C2, -965 to -921; C3, -930 to -886; C4, -895 to -851; C5, -860 to -816; and C6, -825 to -791). NS indicates non-specific competitor. Either 10 pmol (lanes 3, 6, 9, 12, 15, 20, 23, 26, 29, and 32), 5 pmol (lanes 4, 7, 10, 13, 16, 21, 24, 27, and 30), or 1 pmol (lanes 5, 8, 11, 14, 17, 22, 25, 28, and 31) of the competitors were added. SB and FP indicate the shifted band and the free probe, respectively.
Detailed promoter deletion analysis of catB promoter  Detailed deletion analysis was undertaken to confirm these results. Deletion of the catB promoter from -1,000 to -975 (Fig. 3-3, Del. 8), and the site-specific deletion (Del. 12) led to loss of GUS activity in both culture conditions, with and without hydrogen peroxide. Weak activity remained after deletion of the region from -1,000 to -975 and was not affected by further deletions up to -800 (Del. 9-11). These results together with those of the EMSA strongly suggest that the 25-bp region from -1,000 to -975 is the cis-regulatory element essential for strong expression and induction with hydrogen peroxide of the catB promoter.

![Diagram showing promoter deletion analysis](image)

**Fig. 3-3**  GUS activity of the catB promoter with detailed deletion analysis. The numbers, filled bars, open bars, error bars, and culture conditions are as described in the legend to Fig. 3-1.
Discussion

In the initial deletion analysis, the catB promoter was deleted by 200-bp systematically, since 200-bp is the maximum limitation length for a probe length in EMSA. This deletion analysis revealed three regulated regions. The first region, from -1,200 to -1,000, was concerned with repression. The second region, from -1,000 to -800, was necessary for strong expression and induction with hydrogen peroxide. The third region, from -800 to -600, contributed to weak expression. Each region concerned with regulation was distant from the TATA-box or the translation start site, such as agdA gene promoter (Minetoki et al. 1996).

EMSA identified the region from -1,000 to -956 that contained cis-acting elements. Furthermore, detailed promoter deletion analysis indicated that a 25-bp element from -1,000 to -975 was more important. In this 25-bp element, a heat shock elements, (HSE, nTTCnnGAAnnTTCn) and a CCAAT-box, were found (Fig. 3-4). It has been reported that the HSE and its binding protein, a heat shock transcription factor, are highly conserved from yeast to humans (Sakurai and Takemori 2007; Yamamoto et al. 2005; Xiao et al. 1991).

It is well known that heat shock and hydrogen peroxide induces catalase gene expression in Aspergilli (Abrashev et al. 2005; Calera et al. 1997; Kawasaki et al. 1997), and other species (Nakagawa et al. 1999; Nenoi et al. 2001; O’Rourke et al. 2002; Wieser et al. 1991), and that the promoter of each catalase gene has a regulatory element for stress response. However, the regulatory elements are different in species. There is Msn2 or Msn4 binding site in the S. cerevisiae catalase gene CTT1 promoter (O’Rourke et al. 2002), an element A and the Atf1 binding site in the S. pombe catalase gene promoter (Nakagawa et al. 1999), the multi CCAAT-box, and the Sp1 binding site in the human catalase gene promoter (DDBJ/EMBL/GenBank no. AB034940) (Nenoi et al. 2001). Considering these reports, in A.
oryzae, HSE and the CCAAT-box have to do with strong expression and induction with hydrogen peroxide and with a different regulation mechanism of catB in comparison with other species.

In Aspergilli, the catB gene and its homologs are highly conserved and are thought of as housekeeping genes. These genes are regulated by the same mechanism as the glyceraldehyde-3-phosphate dehydrogenase gene (Sano et al. 2001). When the catB gene and its homologous promoters from A. fumigatus, A. nidulans, A. niger, A. terreus, and A. flavus are compared, two highly conserved motifs were found in addition to HSE (Fig. 3-5). The first motif, a CGGAGT-box, which is consistent with the S. cerevisiae transcription factor Adr1p binding motif GG(A/G)G (Cheng et al. 1994), was located in the first region, from -1,200 to -1,000, which was concerned with repression. In the A. oryzae genome database, an ADRI homolog gene was found (AO090023000650, http://www.bio.nite.go.jp/dogan/top). The ADRI homolog gene had high similarity to the DNA binding domain, but no similarity to the activation domain. Further, a DNA binding region and other regions were swapped structurally as compared with ADRI gene, so it is thought that the ADRI homolog gene and its binding CGGAGT-box are concerned with repression of the catB gene. In the third region, from -800 to -600, which contributed to weak expression, a second motif, an ATTACCAAG-box, which was not consistent with any already-known motifs, was located. But neither motifs was detected in EMSA, and a method such as detailed promoter deletion analysis is necessary to identify these motif functions. Since HSE and the two putative motifs were conserved and aliened sequentially in Aspergilli, the catB gene homologs, including A. fumigatus cat1 gene, were regulated in the same way as A. oryzae catB gene described in Section 2.
**Fig. 3-4** Nucleotide sequence of *cis*-acting regions. The HSE motif is underlined. The open box indicates the CCAAT-box.

```
-1,000 GCCAATATTCTCGAAACGTCTCT
      CCAAT-box
```

```
AGCATCAGATCGGATGAGGCC -956
```

**Fig. 3-5** Comparison of *catB* promoters in *Aspergilli*. The numbers denote the distances from the start codon, ATG. The solid box, triangle, open box, and circle indicate the TATA-box, CGGAGT-box, HSE, and ATTACCAAG-box, respectively.
Section 3

Analysis of glucoamylase-encoding gene (glaB) promoter expressed only in solid-state cultures and determination of cis-elements responding to low water activity

The filamentous fungus, *A. oryzae*, is widely used in traditional Japanese fermentations, including sake, soy sauce, *miso*, and *mirin*. These fermentations are conducted using a solid-state cultivation (SSC) process. Protein secretion is enhanced during SSC relative to submerged cultures. Proteomic analysis of extracellular proteins produced as a function of cultivation process identified one set of proteins produced specifically during SSC, a different set produced in submerged cultures, and a third set produced in both cultivation systems (Oda *et al.* 2006). EST analysis showed that the *A. oryzae* gene expression profile changed in a cultivation process-dependent manner (Akao *et al.* 2007). Because submerged culture fermentations have been widely used relative to SSC fermentations, a number of gene expression analyses have focused on the former, involving promoter deletion analysis and EMSA (Ito *et al.* 2004; Toda *et al.* 2001). In spite of the experimental complications involving insoluble substrates (e.g., polysaccharides), several genes have been identified that were expressed specifically during SSC (Kitano *et al.* 2002; Kobayashi *et al.* 2007; Obata *et al.* 2004).

