CHAPTER 3

Functional Genomics of Wine Yeast *Saccharomyces cerevisiae*

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Contents

Abstract

The application of genomic technologies to the analysis of wine strains of *Saccharomyces cerevisiae* has greatly enhanced our understanding of both native and laboratory strains of this important model eukaryote. Not only are differences in transcript, protein, and metabolite profiles being uncovered, but the heritable basis of these differences is also being elucidated. Although some challenges remain in the application of functional genomic technologies to commercial and native strains of *S. cerevisiae*, recent improvements, particularly in data analysis, have greatly extended the utility of these tools. Comparative analysis of laboratory and

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wine isolates is refining our understanding of the mechanisms of genome evolution. Genomic analysis of *Saccharomyces* in native environments is providing evidence of gene function to previously uncharacterized open reading frames and delineating the physiological parameters of ecological niche specialization and stress adaptation. The wealth of information being generated will soon be utilized to construct commercial stains with more desirable phenotypes, traits that will be designed to be genetically stable under commercial production conditions.

I. INTRODUCTION

The completion of the sequence of the genome of the yeast *Saccharomyces cerevisiae* heralded a new era in research on this important industrial and experimental organism (Goffeau *et al.*, 1996). A host of new tools were developed that allow physiological interrogations of this yeast to occur at a remarkable breadth and depth. *S. cerevisiae* is involved in the production of bread, beer, and wine, is used as a food flavorant, and is an additive enhancing the nutritional content of numerous food products. This yeast is also being used and developed for a host of other processes: nutra- and pharmaceutical production and delivery, probiotics, bioremediation, biofuel and bioelectricity generation, and biosensing. It is estimated that *Saccharomyces* has been cultivated by man for several thousand years and can be considered a domesticated yeast (Cavalieri *et al.*, 2003; Fay and Benavides, 2005a; Mortimer, 2000). This yeast is commonly found in association with mankind and civilization, but its true origin in the natural environment has not been determined. It is a minor resident of high-sugar plant surfaces and has evolved to dominate batch fermentations (Boulton *et al.*, 1996). Functional genomic analysis of this organism in its native environments will allow a better understanding of the activities of yeast in important food and beverage production systems and assist in the development of novel yeast-based industrial processes, as well as improve our understanding of this model eukaryote. This review will describe the challenges in the investigation of industrial yeast strains, present a synopsis of the functional genomic technologies available for analysis of *S. cerevisiae* and the issues with their application and interpretation, and finally summarize what has been learned to date about the biology of this yeast in natural and commercial environments as a consequence of the exploitation of these novel experimental tools.

II. CHALLENGES IN THE INVESTIGATION OF NATIVE YEAST STRAINS

There are two main goals for the genomic analysis of wine yeast. The first aim is to understand how wine yeasts differ from laboratory strains and from each other at the genomic level, and to exploit these differences to

broaden our understanding of the fundamental biology of this important model eukaryote. The second aim is to investigate the biological activities of yeast in environments in which they evolved in order to manipulate strain behavior to produce different or more highly valued products (Stephanopoulos *et al*., 2004). The application of genomic technologies to native yeast in wild or production settings poses considerable difficulty. This difficulty arises largely from the widespread differences in genome architecture known to exist among native isolates and laboratory strains and the complexity of the interaction between these yeast and their native environments. The batch fermentation of grape juice exposes *S. cerevisiae* to a wide array of different biotic and abiotic stressors (Bisson, 1999), which is a driving force in the generation of naturally arising strain diversity. There are also technical challenges in the application of genomic technologies to commercial and native strains of *S. cerevisiae*. Specific analytical platforms have not been adequately validated by laboratories employing these technologies. This lack of validation is increasingly being recognized as a major problem in the field (Draghici *et al*., 2006; Grunenfelder and Winzeler, 2002; Lian and Kelemen, 2006; Quackenbush, 2004, 2005; Shields, 2006). Currently applied statistical methods that seek to limit the number of false positives in a data set may be too restrictive instead increasing the level of false negatives and impacting true trait discovery. Methods aimed instead at optimizing the false discovery rate will be of more utility in the analysis of expression profile differences across strains and growth conditions (Storey and Tibshirani, 2003).

