Mycopathologia (2006) 162: 143–153
DOI 10.1007/s11046-006-0049-2

Impact of *Aspergillus oryzae* genomics on industrial production of metabolites

Keietsu Abe[1,2], Katusya Gomi[2,3], Fumihiko Hasegawa[2] & Masayuki Machida[4]
[1]*Laboratory of Enzymology, Division of Life Science, Graduate School of Agricultural Science, Tohoku University, Sendai, 981-8555, Japan;* [2]*The New Industry Creation Hatchery Center, Tohoku University, Sendai, 980-8579, Japan;* [3]*Laboratory of Bioindustrial Genomics, Division of Bioscience and Biotechnology for Future Bioindustries, Graduate School of Agricultural Science, Tohoku University, Sendai, 985-8555, Japan;* [4]*Institute for Biological Resources and Functions, National Institute of Advanced Industrial Science and Technology (AIST), Higashi 1-1-1, Tsukuba, Ibaraki, 305-8566, Japan*

Abstract

Aspergillus oryzae is used extensively for the production of the traditional Japanese fermented foods *sake* (rice wine), *shoyu* (soy sauce), and *miso* (soybean paste). In recent years, recombinant DNA technology has been used to enhance industrial enzyme production by *A. oryzae*. Recently completed genomic studies using expressed sequence tag (EST) analyses and whole-genome sequencing are quickly expanding the industrial potential of the fungus in biotechnology. Genes that have been newly discovered through genome research can be used for the production of novel valuable enzymes and chemicals, and are important for designing new industrial processes. This article describes recent progress of *A . oryzae* genomics and its impact on industrial production of enzymes, metabolites, and bioprocesses.

Key words: *Aspergillus oryzae*, biodegradable plastic, cutinase, DNA microrray, enzyme, EST, genome, hydrophobin, industry, metabolite

Introduction

The *Aspergillus* section *Flavi* complex consists of mycotoxin producers such as *A. flavus* and *A. parasiticus*, which contaminate crops such as maize, peanut, and cotton after harvest at low latitudes and produce potent carcinogens called aflatoxins. These two fungi often overwhelm agribusinesses through the contamination and production of aflatoxins. The section also includes *A. oryzae* and *A. sojae*, both of which are non-toxigenic. Although there is a close taxonomic relationship between the toxigenic and non-toxigenic fungi, the long history of extensive use of the latter two species in the food industries proves their safety [1]. *A. oryzae* and *A. sojae*, called *koji* molds, have been used for indigenous Japanese fermented products such as *sake* (rice wine), *shochu* (spirits), *shoyu* (soy sauce), and *miso* (soybean paste) for over a thousand years. Today, the *koji* molds are also used as host cells for recombinant enzyme production. The beneficial involvement of *koji* molds in Japanese society has been the driving force behind research and development in several fields, including academia, industry, medicine, and agriculture.

Cultures of *A. oryzae* grown on steamed rice grains form an essential ingredient (called *koji*) in *sake* production, and cultures grown on steamed ground soybeans and roasted wheat grains are used for soy sauce. In complex fermentation processes, *A. oryzae* breaks down starches to sugars and proteins to peptides and amino acids, which then are further fermented by yeasts and lactic acid

bacteria. Similar processes with different fungi and bacteria are widely exploited in many Asian countries to produce alcoholic beverages and cakes.

The range of host cells for recombinant protein production was extended from prokaryotes to eukaryotes in the mid-1980s. Because of the potential for hypersecretion of proteins by filamentous fungi, several production systems for recombinant proteins have been developed and industrialized [2]. At first, proteins were restricted to homologs of proteins found in host cells, but heterologous proteins for medical use are now produced.

Recent progress in the development of an *A. oryzae* transformation system [3] has facilitated the production of heterologous proteins by *A. oryzae* in industrial applications [see 4–6as examples]. Nevertheless, basic research on the biology of *A. oryzae* is extremely difficult because of its lack of a sexual life cycle, its multinucleate conidia, and its large genome size. To establish a new research resource for *A. oryzae*, a collaboration of national institutes, universities, and companies in Japan [7], working together with an international group of scientists, has sequenced the genome.

The genomics of industrial Aspergilli has been studied since the late 1990s. Although *A. sojae* is as industrialized as *A. oryzae*, research on *A. sojae* genomics is limited. Therefore, this review covers mainly the recent achievement of *A. oryzae* genomics and its applications in industrial fields.