Significant differences between submerged and solid-state media include water activity, diffusion of nutrients and gasses, continuity of medium distribution, and physical barriers to hyphal extension invading the substrates (Akao *et al.* 2007). With specific reference to SSC-based production of the *koji* mold for sake brewing, control of low water activity and high temperature is of critical importance. One reason is that sake brewing is affected by the glucoamylase activity of the *koji*. The *glaB* gene encoding glucoamylase, one of the most
extensively studied SSC-specific genes, is only expressed during SSC (Ishida et al. 2000, 2006). In Section 3, the author used EMSA and promoter deletion analysis to determine important cis-elements responsive to low water activity stress during SSC.

Materials and methods

Strains, growth conditions  A. oryzae OSI1013 AON-2 was used in all the transformation experiments involving GUS reporter assay. GPY medium (1% peptone, 0.5% yeast extract, 0.1% K$_2$HPO$_4$, 0.05% KCl, 0.05% MgSO$_4$·7H$_2$O, 0.001% FeSO$_4$·7H$_2$O, and 2% glucose or 2% soluble starch, pH 6.0) was used in the transformation experiments. Plate culture (GPY medium containing 2% soluble starch, 1.5% agar, and 0.2 μm mixed cellulose ester membrane (ADVANTEC Inc., Tokyo, Japan)) was used for preparing WCE (Ishida et al. 1998). Steamed rice polished to 70% of total weight (Ishida et al. 2006a) and steamed wheat bran (Kobayashi et al. 2007) were used for SSC. Standard growth conditions for A. oryzae transformants were 30°C for 48 h.

Preparation of WCE and EMSA  WCE were prepared from mycelia of A. oryzae OSI1013 grown on plate culture medium containing 50% maltose at 42°C for 48 h (Machida and Jigami 1994). Oligonucleotides and those labeled with IRDye800 were chemically synthesized and annealed to prepare double-strand probes. The coding region of the A. oryzae pgmA gene (+191 to +240) (DDBJ/EMBL/GenBank accession no. AB032275) was used as a non-specific competitor. DNA fragments that had the same sequences as the probes were used as specific competitors. WCE and probes were incubated for 20 min at room temperature, and were then subjected to EMSA performed using a LI-COR 4200L DNA sequencer (Toda et al. 2001).
Construction of glaB promoter deletion mutants  DNA fragments containing various glaB promoter regions were amplified by PCR using A. oryzae genomic DNA and the appropriate primers listed in Table 4-1. To generate the PstI and SalI sites at both ends of all PCR products, additional sequences, (5'-AAAAACTGCAG-3') and (5'-ACGCGTCGAC-3'), respectively, were added to the 5'-ends of each common upstream (CUS) and common downstream (CDS) primer as shown in Table 4-1. The CUS primer contained the sense nucleotide sequence between -420 and -401 of the glaB promoter. The CDS primer contained the antisense nucleotide sequence between -20 and -1 of the glaB promoter. As an example of how the deleted promoters were constructed, the mutant promoter with a site-specific 30-bp deletion between -382 and -354 was prepared by overlap extension PCR (Higuchi et al. 1988). The upper fragment (-420 to -383) was amplified by PCR using CUS and Del1-u primers, and the lower fragment (-352 to -1) was amplified using the Del1-d and CDS primers. To obtain the final construct containing the site-specific deletion, the two PCRs were combined and subjected to a second PCR using CUS and CDS as primers. All synthesized promoters were digested with PstI and SalI, and individually subcloned upstream of the uidA gene using these same sites in pNGS1.

Transformation of A. oryzae  Mycelia from A. oryzae AON-2 were grown in GPY at 30°C for 24 h and transformed as described (Gomi et al. 1987). Transformants were selected on plates containing sodium nitrate as sole nitrogen source.

GUS assay  Cells obtained from a submerged culture and SSC were frozen in liquid nitrogen and disrupted mechanically using a mortar. The cell-free extracts were suspended in 20 mM sodium acetate buffer (pH 5.0), and GUS activities in the supernatants were measured as described (Jefferson et al. 1986).
Table 4-1  PCR primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′ to 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CUS</td>
<td>AAAACTGCAGAGTCCCGCCTTCTAAGAGCAT</td>
</tr>
<tr>
<td>CDS</td>
<td>ACGCGTCGACGATGTTGTTGACTTCCAAGA</td>
</tr>
<tr>
<td>Del1-u</td>
<td>TAGTTCTCACAGTCTAGTGTAGTCTCTTAAT</td>
</tr>
<tr>
<td>Del1-d</td>
<td>GACTAGGACTGTGAGAATAAGAGAATGGC</td>
</tr>
<tr>
<td>Del2-u</td>
<td>TGCCCGTGCCCTGTTGGAACCCCCATCGGAC</td>
</tr>
<tr>
<td>Del2-d</td>
<td>GTTTCCACCAGGCACGGCAGATGTCGGGA</td>
</tr>
<tr>
<td>Del3-u</td>
<td>AAAAGTGATCAGCCATTCTCTTTAGTTCTCAC</td>
</tr>
<tr>
<td>Del3-d</td>
<td>AGAGAATGGCTGATCACTTTTAAGTCGCTCC</td>
</tr>
<tr>
<td>Del4-u</td>
<td>ATTGAAATCGTCCCGACATCTGCCCGTGCC</td>
</tr>
<tr>
<td>Del4-d</td>
<td>GATGTCCGGACGAGTTTCAATTGGATCCGCC</td>
</tr>
<tr>
<td>Del5-u</td>
<td>ATTTCCGGGAATATTGCTCGGAGCGACTA</td>
</tr>
<tr>
<td>Del5-d</td>
<td>GACGCAATATTCCCGGAAATTAATACCGGA</td>
</tr>
<tr>
<td>Del6-u</td>
<td>TGAAGCGAGATTGCAGCGTGCGCGGATCCAAAT</td>
</tr>
<tr>
<td>Del6-d</td>
<td>CCACGCTGCAATCTGCTTACATGATGGGGCC</td>
</tr>
</tbody>
</table>
Results

**Determination of protein-binding regions** In a previous study, it was suggested that the GC-box and a related region of the *glaB* promoter were the essential *cis*-elements involved in the low water activity response (Ishida *et al.* 2000). Here, the author employed EMSA to detect specific binding of intracellular factors. The probe for EMSA was designed to include a part of the *glaB* promoter region (-352 to -313). A shifted band (arrow adjacent to lane 2, Fig. 4-1) was detected in the sample incubated with WCE from SSC. The shifted band was detected in the presence of non-specific competitors, but disappeared upon the addition of a specific competitor. The shifted band was not detected in the presence of submerged culture-derived WCE.