There are several additional important factors that must be considered in designing a genomic analysis of industrial yeast including the experimental design itself, the biases that may have been inadvertently introduced, and the choice of strain and assay conditions. As detailed in the following sections, challenges posed to the interpretation of genomic data include the complexity of the nutritional environment, strain diversity, varying physical parameters of growth, the difficulty of controlling critical variables in production situations, and, in the special case of wine yeasts, the inability to reproduce the complexities of the diverse microbial populations present normally during fermentation. In addition, brewers, bakers, winemakers, and researchers employ different fermentation management strategies that can have a striking impact on yeast metabolic activities, transcript, and protein expression profiles. It is important to interpret results judiciously making sure that broad conclusions reached from genomic analyses are not in fact restricted to the specific conditions and strains used in the study.

A. The grape juice environment

Grape juice can host a diverse microbial community in a multifaceted and variable growth environment. These growth conditions present a variety of biological challenges to yeast including high sugar, high ethanol,

extremes of temperature, low pH, microbial competition, variable nutrient availability, suboptimal oxygen levels, presence of phenolic compounds and the impact on cellular redox status, and maintenance of metabolic rates under nonproliferative conditions. This variability in chemical and physical environmental parameters obviously poses an experimental design challenge for functional genomics. The observations made and the conclusions reached will be dependent on the growth conditions of the yeast, factors that must be taken into consideration in data interpretation.

Typical grape juice composition is given in Table 1 (Amerine *et al.*, 1980). Grape juice is initially high in sugar content, containing between 200 and 280 g/liter of sugar as an equimolar mixture of glucose and fructose. During fermentation, this high osmolarity decreases as sugar is consumed and is replaced by an equally high concentration of ethanol, decreasing the specific gravity of the environment below that of water. The yeast must contend with this wide range of changes in medium density.

In addition, yeast fermentative metabolism produces significant heat as an end product. For every 100 g of sugar consumed, an increase in temperature of 1.3 °C is obtained (Boulton *et al.*, 1996). Depending on the type of fermentation vessel, ambient temperature, or the use of refrigeration, temperature increases of 12–15 °C or higher are common. In addition, unless mechanical mixing is applied, stratification of temperature develops in batch fermentation conditions. This is particularly true in red wine production where the skins float to the surface once fermentation is initiated due to the presence of carbon dioxide. The ''cap,'' as the layer of skins is called, can retain heat and result in higher metabolic activity. Temperatures can be much higher in this area than in the rest of the tank. Reproducing such nonisothermal conditions in a laboratory in order to study the impact on yeast activities is not a trivial problem. Thus, the yeast must contend with high levels of sugar and ethanol as well as high and variable temperature.

Other environmental factors may also be a source of stress. The pH of grape juice is generally between 3.0 and 4.0, but can vary in this range depending on the metabolic activities of yeast and the other microbes present. In general, wine strains are tolerant to a pH as low as 2.8, and so may be at the edge of this limit during production conditions. As the pH rises above 3.6, a multitude of bacteria that were inhibited at lower pH values can begin to grow (Boulton *et al.*, 1996). At that point, the yeast faces not only competition for nutrients but also must handle the inhibitory effects of the end products of other microbes.

Oxygen is frequently limiting during grape juice fermentation. The lack of oxygen as a participant in biochemical reactions negates the use of some metabolic options for the organism. Indeed, nutrient starvation under anaerobic conditions has been shown to be fundamentally different

TABLE 1 The composition of grape juice (Amerine *et al.*, 1980)

Component	Concentration (g/100 ml)	(mg/liter)
Carbohydrates	8–16	
Glucose	8–16	
Fructose	0.02–0.08	
Inositol	0.01–0.1	
Pectin	0.08–0.2	
Pentoses		
Organic Acids		
Citrate	0.01–0.05	
Malate	0.1–0.8	
Tartrate	0.2–1.0	
Nitrogenous compounds		
Amino N	0.017–0.110	
Amide N	0.001–0.004	
Ammonia	0.001–0.012	
Protein	0.001–0.01	
Minerals and salts		
Aluminum	Trace–0.003	
Boron	Trace–0.007	
Calcium	0.004–0.025	
Chlorine	0.001–0.01	
Copper	Trace–0.0003	
Iron	Trace–0.003	
Magnesium	0.01–0.025	
Manganese	Trace–0.005	
Phosphate	0.02–0.05	
Potassium	0.15–0.25	
Rubidium	Trace–0.0001	
Sodium	Trace–0.02	
Sulfate	0.003–0.035	
Vitamins		
Biotin		0.01–0.06
Folic acid		Trace–0.05
Nicotinic acid		0.3–8.8
Pantothenate		0.25–10.5
Pyridoxine		0.1–2.9
Riboflavin		Trace–1.5
Thiamin		0.1–1.2

