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Characteristics of Sake Yeast Genome and Genes

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Sake is a Japanese traditional alcohol beverage made of steamed rice, using *Aspergillus oryzae*, which is a source of saccharification enzymes, and sake yeast classified as *Saccharomyces cerevisiae*, which produces ethanol from glucose. Sake yeasts have been selected for sake making for a long time and have some unique properties suitable for sake making such as high ethanol productivity, high ethanol tolerance, foam formation, and well-balanced productivities of flavors, which are different from other yeast strains including laboratory strains. Although a few genes to characterize sake yeast were identified using molecular biological techniques in recent years, almost all of them were still unidentified. We have been studying characteristics of sake yeast genome and genes for years. Here, we would like to introduce a part of our works.

A gene involved in foam formation in mash

Almost all sake yeasts form a thick foam layer on sake mash during vigorous fermentation. This foam formation has been used as an indicator of fermentation progress: the foam rises when fermentation becomes strong and it disappears when fermentation becomes weak (Fig. 1). However, this characteristic sometimes reduces the efficiency of sake fermentation because a large part of the fermentation tank is occupied by a thick foam layer. Thus, an absence of foam formation during fermentation is a preferable property. Ouchi and Akiyama developed a method to screen nonfoaming mutants from foaming sake yeast using cell affinity for bubbles, and using this method they isolated a non-foaming mutant from an industrial sake yeast. This mutant had almost the same characteristics as the parental strain except for its nonfoaming property in fermentation. Thus, this method of screening for nonfoaming mutants has been applied to various strains of sake yeasts and the resultant nonfoaming yeasts are now widely used in commercial sake brewing. Comparison of the nonfoaming mutant with its parent revealed that the cell surface of the former is less hydrophobic than that of the latter, suggesting that cell surface hydrophobicity is related to foaming ability. However, the detailed molecular mechanism of foaming ability of sake yeast was unknown. We have cloned a gene from a foam-forming sake yeast that confers foaming ability to a nonfoaming mutant. This gene was named *AWA1* and structures of the gene and its product were analyzed (Fig. 2). The N- and C-terminal regions of Awa1p have the characteristic sequences of a glycosylphosphatidylinositol anchor protein. The entire protein is rich in serine and threonine residues and has a lot of repetitive sequences. These results suggest that Awa1p is

localized in the cell wall. This was confirmed by immunofluorescence microscopy and Western blotting analysis using hemagglutinin-tagged Awa1p. Moreover, an *awa1* disruptant of sake yeast was hydrophilic and showed a nonfoaming phenotype in sake mash. We conclude that Awa1p is a cell wall protein and is required for the foam-forming phenotype and the cell surface hydrophobicity of sake yeast.

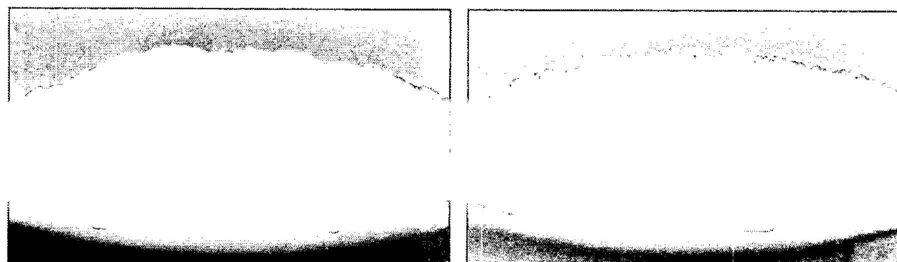

Fig. 1. Foam-forming yeast (left) and nonfoaming yeast (right).

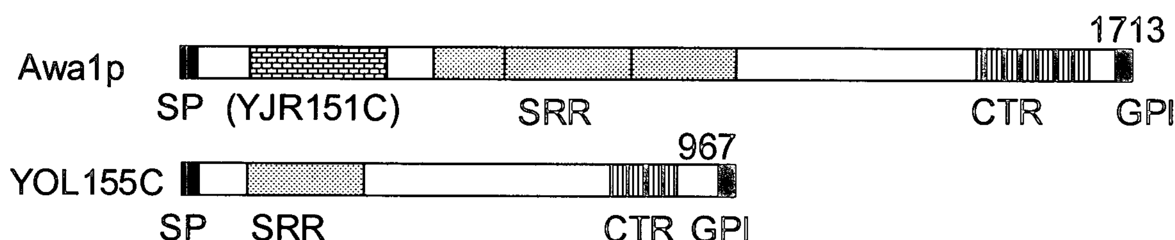

Fig. 2. Structures of the Awa1 protein and YOL155C.
SP, Signal peptide; SRR, Serine rich repeat; CTR, C-terminal repeat; GPI, GPI anchor signal