**Determination of protein-binding neighboring regions** To determine other protein-binding regions, two probes were designed (-382 to -333 and -332 to -283). A shifted band (arrow adjacent to lane 2, Fig. 4-2a, b) was detected with each probe. Further, neither shifted band was affected by the presence of a non-specific competitor, but both disappeared in the presence of a specific competitor. Using the -382 to -333 probe, the shifted band was not affected by the presence of partial competitor GB1 (-352 to -333), but it disappeared in the presence of partial competitor C1 (-382 to -353) (Fig. 4-2a). These results indicate that the 30-bp region from -382 to -353 is the target of the sequence-specific binding factor(s) present in WCE. Using the -332 to -283 probe, the band which was shifted strongly disappeared in the presence of the partial competitor GB2 (-332 to -313) as observed for the specific competitor (Fig. 4-2b). These results also indicate that the 20-bp region from -332 to -313 is the target of the sequence-specific binding factor present in WCE. Using other probes (-420 to -383 and -282 to -254), no additional shifted bands were detected. Using the -253 to -236 probe, a weak and broad shifted band was detected.
**Fig. 4-1** EMSA of GC-box. WCE (5 μg) and 200 fmol of DNA probe derived from the glaB promoter region (-352 to -313) were mixed and incubated. SP and NSP indicate specific competitor and non-specific competitor, respectively. Either 10 pmol (lanes 3 and 6), 5 pmol (lanes 4 and 7), or 1 pmol (lanes 5 and 8) of each competitor were added. SB and FP indicate the shifted band and free probe, respectively.

**Fig. 4-2** EMSA of neighboring regions. SP, NSP, SB, and FP are described in the Fig. 4-1 legend. Partial competitors (GB1, GB2, C1, and C2) are derived from the glaB promoter region (-352 to -333, -332 to -313, -382 to -353, and -312 to -283, respectively). Either 10 pmol (lanes 3, 6, 9, and 12), 5 pmol (lanes 4, 7, 10 and 13), or 1 pmol (lanes 5, 8, 11 and 14) of each competitor were added. a Results obtained using a DNA probe from the glaB promoter region -382 to -333; b Results obtained using a probe from -332 to -283.
Deletion analysis of the glaB promoter  Deletion analysis was undertaken to confirm the EMSA results. Plasmids for deletion analysis were constructed by insertion of site-specific deleted glaB promoter fragments into pNGS1 for fusion with the uidA gene. These plasmids were introduced into the AON-2 strain, and the transformants harboring a single copy of the uidA gene were selected. Initially, each transformant was cultivated in a submerged culture and on steamed rice—an SSC method. Compared to the wild-type glaB promoter (Fig. 4-3, Del. 0), Del. 1, Del. 3, and Del. 6 had less GUS activity. In the submerged culture, Del. 1 and Del. 3 were induced by starch, but Del. 6 was not. Among the deleted promoters, Del. 3 resulted in a drastic reduction, especially during growth on steamed rice. In general, GUS activities obtained from steamed rice culture were higher than those observed in the submerged culture.

Induction by low water activity and high temperature  Based on the deletion analysis, three promoter regions (Del. 1, Del. 3, and Del. 6) were found to play a role in glaB expression as a function of culture condition. To determine which regions might be involved in induction by low water activity and high temperature stress, each transformant was cultured on plate and steamed wheat bran, the easily-controlled SSC conditions. The Del. 1, Del. 3, and Del. 6 constructs, which had less GUS activity than Del. 0, also had much lower activity under these two SSC conditions (Fig. 4-4). Under conditions of low water activity (plate culture with 50 % maltose and steamed wheat bran containing 50 % moisture), only the Del. 3 construct was not induced relative to the controls. At high temperature (growth at 42°C), only the Del. 1 construct was not induced. While generally having lower GUS activity, the Del. 6 construct was induced by both low water activity and high temperature stress.
**Fig. 4-3** GUS activity of the *glaB* promoters in submerged culture and steamed rice culture. The numbers noted in each mutant denote the distance from the start codon ATG. GUS activity in submerged culture is indicated by +Glucose and +Starch for 2% glucose or 2% starch as sole carbon source.

<table>
<thead>
<tr>
<th>Del.</th>
<th>Submerged culture</th>
<th>Steamed rice culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+Glucose</td>
<td>+Starch</td>
</tr>
<tr>
<td>Del. 0</td>
<td>420</td>
<td></td>
</tr>
<tr>
<td>Del. 1</td>
<td>35</td>
<td>203</td>
</tr>
<tr>
<td>Del. 2</td>
<td>42</td>
<td>290</td>
</tr>
<tr>
<td>Del. 3</td>
<td>11</td>
<td>85</td>
</tr>
<tr>
<td>Del. 4</td>
<td>28</td>
<td>301</td>
</tr>
<tr>
<td>Del. 5</td>
<td>30</td>
<td>316</td>
</tr>
<tr>
<td>Del. 6</td>
<td>38</td>
<td>45</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Del.</th>
<th>GUS Activity (U/mg)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>plate culture</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+50% Maltose</td>
</tr>
<tr>
<td>Del. 0</td>
<td>422</td>
<td>2,810</td>
</tr>
<tr>
<td>Del. 1</td>
<td>323</td>
<td>2,230</td>
</tr>
<tr>
<td>Del. 2</td>
<td>360</td>
<td>2,722</td>
</tr>
<tr>
<td>Del. 3</td>
<td>85</td>
<td>65</td>
</tr>
<tr>
<td>Del. 4</td>
<td>378</td>
<td>2,955</td>
</tr>
<tr>
<td>Del. 5</td>
<td>405</td>
<td>2,691</td>
</tr>
<tr>
<td>Del. 6</td>
<td>212</td>
<td>1,374</td>
</tr>
</tbody>
</table>

**Fig. 4-4** Effect of promoter deletions at low water activity and high temperature. GUS activity of the *glaB* promoters in plate culture and wheat bran culture. **a** The control indicates GUS activity in plate culture at 30°C on standard medium with 2% starch as sole carbon source. “+50% maltose” indicates a low water activity condition with 50% maltose. “42°C” indicates the control medium incubated at 42°C. **b** The control refers to GUS activity during wheat bran plate culture at 30°C with 10 g steamed wheat bran and 10 ml water. “50% moisture” indicates low water activity conditions with 5 ml water. “42°C” indicates the control medium incubated at 42°C.
Discussion

The EMSA analysis around the GC-box identified three regions as the targets of sequence-specific binding factors (-382 to -353, -332 to -313, and -253 to -236, designated regions H, S, and R, respectively). The results of the deletion analysis together with the EMSA data strongly suggest that the three regions are cis-regulatory elements responsive to environmental stress.

Because the addition of starch increased GUS activity in submerged cultures, except for the constructs containing region R deletions, the author presumes that region R is involved in starch induction (Fig. 4-3, Del. 6). In fact, region R contains a single CGG triplet, a starch-responsive element recognized in the regulatory transfactor AmyR (Ito et al. 2004). Recently, it was also reported that glaB was not expressed in an amyR gene deletion mutant (Watanabe et al. 2011). These previous studies strongly support the possibility that region R is a starch-responsive element.