Current status of *A. oryzae* genomics

Genome sequencing of A. oryzae

The complete genome sequence of *A. oryzae* strain RIB40 (National Research Institute culture stock ATCC42149) has been determined [7]. This strain shares typical characteristics of morphology, growth, and amylase production with industrial strains used for *sake* brewing, and can produce potent protease activity, which is important for soy sauce fermentation. Although neither genetic nor physical maps were available when the genome sequencing project was launched, a number of gene sequences from strain RIB40 had been characterized, and a large number of expressed sequence tags (ESTs) had been derived [2, 8, 9]. The EST data covered genes expressed both in liquid cultures under various growth conditions and in solid-state cultures. Solid-state fermentation is extensively used in Japan for *sake* brewing, soy sauce fermentation, and industrial enzyme production (Table 1).

The genome sequencing of *A. oryzae* was accomplished by the whole-genome shotgun (WGS) approach. High-quality sequence reads from more than 500,000 WGS clones yielded 7× depth of coverage of the genome. Approximately 20,000 cosmid and 9000 BAC clones were used to join the WGS contigs. Subsequently, the sequences were assembled into contigs comprising 36.9 Mbp in total with approximately 9× depth of coverage. More than 99.15% of the assembly was supported by two or more (more than two) independent BAC or cosmid clones. Chromosome assignments for the contigs and their relative positions on each chromosome were based on Southern hybridization of the chromosomes separated by pulse-field gel electrophoresis (PFGE) and by the fingerprinting method [10].

A combination of several gene-calling programs was used for the prediction of genes. BLASTX searches followed by homology-based gene structure prediction with ALN algorithm [11] were used to identify genes encoding homologs of proteins in the public databases. Novel genes were predicted by the *ab initio* gene finders GeneDecoder [12] and GlimmerM [13] using 489 genes predicted by ALN with high confidence as a

Table 1. A. oryzae ESTs and their libraries

Culture condition	Analyzed ESTs
Liquid complete medium (+glucose)	2693
Liquid complete medium (+glucose, 37 °C)	2072
Liquid synthetic medium (−glucose)	1953
Liquid complete medium (+maltose)	932
Liquid complete medium (pH 10)	751
Solid-state cultivation (wheat bran)	6309
Solid-state cultivation (*shoyu koji* 25 °C)*	1049
Liquid complete medium (germination)	1049
Total	16,808

*Solid-state cultivation is the same as that using wheat bran except that a mashed and steamed complex of soybean and wheat was used as the medium instead of mashed and steamed wheat.

learning set. The predicted genes were validated against SIM4-generated alignments [14] with *A. oryzae* ESTs [2] and *A. flavus* ESTs [9]. All predicted protein sequences were annotated by searching against the COG database [15] using BLASTP, followed by manual correction. Transfer RNAs were identified with tRNAScan-SE [16], and repeated sequences were detected with RepeatMasker (http://www.repeatmasker.org).

The *A. nidulans* and *A. fumigatus* genome sequences used for comparison are available at http://www.broad.mit.edu/annotation/fungi/aspergillus/ and http://www.tigr.org/tdb/e2k1/afu1/. Fully annotated versions of the *A. oryzae*, *A. nidulans*, and *A. fumigatus* genomes are available following publication of the genome analysis papers [7, 17, 18].

Overall structure of the A. oryzae genome

The *A. oryzae* genome consists of eight chromosomes, numbered I to VIII in order of decreasing size, with lengths of 6.3, 6.2, 5.0, 4.8, 4.4, 4.1, 3.4 (including 0.7-Mbp rDNA repeats), and 3.3 Mbp, resulting in a total genome size of 37.6 Mbp (Figure 1) [7]. The mitochondrial DNA measures 28.9 kbp. In accordance with the existence of eight chromosomes, 16 DNA fragments containing the telomeric repeat TTAGGGTCAACA were identified [19]. Note that the chromosome numbering in this manuscript is different from that reported previously [20]. The longest DNA fragment identified by PFGE, previously assigned to chromosome I [20], consists of chromosomes I and II [7], whereas the shortest fragment, previously shown to contain two chromosomes (VII and VIII) [20], consists of a single chromosome (chromosome VIII) [7]. Centromeric sequences have not yet been obtained because of unsuccessful cloning attempts that are most likely due to the extremely high AT-content or DNA curvature of these segments [21]. The *A. oryzae* genome size is very close to those of *A. niger* and *A. flavus* [22; Yu J. et al., SRRC/USDA personal communication, 2005], and 20–30% longer than those of *A. nidulans* [17] and *A. fumigatus* [18].

The total number of predicted *A. oryzae* genes that encode proteins longer than 100 amino acids is 13,572. Thus, the average gene density is 2.8 kb/gene, which is approximately 1.4 times that of *Saccharomyces cerevisiae* (2 kb/gene) [7, 22, 23].