These results suggest that nonfoaming mutants have mutated *AWA1* genes that cannot make the cell surface hydrophobic. To confirm this, we cloned and characterized the *AWA1* gene in a nonfoaming mutant strain K701 derived from a foam-forming sake yeast strain K7. K701-*AWA1* was cloned in a cosmid and its sequence was compared with that of K7-*AWA1*. Although the 5' half of K701-*AWA1* was identical to that of K7-*AWA1*, the 3' half of K701-*AWA1* was different from that of K7-*AWA1*, resulting in a loss of the C-terminal hydrophobic sequence of Awa1p. Since this sequence is required for the anchoring of Awa1p to the cell wall, K7-Awa1p can not confer both cell surface hydrophobicity and foam-forming ability to strain K701 cells. Since the change found in K701-*AWA1* was not a point mutation but a larger scale event, we analyzed chromosome rearrangement by pulsed-field gel electrophoresis Southern blot analyses. The results suggest that the left subtelomeric region of chromosome IX in strain K7 was translocated to the *AWA1* gene in chromosome XV by a nonreciprocal recombination (Fig. 3).

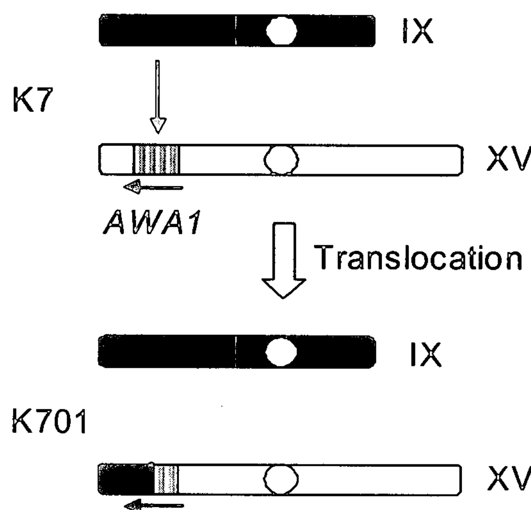

Fig. 3. Chromosome rearrangement found in the nonfoaming mutant (K701-AWA1).

Characterization of sake yeast genome by DNA microarray

Although DNA microarray is usually used for expression profiling, it is also a powerful technique for whole genome analysis, especially for detection of partial duplications and deletions of chromosomes. We used a commercial DNA microarray manufactured based on genome sequencing of S288C, a laboratory strain, to characterize genome of sake yeast.

DNA samples prepared from sake yeast strain K7 and strain S288C were labeled with different fluorescent dyes and were hybridized with the DNA microarray. Fluorescent signals of two dyes were quantified by a microarray scanner. As described in a later section, since the genome sequence of strain K7 is almost identical to that of strain S288C, signal ratios of strains K7 to S288C were approximately one. However, we have found several regions in which deletion or duplication occurred in strain K7 compared to strain S288C.

First, we found that strain K7 had less copies of *CUP1*, *ENA1* and *ASP3*. We confirmed that strain K7 had only one copy of these genes whereas strain S288C had ten copies of *CUP1*, five copies of *ENA1* and four copies of *ASP3* by Southern blot analysis. It was reported that *CUP1* is required for Cu^{2+} ion tolerance and *ENA1* is required for Na^{+} ion tolerance. Actually, strain K7 cells are more sensitive to $CuCl_2$ and NaCl than strain S288C.

Second, we found that the subtelomeric region from YPR192W to YPR210W in the chromosome XVI right arm of strain K7 was duplicated three times (Fig. 4). To analyze how these duplication occurred, we cloned cosmids containing these sequences and analyzed their sequences. As a result, the subtelomeric region of the chromosome XVI right arm was translocated to the subtelomeric region of the chromosome IV right arm and the subtelomeric region of the chromosome XIII left arm by nonreciprocal manner. We further found that this duplication was not specific to strain K7 but found in other commercial sake yeast strains including strains K6, K9 and K10 (Fig. 5). These strains and their mutants are widely used in commercial sake brewing. Interestingly, other yeast strains including old sake yeast strains that are not used anymore and wild contaminant sake yeast strains do not have this characteristics. This suggests that the duplication of YPR192W to YPR210W is important for survivals of yeast cells in sake brewing environment although functions of these genes in sake brewing are yet unknown. Alternatively, strains K6, K7, K9 and K10 may be evolved from a common ancestor.

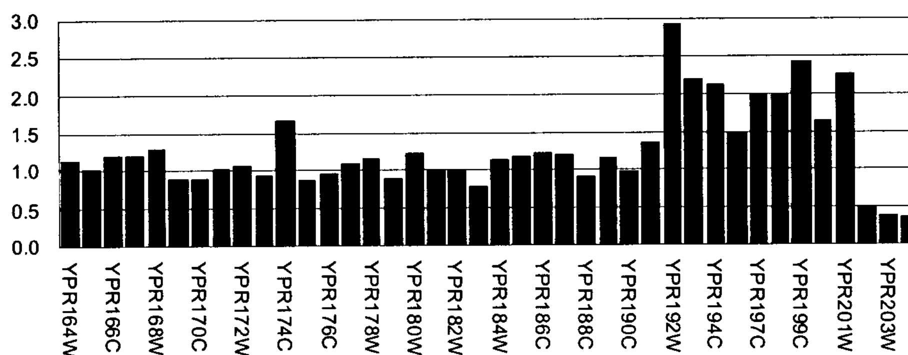

Fig. 4. The subtelomeric region of the chromosome XVI right arm was duplicated in strain K7. The vertical axis indicates signal ratio of strains K7 to X2180 of each gene by DNA microarray analysis.