During SSC at high temperature, GUS activity increased except for the construct with a deleted region H (Fig. 4-4, Del. 1), suggesting that region H is involved in induction by high temperature. In region H, HSE like motif (TTCnmmnGATnmmmTTC) was found (Matsushita et al. 2009). In the previous study, f-HSE (-349 to -345) or b-HSE (-340 to -336) substitution is highly effective in GUS activities about 2-fold lower (plate culture) and 2-fold upper (SSC), respectively, but our EMSA results show that no proteins bind these regions (Ishida et al. 2000). Perhaps, these substitutions affect of sequence-specific binding factors on their binding ability to cis-regulatory elements.
During SSC, deletion of region S caused a dramatic decrease in GUS activity, suggesting that region S is essential for strong glaB expression. Although GUS was inducible by starch and high temperature in the region S deletion mutant, it was not affected by low water activity (Fig. 4-4, Del 3). Unlike regions R and H, no known motifs were found in region S. While glucoamylases in Aspergilli generally contain catalytic and starch-binding domains, the glucoamylase encoded by glaB only contains a catalytic domain. Recent genomic analysis found that a glaB ortholog exists in Aspergilli including A. clavatus (DDBJ/EMBL/GenBank accession no. XM_001272680) and A. terreus (XM_001215158). When the glaB promoter was compared to those of orthologs from A. clavatus and A. terreus, a highly conserved motif CGGnnAGnnGTCGGG (SSC-motif) was found (Fig. 4-5). In a previous study, the sequence was identified as CGG-N8-CGG, the AmyR-binding motif (Petersen et al. 1999). More recent work identified the functional binding motif as CGGnnnTTTnTCGG (Wang et al. 2012). Together with these previous studies, our results strongly suggest that a transfactor(s) other than AmyR binds to this motif in A. oryzae. In Section 3, the author identified region S which

![Fig. 4-5](image-url)
contains *cis*-elements responding to low water activity stress. Because the *glaB* gene is found on a syntenic block based on comparative genomics (Machida *et al.* 2008), the author compared the sequences of several adjacent genes. These genes are partially conserved in *A. oryzae*, *A. clavatus*, and *A. terreus* (Fig. 4-6). Although six genes including *glaB* are located in this region, insertion, reversal, and transfer events could have occurred, there is no evidence for insertion or reversal events within the first promoter region between *glaB* and an integral membrane protein-encoding gene (*impB*) in these three species. This holds true for the second promoter region between an ER membrane protein-encoding gene (*pkr1*) and a monosaccharide transporter-encoding gene (*mstB*). When the two regions were compared, a region S-like sequence was found (5′-GGTGGAATGGGCAGATATGGTG-3′) in the second promoter region. Because the length of the promoter regions was 1,339 and 2,066-bp, respectively, it is likely that only one region S sequence was shared by the *glaB* and *impB*. The author speculated that the *pkr1* and *mstB* genes also shared the region S-like promoter sequence. Region S-like sequences were not found in the promoters of the anaphase-promoting complex subunit-encoding gene (*apcB*) or taurine catabolism dioxygenase-encoding gene (*tauD*). These findings suggest that gene insertion, reversal, or transfer events may have resulted in loss of region S-like sequences. Further, because the fungal-specific transcription factor-encoding gene in *A. oryzae* (Fig. 4-6, *T1*, *fstA*) and transcription factor-encoding gene in *A. clavatus* (Fig. 4-6, *T2*, *rfeD*) are found on non-syntenic blocks, the transcription factor-encoding gene subject to region S regulation would be transferred to another syntenic block.

Section 3 is the first to analyze *cis*-elements in the *glaB* promoter that are responsive to SSC-specific conditions (e.g., low water activity) by EMSA and deletion analysis. Further, our combined approach involving WCE preparation, easily-controlled SSC conditions, and
comparative genomics can be applied directly to identify other genes whose promoters are expressed specifically during SSC.

Fig. 4-6 Comparative genomics of the *glaB* gene region in *A. oryzae*, *A. clavatus*, and *A. terreus*. *GB*, *I*, *E*, *M*, *A*, *D*, *T1*, and *T2* indicate the *glaB* gene (DDBJ/EMBL/GenBank accession no. XM_001819466 in *A. oryzae*), integral membrane protein-encoding gene (*impB*, XM_001819467), ER membrane protein-encoding gene (*pkr1*, XM_001819469), monosaccharide transporter-encoding gene (*mstB*, XM_001819470), anaphase-promoting complex subunit-encoding gene (*apcB*, XM_001819471), taurine catabolism dioxygenase-encoding gene (*tauD*, XM_001819472), fungal-specific transcription factor-encoding gene (*fstA*, XM_001819473), and transcription factor-encoding gene (*rfeD*, XM_001272671 in *A. clavatus*), respectively. Box-like arrows indicate gene orientation.
Chapter III

High production of llama variable heavy-chain fused to glucoamylase (GlaB) in submerged culture

Antibodies and their derivatives are proteins that possess specific and high-affinity binding ability. These abilities have been applied successfully to human therapy in the form of humanized antibodies, e.g., tocilizumab (Nishimoto et al. 2003), omalizumab (Spector 2004), and ustekinumab (Nora et al. 2010). Antibodies have traditionally and widely been used as reagents for the detection and purification of antigens. In the area of food safety, reliable and cost-effective detection of caffeine (Carvalho et al. 2010), aflatoxin (Uchigashima et al. 2009), and botulinum toxin (Ponmariappan et al. 2011) requires highly stable antibodies that can be produced at low cost.

Hamers-Casterman et al. (1993) reported a novel class of two heavy chain immunoglobulins, in addition to conventional four-chain immunoglobulins, in members of the camelid family (including camels and llamas). Because these antibodies are devoid of light chains and do not require a constant heavy chain domain to bind antigens, their heavy-chain variable domain (VHH) has been proposed as a potentially valuable biotechnological tool (Muyldermans 2001). Recently, other two heavy-chain immunoglobulins were found in various vertebrates, including cartilaginous fish (ratfish, shark, skates, and rays), monotreme (platypus), and marsupial (koala) (Flajnik et al. 2011). Because an antibody consisting of a single polypeptide is more easily manipulated through protein engineering, this class of antibodies has been the subject of significant research effort, e.g., improved thermal stabilization (Hagihara et al. 2007; Hussack et al. 2011).

Aspergilli have a long history of use in biotechnology as expression platforms for the
production of various excreted homologous and heterologous proteins (Meyer et al. 2011). In a first report, non-fused VHH were produced by *Aspergillus awamori*, resulting in functional VHH excreted into culture medium (Joosten et al. 2005a). Following this early study, VHH fusion proteins (e.g., VHH-peroxidase) were produced (Joosten et al. 2005b). These studies have shown that VHH and fused VHH fusion proteins could be produced, but at low levels (7.5 and 30 mg protein/l culture medium, respectively), which is inadequate for large-scale industrial application.