Primary and secondary metabolites

Genes for primary metabolism and hydrolytic enzymes

Within the *koji*, *A. oryzae* grows on the surface of steamed rice or ground soybean, where amino acids and sugars are initially deficient. The need for *A. oryzae* to gain access to external nitrogen and carbon sources after degradation of protein and

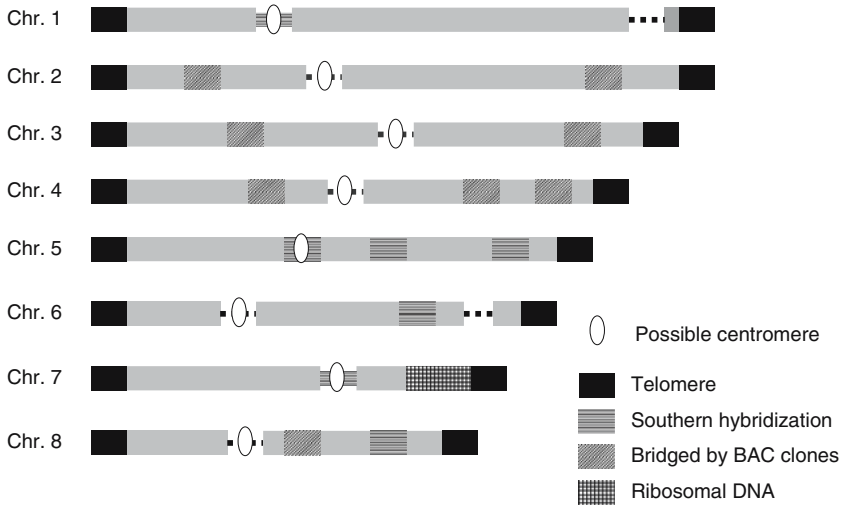

Figure 1. Structure of the *A. oryzae* genome. Ovals, possible centromeres; black bars, putative telomeres; gray bars, ribosomal DNA. Thinner hatched lines and hatched bars represent the unsequenced regions bridged by Southern hybridization and BAC clones, respectively. Thin dotted lines designate the region where no clones have been obtained.

starch is consistent with its greater numbers of metabolic and transporter genes than are found in other fungi [7]. Gene family expansion in *A. oryzae* was also observed with genes involved in phenylalanine/tryptophane degradation, and those participating in toluene/m-cresol/p-cymene degradation [7]. Genes which contribute to the biosynthesis and degradation of hydrophobic amino acids, lysine and serine are also expanded [7].

As judged by EST data, the genes for alcohol dehydrogenase, pyruvate decarboxylase, and sugar transporters are typical examples of strongly transcribed genes [8]. The strong expression of such genes might have been enhanced by adaptation [24] during domestication.

Since *A. oryzae* has been exploited for the degradation of starch and proteins in the traditional Japanese fermentation industries, the hydrolytic enzymes have been studied most extensively. *A. oryzae* produces many enzymes which degrade various biomolecules of high molecular mass such as carbohydrates, polypeptides, and nucleic acids. α-amylase randomly breaks down α-1,4-glycosyl bonds in starch to yield dextrin. Glucoamylase removes a glucose unit from the non-reducing terminus of dextrin chains, and the product serves as a carbon source for alcohol production by yeast. The *A. oryzae* strains commonly used in research possess three α-amylase genes in the genome [20], probably because *A. oryzae* strains with strong glucose-producing activity have been selected down through the ages for efficient alcohol production in *sake* brewing.

Endo-proteinases and exo-peptidases play the most important roles in yielding amino acids during soy sauce production from the proteins in soy beans and wheat grains. Since soy sauce productivity depends on how efficiently the proteins are utilized, strains possessing strong proteinase and peptidase activities have been screened and selected. In addition, many hydrolytic enzymes, including nucleases, lipases, and amylases, play important roles in efficient production and in the taste and flavor of the products. Although the yeasts and lactic acid bacteria that subsequently ferment after *A. oryzae* also significantly affect productivity and product quality, fermentation by *A. oryzae* is a key to the entire process.

Approximately 25% of all genes in the genomes of lower eukaryotes such as yeast are unexpressed or uninduced under ordinary culture conditions in the laboratory [25]. This means that 2000–3000 genes in *A. oryzae* may remain unused under these conditions. Therefore, these genes have little chance to be detected by EST analysis, but could be identified by whole-genome sequencing. It is expected [7] that genes encoding valuable enzymes that break down ingredients other than starch or protein may be found in the genome, because filamentous fungi can degrade a variety of materials [8]. These hitherto unidentified genes will be of great value to industry for improvement of productivity or development of new products, because of the safety of *A. oryzae*.