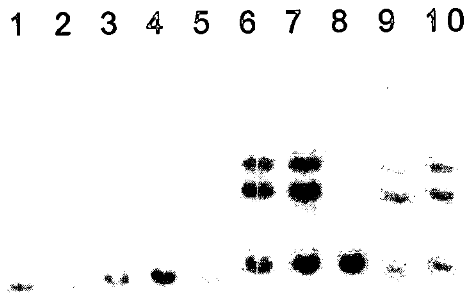

Fig. 5. Genomic Southern blot analysis of YPR192W (chromosome XVI).
Strains K6, K7, K9 and K10 have three copies of YPR192W (lanes 6, 7, 9 and 10).

Genome analysis of sake yeast and identification of a new gene specific to sake yeast

To characterize sake yeast more clearly, we carried out partial genome sequencing of sake yeast strain K7. We made a plasmid shot-gun library from strain K7, and sequenced them randomly to get approximately 20,000 sequences. Sequencing redundancy against a haploid genome was about 0.5. Sequence comparison with *Saccharomyces* Genome Database (SGD) revealed that average sequence identity between strains K7 and S288C is about 99%. Some scientists argued that sake yeast should be classified as a different species from *Saccharomyces cerevisiae* since sake yeasts have many different characteristics from other *Saccharomyces* cerevisiae strains, e.g. foam formation in sake mash, higher ethanol tolerance and biotin autotrophy. However our results clearly showed that sake yeast belongs to *Saccharomyces cerevisiae* and that characteristics of sake yeast depend on this small difference.

Furthermore, we constructed a cosmid library of strain K7 genomic DNA and sequenced inserts of approximately 1,000 cosmids from both directions to identify chromosomal rearrangements in sake yeast. When a cosmid contains two sequences corresponding to different chromosomes after sequence comparison with SGD, it was further analyzed by chromosome Southern blotting using pulsed filed gradient gel electrophoresis as a candidate of chromosome rearrangement. By this screening, we identified a cosmid clone of which one side showed very low homology to SGD. By assembling this sequence and data from the shot gun sequencing, we identified a new open reading frame. We named this gene *BIO6* since it is homologous to *Escherichia coli bioA* and *Saccharomyces cerevisiae BIO3*. Chromosome Southern blotting analysis of strain K7 using *BIO6* as a probe revealed that at least four copies of *BIO6* homolog were present in different chromosomes (Fig. 6). Moreover, almost all sake yeasts had several copies of *BIO6* homolog in different

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Fig. 6. Chromosome Southern blotting analysis of *BIO6*.
Lane 1, laboratoryyeast strain X2180; lane 2, sake yeast strain K7

chromosomes whereas most wine and brewing yeasts had no *BIO6*. Interestingly, strains having *BIO6* homolog did not require biotin for their growth. Since a laboratory strain A364A had only one copy of *BIO6*, we disrupted *BIO6* in this strain and found that the disruptant showed biotin auxotrophy. These result indicate that the *BIO6* gene is essential for biotin synthesis at least in strain A364A. Standard laboratory strains of *Saccharomyces cerevisiae* are known to require biotin for their growth. Our finding of *BIO6* suggest that biotin prototrophy of sake yeasts may be caused at least partially by the presence of *BIO6*.

Evolution of sake yeasts

Sake yeast strains K6, K7, K9, K10 and their mutants are widely used in commercial sake brewing because of their productivity and high quality of sake produced by them. These strains have several common unique properties as described in the previous sections. First, they have several copies of *BIO6* in different chromosomes and they do not require biotin for their growth. Second, they have three copies of the subtelomeric region of the chromosome XVI right arm. Third, they are foam forming in mash and have the *AWA1* gene in their genome although their *AWA1* lengths are different. From these facts, I propose a hypothesis of evolution of sake yeast. The first and oldest events might be the acquisition of *AWA1* in sake yeasts and losses of *BIO6* and biotin synthesis pathway in other yeast strains. These characteristics distinguish almost all sake yeasts from other *Saccharomyces cerevisiae* strains. The second event might be the duplication of the subtelomeric region of the chromosome XVI right arm, which differentiate sake yeast strains K6, K7, K9 and K10 from other sake yeast. The final event might be changes of *AWA1* size, which are diverse even in these strains. It is noteworthy that all genes mentioned above exist in subtelomeric regions. These regions are very susceptible for chromosome rearrangement including translocation and produce genomic diversity allowing yeast cells to adapt to various environments. Of course, meanwhile many mutations were simultaneously accumulated in sake yeast genome, forming unique characteristics of sake yeasts. It is likely that these evolutional events were facilitated by geographical isolation of Japan or unique microbiological environment of sake brewing. I believe studies on sake yeast genome would produce fruitful results including these issues.

References

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