*A. oryzae* is capable of secreting a significant amount of proteins into a culture medium (Archer and Peberdy 1997). Production of a heterologous target protein fused to homologous secreted proteins such as Taka-amylase and glucoamylase A (Hirayama et al. 2010; Ohno et al. 2011) is one means of overcoming the problem of low productivity. In Chapter III, the author describes the expression of VHH against human chorionic gonadotropin (hCG) in *A. oryzae* by fusing VHH to the C-termini of four highly secreted homologous leader proteins. C-terminal His-tag sequences were added to confirm that full-length VHHs were produced. The VHH proteins were assayed for native binding ability, and evaluated for overproduction.

**Materials and methods**

**Strains and growth conditions** The *A. oryzae* OSI1013 leu-5, a *leuA* mutant (deposited with IPOD as FERM P-20079), derived from wild-type *A. oryzae* OSI1013 was used throughout the transformation experiments for target gene expression and production (Ishida et al. 2006b). GPY medium was used for growing the *A. oryzae leuA* mutant and transformants. *E. coli* HST08 strain (TaKaRa Bio) was used for DNA manipulations.
Plasmid construction and transformation of *A. oryzae*  The plasmids constructed and used in Chapter III are summarized in Table 5-1 and Table 5-2, respectively. Plasmid phlACB which contains the *hlyA* promoter and *glaB* terminator (Bando et al. 2011) was used as the target gene expression cassette. All target genes and vectors were amplified by PCR using *A. oryzae* genomic DNA, the phlACB plasmid, and a synthetic VHH-encoding gene (Hokkaido System Science, Sapporo, Japan; Fig. 5-1), to construct the plasmids in Chapter III. As an example of how the *amyA-His* (*amyA*, DDBJ/EMBL/GenBank accession no. M33218) expression vector pAA was constructed, the leader protein-encoding gene and common expression vector (CEV) were amplified by PCR separately, after which the protein-encoding DNA fragment was subcloned into CEV. Initially, the CEV was amplified by PCR using vec-u and vec-d primers and phlACB plasmid as a template. Next, the *amyA* gene fragment was amplified by PCR with AA-u and AA-d primers and *A. oryzae* OSI1013 strain genomic DNA as template. The *amyA* gene fragment was then subcloned into the CEV using an In-Fusion™ Advantage PCR cloning kit (TaKaRa Bio) to generate the expression vector pAA plasmid. In the case of VHH expression, e.g., *amyA-VHH-His*, the *amyA* gene was amplified using primer AA-d-VHH instead of AA-d. The common VHH-encoding gene fragment (CV) was amplified using VHH-u and VHH-d primers and the synthetic VHH-encoding gene as template. The *amyA* gene fragment and CV were then subcloned into the CEV using the same cloning kit to generate expression vector pAAV. Other expression vectors were generated similarly by PCR using the appropriate primers.

*A. oryzae* was transformed as described by Bando et al. (2011). DNA used for transformations was generated as described below. Each expression cassette was amplified by PCR using M13-P7 and M13-P8 primers and appropriate constructed plasmids as templates. The isopropylmalate isomerase-encoding gene (*leuA*) from *A. nidulans* was amplified by PCR
using the same primer noted above in Bando et al. (2011). The resultant transformants were subcultured and selected on solid Czapek-Dox medium.

**Table 5-1** Genes, plasmids and relevant proteins used in Chapter III

<table>
<thead>
<tr>
<th>Gene</th>
<th>Source</th>
<th>Relevant protein</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>amyA</em></td>
<td>M33218&lt;sup&gt;a&lt;/sup&gt;</td>
<td>α-amylase (<em>AmyA</em>&lt;sup&gt;b&lt;/sup&gt;)</td>
</tr>
<tr>
<td><em>glaB</em></td>
<td>AB007825&lt;sup&gt;a&lt;/sup&gt;</td>
<td>glucoamylase (<em>GlaB</em>&lt;sup&gt;b&lt;/sup&gt;)</td>
</tr>
<tr>
<td><em>celA</em></td>
<td>D83731&lt;sup&gt;a&lt;/sup&gt;</td>
<td>endo-1,4-β-glucanase (<em>CelA</em>&lt;sup&gt;b&lt;/sup&gt;)</td>
</tr>
<tr>
<td><em>celB</em></td>
<td>D83732&lt;sup&gt;a&lt;/sup&gt;</td>
<td>endo-1,4-β-glucanase (<em>CelB</em>&lt;sup&gt;b&lt;/sup&gt;)</td>
</tr>
<tr>
<td><em>VHH</em></td>
<td>1G9E_A&lt;sup&gt;c&lt;/sup&gt;</td>
<td>heavy-chain antibody fragment (<em>VHH</em>&lt;sup&gt;b&lt;/sup&gt;)</td>
</tr>
<tr>
<td><em>His</em></td>
<td></td>
<td>6xHis-tag (<em>His</em>&lt;sup&gt;b&lt;/sup&gt;)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Expressed gene</th>
<th>Relevant protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAA</td>
<td><em>amyA</em>-His</td>
<td><em>AmyA</em>-His</td>
</tr>
<tr>
<td>pAAV</td>
<td><em>amyA</em>-VHH-His</td>
<td><em>AmyA</em>-VHH-His</td>
</tr>
<tr>
<td>pGB</td>
<td><em>glaB</em>-His</td>
<td><em>GlaB</em>-His</td>
</tr>
<tr>
<td>pGBV</td>
<td><em>glaB</em>-VHH-His</td>
<td><em>GlaB</em>-VHH-His</td>
</tr>
<tr>
<td>pCA</td>
<td><em>celA</em>-His</td>
<td><em>CelA</em>-His</td>
</tr>
<tr>
<td>pCAV</td>
<td><em>celA</em>-VHH-His</td>
<td><em>CelA</em>-VHH-His</td>
</tr>
<tr>
<td>pCB</td>
<td><em>celB</em>-His</td>
<td><em>CelB</em>-His</td>
</tr>
<tr>
<td>pCBV</td>
<td><em>celB</em>-VHH-His</td>
<td><em>CelB</em>-VHH-His</td>
</tr>
</tbody>
</table>

<sup>a</sup> DDBJ/EMBL/GenBank accession no.  <sup>b</sup> Abbreviation  <sup>c</sup> PDB accession no.