Secondary metabolites and product safety from the viewpoints of conventional and genomic approaches

Some strains of *A. oryzae* produce secondary metabolites that are directly or indirectly toxic such as 3-nitropropionic acid [26]. However, kojic acid and many others may not be true mycotoxins [26]. Production of secondary metabolites in *A. oryzae* is strain-specific and environment-dependent. The extent of mycotoxin testing in food enzyme preparations should be judged on a case-by-case basis, through a careful evaluation based on knowledge of taxonomy, biochemistry, and genetics. In many cases, the testing scope at the level of genus should be sufficient. In other cases, the scope should be narrowed on the basis of scientific knowledge and assessment of a species and its history.

A. oryzae and *A. sojae* are important species in industrial fermentation for the production of various substances. The products have wide application in numerous industrial fields, including the food, chemical, and pharmaceutical industries. A few examples are fermented foods produced with *A. oryzae* and *A. sojae*, and a number of enzyme preparations produced by either traditional or modern biological technologies [27]. The safety of any food-grade product is carefully evaluated before its commercialization and is assured throughout the manufacturing, processing, transportation, storage, and use of the product. Safety assurance measures range from the selection of manufacturing raw materials to a series of toxicological tests. To ensure the safety of food-grade enzymes, the Joint FAO/WHO Expert Committee on Food Additives [28]) required that food enzyme

preparations derived from fungal sources should not contain detectable amounts of aflatoxin B1, ochratoxin A, sterigmatocystin, T-2 toxin (a major trichothecene toxin), or zearalenone. The safety of *A. oryzae* and *A. sojae* as production organisms for food-grade products has long been recognized.

To ensure the safety of the products, the industry carefully selects, maintains, and sometimes modifies the production strains. Fermented foods produced by *A. oryzae* and *A. sojae* have been shown to be aflatoxin-free [29–31]. No soy sauce production strains of *A. oryzae* tested produced detectable amounts of cyclopiazonic acid [32]. The US EPA concluded that commercial strains of *A. oryzae* "apparently do not produce maltoryzine" [26].

In the field of enzyme production, the safety and toxigenicity of *A. oryzae* as a production organism have been assessed [33]. Toxicity tests of several enzyme preparations derived from *A. oryzae* indicate that *A. oryzae* is safe for use in its intended applications [34–36]. In the USA, a number of enzyme preparations derived from *A. oryzae* have been granted "generally recognized as safe" status in various food-processing applications on the basis of publicly available information and scientific studies (GRN89; GRN90; GRN113; 21 CFR §184.1250). When a new production strain of *A. oryzae* or *A. sojae* is developed, it is of primary importance that the strain be tested for the production of toxicologically significant amounts of relevant secondary metabolites. This is especially important if the microorganism or its products are to be used in the food industry. Care must be taken to ensure that these production strains do not produce mycotoxins under commercial enzyme production conditions. The toxigenicity of *A. oryzae* and *A. sojae* is controllable, these organisms are safe to use as production organisms, and the derived products are safe to use, given that (a) the production species is correctly identified; (b) the production strain is carefully selected, manipulated, and tested to ensure its lack of relevant secondary metabolite production; (c) the research, development, and manufacturing processes, including the establishment of a validated seed bank, are carefully designed, operated, and monitored; and (d) the product is routinely tested for mycotoxin contamination, although there has never been any report of spontaneous transformation of non-toxic production strains into toxic ones. It should be stressed that most industrial production strains of *A. oryzae* and *A. sojae* have successfully demonstrated a long history of safe use.

The genome sequence has provided new insights into the safety of *A. oryzae*: no ESTs for aflatoxin biosynthetic genes were detected except for *aflJ* and *norA* encoding a co-activator necessary for transcription of aflatoxin biosynthetic gene cluster and norsolorinic acid reductase respectively [Machida M. et al., 2005, National Institute of Advanced Industrial Science and Technology (AIST), JAPAN, unpublished results], whereas ESTs for all 25 aflatoxin pathway genes were found in *A. flavus* [37]. The long history of industrial use of *A. oryzae* and *A. sojae* may have deselected genes unfavorable for human consumption, or the two industrial fungi may have been selected as non-toxigenic from the beginning. *A. oryzae* may possess a silencing mechanism similar to that observed in the regulation of aflatoxin biosynthesis in *A. sojae* [30, 31], or there may have been mutations in a global regulator of secondary metabolic genes such as *laeA* [38]. AflR regulates transcription of a gene cluster for the biosynthesis of aflatoxin [39]. In *A. nidulans*, AflR controls the production of sterigmatocystin, a compound related to aflatoxin [40]. The *aflR* gene is present within the aflatoxin biosynthesis cluster in these organisms and *A. oryzae*. However, *aflR* is not expressed or AflR is non-functional in *A. oryzae* and *A. sojae* [41–43].