**Fig. 5-1** Nucleotide sequence of a synthetic VHH-encoding gene (PDB accession no. 1G9E_A).
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>vec-u</td>
<td>GGTTTTGTGGTTGTAAGGTTAGTTGATGTG</td>
</tr>
<tr>
<td>vec-d</td>
<td>CACCATCCACACACCAAACACCCTAACATGTCATTTCCAGTGGTCGTATGTCTACTCT</td>
</tr>
<tr>
<td>AA-u</td>
<td>TCACACCACAACACCATGATGGTCGGTTGGTCTCTATTTTCTG</td>
</tr>
<tr>
<td>AA-d</td>
<td>GTGGTTGGTAGTGTCGTGGTACATCTACAGATCTTGGCTACCTGCAAA</td>
</tr>
<tr>
<td>GB-u</td>
<td>TCACACCACAACACCATGCGAGAAACAACCTTCTTTTCTTTCCCCTCAAT</td>
</tr>
<tr>
<td>GB-d</td>
<td>GTGGTTGGTAGTGTCGTGGTAGTAGGGTAGTGACTACT</td>
</tr>
<tr>
<td>CA-u</td>
<td>TCACACCACAACACCATAGTCTGACTGCTCCCTTTGTCAGTC</td>
</tr>
<tr>
<td>CA-d</td>
<td>GTGGTTGGTAGTGTCGTGGTAGGCTCAGTGGGAAACGT</td>
</tr>
<tr>
<td>CB-u</td>
<td>TCACACCACAACACCATGATCGACTGCTCCCTTTGTCAGTC</td>
</tr>
<tr>
<td>CB-d</td>
<td>GTGGTTGGTAGTGTCGTGGTAGGCTCAGTGGGAAACGT</td>
</tr>
<tr>
<td>VHH-u</td>
<td>CAGTTCCAGCTCCAGGAGTCCGGTTGGT</td>
</tr>
<tr>
<td>VHH-d</td>
<td>GTGGTTGGTAGTGTCGTGGTAGGCTCAGTGGGAAACGT</td>
</tr>
<tr>
<td>AA-d-VHH</td>
<td>CTGGAGCTGGACCTGCGAGCTACAGATACCTTGGCTACCTGCAAA</td>
</tr>
<tr>
<td>GB-d-VHH</td>
<td>CTGGAGCTGGACCTGCGAGCTACAGATACCTTGGCTACCTGCAAA</td>
</tr>
<tr>
<td>CA-d-VHH</td>
<td>CTGGAGCTGGACCTGCGAGCTACAGATACCTTGGCTACCTGCAAA</td>
</tr>
<tr>
<td>CB-d-VHH</td>
<td>CTGGAGCTGGACCTGCGAGCTACAGATACCTTGGCTACCTGCAAA</td>
</tr>
<tr>
<td>M13-P7</td>
<td>GGTTAAGCGGACGGTTTCCAGTCACGAC</td>
</tr>
<tr>
<td>M13-P8</td>
<td>GTGAGCGGATAACATTTCACACAGGAAAC</td>
</tr>
</tbody>
</table>
Purification of His-tagged proteins  Transformants were grown in 100 ml GPY medium at 30°C for 3 d. To recover culture media, mycelia were removed by filtration using a 0.45-μm filter. The culture medium was adjusted to pH 7.4, and loaded onto a His GraviTrap™ system (GE Healthcare Bio-Sciences, Little Chalfont, United Kingdom) from which the His-tagged proteins were purified as described by the manufacturer. Impurities were first removed by use of a wash buffer (20 mM sodium phosphate, 500 mM NaCl, 10 mM imidazole, pH 7.4). His-tagged proteins were then recovered by elution with 20 mM sodium phosphate, 500 mM NaCl, and 500 mM imidazole, pH 7.4. Desalting and buffer exchange were performed by loading eluates onto PD-10 desalting columns (GE Healthcare Bio-Sciences) as described by the manufacturer.

Deglycosylation of purified proteins with endoglycosidase H (Endo H)  The purified protein was deglycosylated with endoglycosidase H (Endo H from Streptomyces plicatus; Merck KGaA, Darmstadt, Germany) using the modified protocol. The protein was diluted to 0.5 mg/ml using denaturing reaction buffer (50 mM sodium phosphate, 0.1% sodium dodecyl sulfate [SDS], and 50 mM β-mercaptoethanol, pH 5.5), and incubated at 80°C for 10 min. The denatured protein sample was then treated with Endo H (final concentration 0.1 U/ml) at 37°C for 3 h.

Binding assay of fused VHH protein against hCG  The purified proteins were immobilized on CNBr-activated sepharose™ 4B (GE Healthcare Bio-Sciences) using the standard protocol supplied by the manufacturer. Each purified protein (40 μg) and the activated sepharose beads, 20 μl, were mixed to immobilize the purified protein. The binding assay using the immobilized sepharose beads and human chorionic gonadotropin (hCG, Wako Pure Chemical, Osaka, Japan) was performed as described (Hagihara et al. 2005). The protein-immobilized sepharose beads, 20 μl, and hCG, 10 μg, were incubated for 90 min at
room temperature in binding buffer (20 mM Tris-HCl, 150 mM NaCl, pH 8.0). After the beads were washed with binding buffer three times, they were boiled for 15 min at 95°C with 10 μl of SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer. The supernatant recovered from the beads was loaded onto an SDS-PAGE gel (Mini-PROTEAN TGX™ Any kD gels, Bio-Rad Laboratories) and stained with Coomassie Brilliant Blue (CBB; GelCode Blue Safe Protein Stain™, TaKaRa Bio).

**His-tagged protein staining in polyacrylamide gel** His-tagged proteins in polyacrylamide gels were stained using a commercial staining kit (InVision™ His-tag In-gel Stain, Life Technologies, Carlsbad, CA). Stained bands were detected using a UV transilluminator and signal intensity was quantified by Image J software (Abramoff et al. 2004).

**Detection of hCG-binding activity of fused VHH protein by enzyme-linked immunosorbent assay (ELISA)** A microtiter plate (type H 96-well microtiter plate; Sumitomo Bakelite, Tokyo, Japan) was coated overnight at 4°C with 20 μg hCG (20 mM Tris-HCl, pH 8.0) and then washed three times with 20 mM Tris-HCl buffer (pH 8.0). After the plate was blocked with blocking buffer (1% BSA, 20 mM Tris-HCl, pH 8.0) for 3 h at room temperature, it was washed three times with binding buffer. After addition of the purified protein, the plate was incubated for 3 h at room temperature followed by another wash with binding buffer. This was then followed by addition of substrate solution (50 mM sodium citrate, 5 mg/ml maltose, 15 ng/ml 3,3’,5,5’-tetramethylbenzidine [TMB], 10 U/ml glucose oxidase from *A. niger*, 10 U/ml peroxidase from horseradish, pH 5.0). After incubation for 30 min at room temperature, 1 M sulfuric acid solution was added to stop the reaction (Christensen et al. 1995).
Results

Overexpression and overproduction of leader proteins and fused VHH proteins
The VHH gene was fused to genes encoding leader proteins amylase (AmyA-His), glucoamylase (GlaB-His), and endo-glucanases (CelA-His, and CelB-His) to generate cassettes for expression in *A. oryzae* (AmyA-VHH-His, GlaB-VHH-His, CelA-VHH-His, and CelB-VHH-His, Table 5-1). Transformants harboring each expression cassette were cultured in GPY medium at 30°C for 3 d, after which culture media were analyzed by SDS-PAGE analysis (Fig. 5-2). Although the CelA-VHH-His protein was produced at a very low level, the other proteins were produced more efficiently, as the apparent molecular weight of fused VHH protein increased. Each VHH fusion protein was about 10 kDa larger than the corresponding leader protein.