From the results of recent genome analysis of *A. oryzae*, *A. fumigatus*, and *A. nidulans*, *A. oryzae* possesses more genes predicted to play a role in secondary metabolism (for instance, cytochrome P450s and polyketide synthase) [7, 17, 18], although most of the genes are not transcribed according to EST and DNA microarray analyses [7]. Details are described elsewhere [7].

A. oryzae DNA microarrays and their applications

In 2001, we manufactured a first-generation *A. oryzae* DNA microarray consisting of approximately 2000 cDNAs amplified from EST clones [8]. The 2000 cDNAs were highly expressed among 6000 non-redundant EST clones prepared in the *A. oryzae* EST project of a Japanese EST consortium [2, 9]. In 2003, Yamada et al. at

the National Research Institute of Brewing in Higashihiroshima manufactured second-generation cDNA microarrays (the NRIB 3000 DNA microarrays), which cover another 3000 EST clones from the 6000 non-redundant *A. oryzae* contigs (Yamada et al. unpublished results 2003). In 2004, cDNA microarrays with approximately 5000 independent EST clones were constructed by a Japanese consortium [Masuda et al. Noda Institute of Industrial Science, unpublished results 2004], and Machida et al. at AIST constructed oligonucleotide DNA microarrays that cover over 11,000 genes. The oligonucleotide DNA arrays are commercially available from the Japanese company FarmLab Inc. (Tokyo, Japan) in 2005.

Microarrays as a monitoring tool for fermentation

Maeda et al. [8] described the potential of a cDNA microarray with 2000 cDNAs as a monitoring tool for fermentation processes. The microarray contains mostly genes coding for the glycolytic pathway, the tricarboxylic acid (TCA) cycle, and part of the electron-transfer (ET) chain. Using the cDNA microarrays, they analyzed the gene expression profiles of *A. oryzae* cells grown in glucose-rich and glucose-poor liquid culture conditions [2, 8]. The sets of genes identified by the cDNA microarray as highly expressed under each culture condition agreed well with the highly redundant ESTs obtained under the same conditions. In particular, transcription levels of most catabolic genes of the glycolytic (EMP) pathway and TCA cycle were higher under glucose-rich than glucose-poor conditions, suggesting that *A. oryzae* uses both EMP and TCA pathways for glucose metabolism under glucose-rich conditions (Figure 2).

Maeda et al. [8] further studied the expression of genes for hydrolytic enzymes by using cDNA microarrays and mRNAs prepared from cells grown on three industrial solid-phase media, namely wheat bran, rice bran, and soybean refuse. Cells grown on the wheat bran medium expressed a larger number of genes for the hydrolytic enzymes than those grown on the other two solid media, supporting the traditional use of wheat bran in industrial fungal enzyme production in Japan. Expression profiles of genes for energy catabolism were compared under the same culture conditions. The wheat bran culture gave the lowest expression levels of the catabolic genes (EMP, TCA) among the three media. The low expression levels of catabolic genes in the wheat bran culture may release catabolite repression, consequently leading to the expression profiles in which a larger number of genes encoding hydrolytic enzymes are transcribed. The gene expression profiles observed in the wheat bran solid-phase culture imply that the solid-phase culture conditions mimic the C- or N-limiting culture conditions in liquid culture systems that are often used in industrial enzyme production to obtain maximum productivity. These results show the potential of DNA microarrays as monitoring tools for industrial fermentation processes.

Microarrays as a discovery tool for designing new bioprocesses

When a protein shows enzyme activity, it can be detected and screened by measurement of its activity. If a protein does not show enzyme activity and no antibody against it is available, DNA microarrays are useful for discovering such proteins, as shown in the following example.

To achieve zero emissions and sustainable material recycling in our society, industry has developed biodegradable plastics as replacements for non-biodegradable plastics [44]. Because the production and consumption of biodegradable plastics are predicted to increase enormously in the near future, large-scale industrial facilities such as composters or fermenters will be required for the biological degradation of these plastics and for recovery of the component monomers or oligoesters. Recently, solid-phase fermentation systems using the industrial fungi *A. oryzae* and *A. sojae* have been introduced for the production of industrial enzymes and speciality chemicals [9]. These efficient systems should be able to recycle biodegradable plastics (Figure 3).