Fig. 5-2  Overproduction of VHH fusion proteins by *A. oryzae* transformants. Supernatants (7.5 μl) from 3-day culture media were analyzed by SDS-PAGE. Control (lane 1), leader proteins AmyB-His (lane 2), GlaB-His (lane 4), CelA-His (lane 6), and CelB-His (lane 8) and VHH-leader fusion proteins AmyB-VHH-His (lane 3), GlaB-VHH-His (lane 5), CelA-His (lane 7), and CelB-VHH-His (lane 9).
Purification of VHH fusion proteins and deglycosylation  VHH fusion proteins were purified using a commercial His-tag column. Concentrations of the purified proteins were determined using BSA as a standard. The GlaB-VHH-His protein was recovered at the highest concentration, about 0.155 mg protein/ml culture medium. AmyA-VHH-His and CelB-VHH-His were recovered at 0.033 and 0.101 mg protein/ml culture medium, respectively. Very little of the CelA-VHH-His protein was detected, less than 0.005 mg protein/ml culture medium.

The apparent molecular weight of the purified proteins was larger than the calculated molecular weight (Fig. 5-3). To determine exact molecular weight of the fused VHH proteins, a deglycosylation treatment was performed. AmyA-VHH-His, GlaB-VHH-His, and CelB-VHH-His (calculated molecular weight of 65.8, 64.0, and 56.0 kDa, respectively) were detected by SDS-PAGE analysis (Fig. 5-3). These results indicate that the full-length fused VHH proteins were produced by *A. oryzae*.

**Fig. 5-3** Deglycosylation of VHH fusion proteins by Endo H treatment. Purified VHH fusion proteins incubated with Endo H (lanes 4-6), and without Endo H (lanes 1-3) were analyzed by SDS-PAGE. Lanes 1 and 4, purified AmyA-VHH-His; lanes 2 and 5, purified GlaB-VHH-His; lanes 3 and 6, purified CelB-VHH-His.
Binding assay of VHH fusion proteins with the hCG antigen  The GlaB-His and purified VHH fusion protein GlaB-VHH-His were immobilized on sepharose, and the presumptive immobilized sepharoses were incubated with hCG, washed, and then treated with SDS-PAGE sample buffer to elute the bound hCG. hCG was detected in the eluent from the GlaB-VHH-His immobilized sepharose by SDS-PAGE analysis. hCG was detected in the eluent from the GlaB-VHH-His-immobilized sepharose, but not in those from the GlaB-His-immobilized and non-immobilized (Fig. 5-4). These results indicate that hCG binds specifically with the VHH region of the VHH fusion protein. Similar results were obtained with the other VHH fusion proteins, AmyA-VHH-His and CelB-VHH-His.

![Figure 5-4](image)

**Fig. 5-4**  Binding of the hCG antigen to immobilized and purified VHH fusion proteins. Leader proteins AmyB-His (lane 3), GlaB-His (lane 5), and CelB-His (lane 7) and VHH fusion proteins AmyB-VHH-His (lane 4), GlaB-VHH-His (lane 6), and CelB-VHH-His (lane 8) were immobilized on sepharose beads. Beads and hCG were incubated at room temperature for 90 min. After washing and elution, bound hCG was subjected to SDS-PAGE.
Detection of the hCG antigen by ELISA using glucoamylase activity  

Because purified GlaB-VHH-His had glucoamylase activity, the author demonstrated the coupled glucoamylase assay involving glucoamylase, glucose oxidase, and peroxidase in an ELISA-based format to confirm binding ability of GlaB-VHH-His. While addition of GlaB-His did not cause color formation in the microtiter plate coated with hCG, addition of GlaB-VHH-His did at least 0.1 μg (Fig. 5-5).

<table>
<thead>
<tr>
<th>Amount of purified protein (μg)</th>
<th>3.0</th>
<th>1.0</th>
<th>0.3</th>
<th>0.1</th>
<th>0.03</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlaB-His</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GlaB-VHH-His</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Fig. 5-5  Detection of the hCG antigen with GlaB-VHH-His using glucoamylase activity. The hCG-binding activity of GlaB-VHH-His was tested by ELISA. Microtiter plates were coated with hCG and various amounts of purified GlaB-VHH-His were subsequently loaded onto the plates. Bound GlaB-VHH-His was detected using the coupled enzymatic assay (based on addition of glucose oxidase and peroxidase and substrates maltose and TMB). Purified GlaB-His was used as a control to confirm non-specific binding.
Production of GlaB-VHH-His  Because higher amount of GlaB-VHH-His was produced than any of the other fusion proteins, the author analyzed GlaB-VHH-His productivity by the A. oryzae transformant. Ammonium sulfate (final concentration, 1% w/v) or potassium phosphate (final concentration 100 mM) was added to the culture medium to prevent proteolysis, or to improve secretion efficiency, respectively (Sato et al. 2011). The amount of GlaB-VHH-His was determined by SDS-PAGE analysis, using purified GlaB-VHH-His as a standard. Using CBB staining, low molecular weight signals were detected in addition to the expected band corresponding to the standard. Staining with the His-tag specific reagent generated signals only corresponding to the expected band for GlaB-VHH-His. By CBB staining, 7-day culture media with or without potassium phosphate addition indicated the highest productivity. Using the His-tag-specific stain, the 5-day culture medium with potassium phosphate addition had the highest productivity (1.35 mg protein/ml, Fig. 5-6). Using the His-tag column, GlaB-VHH-His was recovered at 0.610 mg protein/ml above-mentioned culture medium. Addition of ammonium sulfate caused lower productivity than non-addition and addition of potassium phosphate.
**Fig. 5-6** Time course of GlaB-VHH-His production by *A. oryzae* transformant harboring *glaB-VHH-His* expression cassette. Supernatants (2.5 μl) from culture media were analyzed by SDS-PAGE by CBB staining (a) and by His-tag-specific staining (b). Signal intensity was estimated with imaging software (c). Purified GlaB-VHH-His was used as a standard (lane 1, 1 μg; lane 2, 4 μg). 3-day cultivation (lanes 3-5), 5-day cultivation (lanes 6-8), 7-day cultivation (lanes 9-11), and 10-day cultivation (lanes 12-14). Lanes 3, 6, 9, and 12, GPY medium with no addition; lanes 4, 7, 10, and 13, GPY medium with ammonium sulfate addition; lanes 5, 8, 11, and 14, GPY medium with potassium phosphate addition; lane M, protein ladder. In c, the empty box indicates the calculated amounts of GlaB-VHH-His from CBB staining while the filled box indicates calculated amounts of GlaB-VHH-His from His-tag-specific staining.
Discussion