Because of the need to study and develop large-scale biological monomer-recycling systems for biodegradable plastics such as polybutylene succinate-coadipate (PBSA), we examined whether *A. oryzae* can degrade PBSA and found that it can [45]. Next, we looked for the proteins involved in PBSA degradation and found that when *A. oryzae* was cultivated in liquid culture containing PBSA microparticles as the sole carbon source, the cutinase CutL1 appeared in the culture broth as

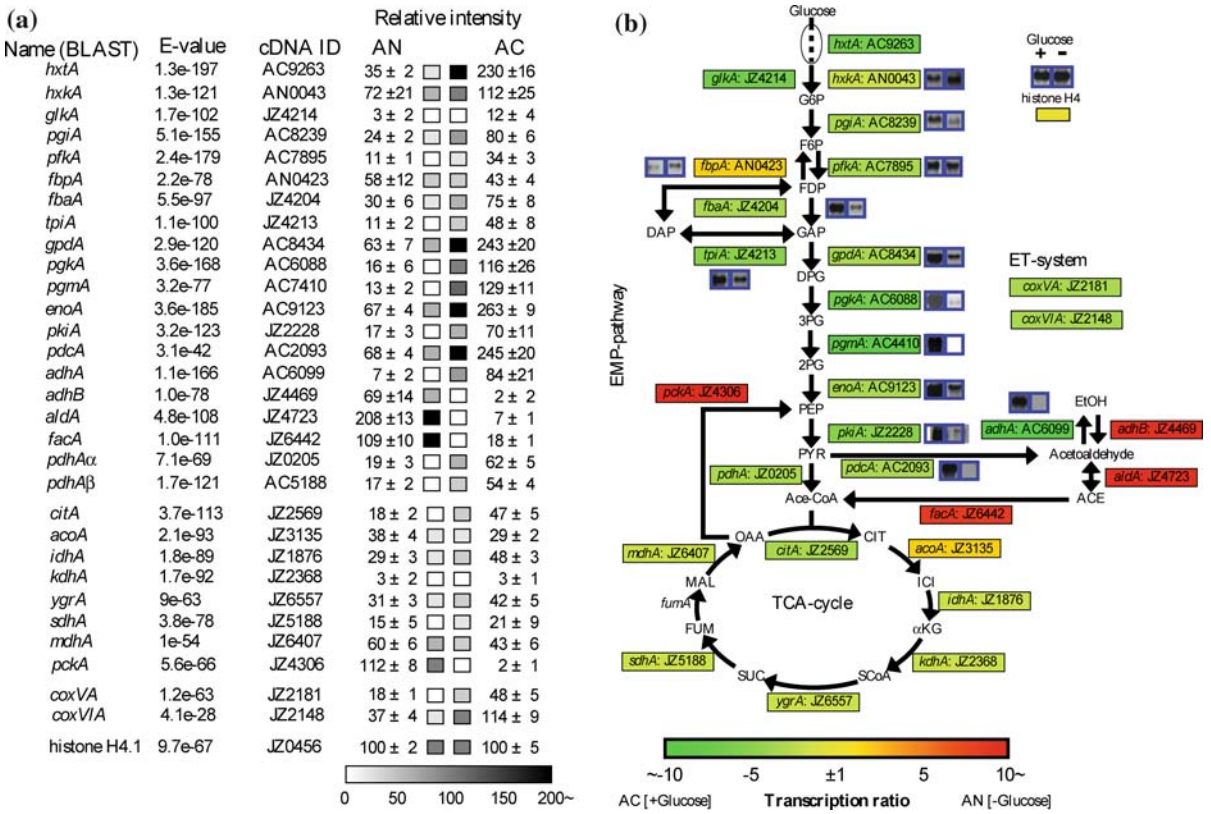

Figure 2. Expression profiles of genes encoding enzymes for the glycolytic (EMP) pathway, the tricarboxylic acid (TCA) cycle, and the electron-transfer (ET) system in *A. oryzae* grown in glucose-rich (AC) and glucose-depleted (AN) media. Transcripts from cells grown under AC conditions were labeled with Cy3 (green), and those grown in AN medium were labeled with Cy5 (red). (A) Transcription levels of genes involved in the glycolytic pathway, the TCA cycle, and cytochrome c oxidase subunits V/VI under the AC and AN conditions, shown as relative intensities normalized to histone H4.1. 'Name (BLAST)' indicates that the name of the cDNA is based on the best match in the database obtained by BLASTX. (B) Transcription ratios (AN [Cy5-red]/AC [Cy3-green]) of genes of the glycolytic pathway, the TCA cycle, and cytochrome c oxidase subunits V/VI are indicated on a metabolic map. Signal ratios of (AN/AC) < 1.0 were inverted and multiplied by −1 to facilitate their interpretation. Northern hybridization analyses of several glycolytic genes were carried out with the same transcripts obtained from the AC and AN cultures. *hxtA*, hexose transporter; *hxkA*, hexokinase; *glkA*, glucokinase; *pgiA*, phosphoglucose isomerase; *pfkA*, phosphofructokinase; *fbpA*, fructose bisphosphatase; *fbaA*, fructose bisphosphate aldolase; *tpi*, triose phosphate isomerase; *gpdA*, glyceraldehyde-3-phosphate dehydrogenase; *pgkA*, phosphoglycerate kinase; *pgmA*, phosphoglycerate mutase; *enoA*, enolase; *pkiA*, phospho*enol*pyruvate kinase; *pdcA*, pyruvate decarboxylase; *adhA*, alcohol dehydrogenase I; *adhB*, alcohol dehydrogenase II; *aldA*, acetaldehyde dehydrogenase; *facA*, acetyl coenzyme A (CoA) synthase; *pdhA*, pyruvate dehydrogenase; *citA*, citrate synthase; *acoA*, aconitase; *idhA*, isocitrate dehydrogenase; *kdhA*, 2-ketoglutarate dehydrogenase; *ygrA*, succinyl CoA synthase; *sdhA*, succinate dehydrogenase; *fumA*, fumarate dehydratase; *mdhA*, malate dehydrogenase; *pckA*, phosphoenolpyruvate carboxykinase; *coxVA*, cytochrome oxidase subunit V; *coxVIA*, cytochrome oxidase subunit VI.