In Chapter III, four highly secreted proteins were used as leader proteins fused to VHH in order to achieve high-level production and secretion in *A. oryzae*. The productivity of the fusion proteins was greatly affected by the choice of the leader protein. As the apparent molecular weight of the leader protein increased, productivity improved. The author speculated that the apparent molecular weight of the leader protein might play a role in protection against proteolysis, because the productivity of the fusion proteins was higher than that of VHH alone produced by *A. awamori* as reported in previous studies (Joosten *et al.* 2005a, b).

The ability of the VHH fusion proteins to bind hCG was determined using the same method tested for constructs previously expressed in *E. coli* (Hagihara *et al.* 2005). The author detected similar binding ability for constructs expressed in *A. oryzae*.

To confirm binding ability of the fused VHH proteins in a practical ELISA-based format, a coupled enzymatic assay was developed for the GlaB-VHH-His construct that had glucoamylase activity. By this assay, as little as 0.1 μg per well of the GlaB-VHH-His fusion protein was detectable. An ELISA requires a first antibody to bind its target antigen and a secondary antibody typically conjugated to peroxidase or alkali phosphatase for detection of binding. Although the first antibody was replaced by VHH alone produced by *E. coli* and *A. awamori*, a secondary antibody was found necessary for detection in previous work (Hagihara *et al.* 2005; Joosten *et al.* 2005a). In contrast, our results here show not only that a secondary antibody was not necessary for detection, but also that the GlaB-VHH-His was more suitable for practical use than peroxidase-VHH fusion protein (Joosten *et al.* 2005b).

For large-scale industrial application, more than 1 g of VHH fusion protein/l is required
The author examined a high-level production of the GlaB-VHH-His construct. Although 1.35 mg of protein/ml culture medium was obtained for full-length GlaB-VHH-His by His-tag staining, yields of the purified protein using His-tag column were much lower. In *A. niger*, it was reported that a C-terminal His-tag was easily degraded, resulting in low binding ability to His-tag affinity columns. In fact, immunoblot analysis using an anti-His-tag antibody was able to detect non-adsorption of His-tagged proteins in the flow through fractions from the affinity columns (Roth and Dersch 2010), indicating that actual GlaB-VHH-His productivity was close to that estimated using the His-tag-specific stain. An ELISA-based estimate using the glucoamylase activity indicated more than 1.0 mg protein/ml culture medium.

Degradation of GlaB-VHH-His could have occurred over long-term cultivation (Fig. 5-5a, b) as described for a peroxidase-VHH fusion protein in *A. awamori* (Joosten *et al.* 2005b). The host strain used for production of GlaB-VHH-His in Chapter III possesses proteases that might be responsible for degradation of expressed GlaB-VHH-His. Hence the use of a protease-deficient host might increase protein production dramatically (Yoon *et al.* 2011). In Chapter III, the author demonstrated high-level production of a VHH fusion protein, approaching industrially relevant levels. Not all tested leader proteins were found to be effective in terms of yields of the fusion constructs. Incorporation of an enzymatically active leader protein allowed development of a novel ELISA for sensitive detection of the desired protein as an additional advantage of this approach. The author anticipates that the method described here should be promising also for the overproduction of valuable heterologous proteins and peptides.
Conclusions

A final and long-cherished goal of fermentation industries in Japan is to manufacture a high quality of “liquid koji” that contains a significant amount of saccharifying enzymes such as glucoamylase and α-amylase for use in modern industrial manufacture of good quality of traditional Japanese fermentation foods such as sake, mirin, miso and soy sauce. Due to lack of detailed information about the mechanisms and regulation of expression of saccharifying enzyme-encoding genes in Aspergillus oryzae, an essential microbe for koji manufacture, a good quality of koji has so far been and can only be manufactured in a solid-state culture with a delicate and complicated process by craftsmanship of skilled brewers. Therefore, it is critically important to understand how the gene expression is controlled depending on culture conditions in A. oryzae, a specifically bred fungus over years for koji manufacture (solid-state culture). The present study describes gene expression and promoter analysis of the genes that are expressed in a culture-condition dependent manner in A. oryzae. This study revealed in Chapter I that the expression of two catalase isozyme-encoding genes, catA and catB, is controlled independently by culture conditions and in response to H$_2$O$_2$.

Since gene expression is regulated directly by its promoter, the analysis of promoters is important in understanding the regulation mechanism of culture-condition-specific gene expression. The promoter analysis of submerged (liquid) culture-specific expression gene (sodM) showed that the promoter carried unique cis-elements that are not found in other Aspergilli, suggesting that A. oryzae would be bred specifically for use in fermentation as a fungus of high safety. In contrast, the promoter analysis of the gene expressed under various culture conditions (catB) showed that the promoter held universal cis-elements conserved widely in other Aspergilli and that the gene was regulated similarly. Lastly, the analysis of the
promoter of solid-state culture-specific expression gene (glaB) revealed that the promoter harbored unique cis-elements that are found in a limited number of species of Aspergilli, a genetic trait suggesting that A. oryzae have been intentionally selected for sake brewing among Aspergilli. The results of the promoter analyses of three genes revealed several unique features of A. oryzae in Aspergilli as a result of breeding over years for sake brewing, and suggested the potential of A. oryzae as a benign and useful microbe of high safety and fidelity for liquid koji manufacture in the future that is suitable for highly mechanized fermentation industries.

A. oryzae is also an attractive mold as a host for production of valuable proteins from different organisms. A llama variable heavy-chain antibody (VHH) fused with glucoamylase was produced and secreted in submerged culture of A. oryzae. The fusion protein was functional and had an antigen binding ability as the original VHH. Furthermore, the glucoamylase activity derived from the fusion leader protein was employed successfully as a marker for single protein ELISA. The expression and secretion of the fusion protein as much as over 1 g/l was achieved in a liquid culture. These results expand the scope of A. oryzae as a valuable host for production of heterologous and useful proteins. This might lead to a “functional sake” with an additional health benefit as a medicated beverage by including proteins useful for keeping healthy conditions.

The author believes that the results and expertise shown in this study should give an important step toward transition from traditional solid-state koji to more sophisticated submerged (liquid) koji for efficient and elegant manufacture of sake and other traditional Japanese fermentation foods.
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Publications

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