an induced enzyme that hydrolyzes PBSA [45]. Cutinases, which are produced by pathogenic fungi (including *Fusarium*), hydrolyze cutin, a heterologous complex of wax esters found on the surfaces of plants [46]. To investigate additional proteins involved in PBSA degradation, we used *A. oryzae* cDNA microarrays to examine the gene expression profiles of *A. oryzae* cells grown in liquid culture containing PBSA particles as the sole carbon source [47]. The *rolA* (ro*dA*-like) gene, encoding RolA, a hydrophobin orthologous to *A. nidulans* RodA protein, was highly transcribed under these conditions [47]. (*A. nidulans* expresses RodA during conidiation under plate culture conditions (solid-phase culture) [48].) Hydrophobins are small proteins containing eight conserved cysteine residues, and are ubiquitous among filamentous fungi [49–51]. We found that RolA binds to the solid PBSA surface and specifically recruits CutL1 to the surface and consequently facilitates PBSA degradation [47]. Quartz crystal microbalance [52] and immunostaining analyses with purified RolA

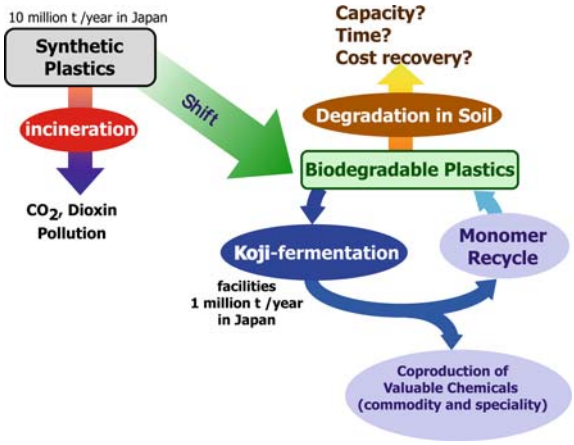

Figure 3. Concept for recycling of monomers from biodegradable plastics in solid-phase *koji* fermentation.

and CutL1 clearly demonstrated specific interactions between RolA adsorbed on hydrophobic surfaces and CutL1 [47]. We found another novel physicochemical property of RolA: Because RolA recruited CutL1 onto PBSA films and stimulated PBSA hydrolysis catalyzed by CutL1, we speculated that RolA on the surface of PBSA films may have some degree of mobility. Therefore, we used fluorescence recovery after photobleaching (FRAP) analysis [53] to study PBSA films coated with fluorescein isothiocyanate (FITC)-labeled RolA. High levels of fluorescence recovery were observed after photobleaching of FITC-labeled RolA adsorbed on PBSA films, which suggests lateral mobility of RolA on the PBSA surface.

Figure 4 depicts a schematic model of the lateral mobility of RolA on PBSA and the subsequent recruitment of CutL1. We propose that RolA molecules spontaneously self-assemble and form either a monolayer or a multilayer on the PBSA films. If they form a monolayer, then hydrophobic segments in each RolA molecule are adsorbed to the PBSA surface, where they can slide back and forth (Figure 4A, left). If they form an amphipathic multilayer [53–55], then RolA molecules in the middle or upper layer move laterally on the bottom layer, which is immobilized on the PBSA surface (Figure 4B, left). When molecules of the PBSA degradation enzyme CutL1 interact with RolA adsorbed to PBSA, the lateral mobility of RolA molecules is decreased (Figure 4B, right). Consequently, molecules of CutL1 accumulate at the interface between the PBSA surface and the water phase, resulting in stimulation of PBSA hydrolysis. The fact that CutL1 is specifically recruited to RolA molecules immobilized to the PBSA surface suggests that a novel molecular mechanism for degradation of natural hydrophobic solids may exist. This mechanism appears to involve cutinase recruitment to a solid surface, dependent on the conformation of the RolA bound to the hydrophobic surface, and

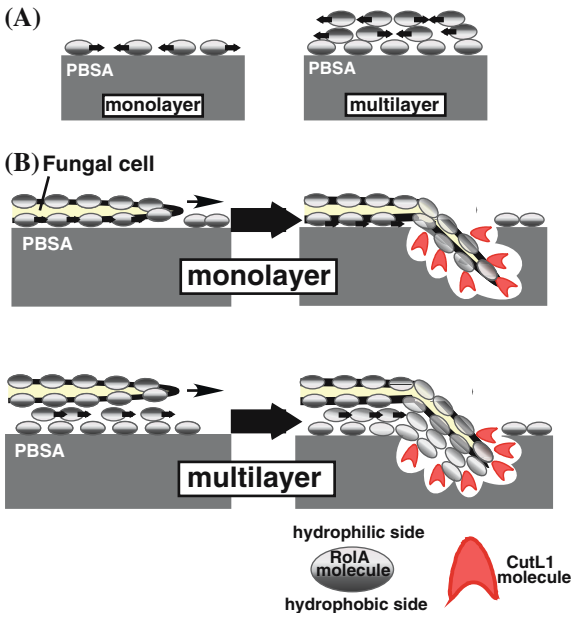

Figure 4. Proposed roles of hydrophobin RolA in degradation of plastics. (A) Schematic models of lateral mobility of RolA on the PBSA surface. The results of fluorescence recovery after photobleaching (FRAP) analyses suggested that RolA is laterally mobile on the PBSA surface. RolA molecules are predicted to form a monolayer or a multilayer on PBSA. If RolA molecules are mobile as a monolayer, putative hydrophobic segments of RolA, which have not yet been determined, are attached to the PBSA surface and can slide on the PBSA (left). In the multilayer model (right), RolA molecules self-assemble spontaneously on the PBSA surface and form an amphipathic multilayer, in which RolA molecules in the middle or upper layers are laterally mobile. (B) In the presence of PBSA, *A. oryzae* secretes RolA molecules, which are displayed on the cell surface and also spontaneously form a monolayer or a multilayer on the PBSA surface. Since RolA molecules are predicted to be laterally mobile on the PBSA surface (as described in A), RolA contributes not only to adhesion of fungal cells to the PBSA surface, but also to spreading of fungal cells on the PBSA. Then the PBSA-degrading enzyme CutL1, a cutinase, secreted by *A. oryzae*, is specifically recruited to the interface of the PBSA–fungal cell surface by RolA, resulting in condensation of CutL1 onto the PBSA surface and stimulation of PBSA hydrolysis. Thus, the recruited CutL1 molecules promote penetration of the fungal cells into the PBSA.

implies a novel tactic for fungal invasion of plants, insects, and other animals. That is, hydrophobins not only function as adhesive molecules at the interface between fungal cells and their host cells, but also recruit the hydrolytic enzymes required for penetration into the hosts. Work on identifying other enzymes that can interact with the adsorbed form of RolA to hydrophobic surfaces is now in progress. We are testing the novel mechanism of plastic degradation in combination with RolA and CutL1 in a large-scale plastic recycling system.

As shown in the above example, the DNA microarray is useful for discovering proteins that have no enzymatic activities or whose antibodies are not available.

Conclusion

Although *A. oryzae* has been extensively used in the fermentation industries and thus is economically important, detailed knowledge of its biology has been limited by difficulties in studying it by conventional genetic manipulation; this conventional approach is also limited by the fact that *A. oryzae* forms multinucleate conidia and lacks a sexual life cycle. To establish an alternative way to study *A. oryzae*, national institutes, universities, and companies in Japan, working together with international groups, recently sequenced *A. oryzae* ESTs and the whole genome. The results of this research are revealing the potential of the fungus as a new source of many industrial enzymes, and of a number of primary and secondary metabolites. Comprehensive transcriptional analyses using DNA microarrays indicate their potential as a tool for development of industrial systems. *A. oryzae* has various beneficial characteristics for industrial application. A huge amount of information on *A. oryzae* culture has been accumulated during its long history of industrial use in Japan, and a number of factories already grow it. The genome data will accelerate industrial applications of the fungus.

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Address for correspondence: Keietsu Abe, Laboratory of Enzymology, Department of Molecular and Cell Biology, Graduate School of Agricultural Science, Tohoku University, 1-1 Amamiya, Tsutsumi-dori, Aobaku, Sendai 981-8555, Japan
Phone: +81-22-717-8777; Fax: +81-22-717-8778
E-mail: kabe@biochem.tohoku.ac.jp