from starvation under aerobiosis (Thomsson *et al.*, 2005). Under aerobic conditions, yeasts tolerate carbon limitation better than nitrogen limitation, but under anaerobic conditions, the opposite is true (Thomsson *et al.*, 2005). Carbon limitation anaerobically leads to a large drop in ATP levels that is not observed aerobically where respiratory metabolism can be employed to boost ATP production. Growth conditions strongly influence the ability of yeast to adapt to a changing environment and delineate the nature of what that adaptation may be. How the yeasts are pregrown can also have a dramatic impact on the response to stress in the environment. The basal levels of expression of stress response genes will affect the tolerance to specific stress conditions encountered by the yeasts and impact the observations detected at the genomic level (Davidson and Schiestl, 2001; Gasch, 2003; Ivorra *et al.*, 1999; Zuzuarregui and del Olmo, 2004). Strains that show a low-fold induction of stress genes often are more tolerant than those showing a high-fold induction (Nugent, Mangahas, and Bisson, unpublished observations) because the difference between basal level and maximally expressed level is not as important as the basal level itself (Siderius and Mager, 2003).

Grape juice also contains a plethora of phenolic compounds (Table 2), many of which have been shown to be bioactive in humans. An early study suggested that the presence of these compounds dramatically influenced yeast metabolic activities (Cantarelli, 1989). The impact of these compounds on the redox status of the yeast cells, and the impact of redox conditions in the grape milieu on wine yeast, has gone largely unexplored. One of the largest gene families in *Saccharomyces* is the multidrug-resistant transporter family (Goffeau *et al.*, 1997). The true role of these genes may be in maintaining the redox status of the cell. Alternately, the growth, metabolic activities, and stress tolerance of many types of yeast are increased in the presence of phenolic compounds. The roles of these bioactive molecules in yeast may be varied and either beneficial or deleterious. In any event, they are present in the environment and therefore must be dealt with by the organisms.

TABLE 2 The phenolic composition of grape juice (Amerine *et al.*, 1980)

	Concentration (mg/liter)	
Component	Red grapes	White grapes
Benzoic acids	50–100	1–5
Cinnamic acids	50–100	2–10
Flavonols	10–15	Trace
Anthocyanidins	20–500	0
Flavan-3-ols	50–5000	0–100
Flavan-3,4-diols	Trace	Trace

The chemical composition of grape juice is likewise highly variable. In the native environment, yeasts frequently encounter nutrient excess or starvation, and with the potential for rapid shifts in between. Industrial yeasts have been selected over time that can withstand these swings in nutritional status. The response to sudden starvation is dependent on previous growth conditions. This has important implications in the design of genomic experiments and in the comparison of results across laboratories. Direct application of observations made using laboratory yeast in laboratory conditions is challenging. Much of the ''dogma'' surrounding our understanding of the biology of *S. cerevisiae* based on exclusive examination of laboratory strains is not valid in the interpretation of behavior of native and commercial strains in the absence of an appreciation of the differences in environmental forces affecting metabolic activities of these organisms. For example, the current model of cell cycles and growth based on laboratory strains in laboratory media poses nutrient uptake in G1, with accumulation of sufficient nutrients signaling start of a new cell cycle. At the end of that cycle, nutrients are again accumulated for the next cycle, and so on. Vacuoles accumulate in mass at the point of glucose exhaustion in stationary phase. In contrast, in wine yeast under wine production conditions, stationary phase-type vacuoles are present during active growth. They serve an important purpose as sites of storage of medium nitrogen. Yeasts efficiently deplete nitrogen from the medium, storing it internally, and can undergo several rounds of replication without further nutrient addition. This divergence is likely due to the vast differences between sugar levels in laboratory media and in the natural environment. Laboratory yeasts have largely been examined under a single condition: limitation for a carbon and energy source while in the wild, other nutrients may be limiting initially with growth eventually being impacted by the accumulation of ethanol. We found that in the absence of other stress factors, laboratory industrial and native isolates of *Saccharomyces* grow quite well even at ethanol concentrations of 10% if sufficient other nutrients have been provided and a sugar substrate is available. This is also supported by analyses of gene expression in isolated native strains of *S. cerevisiae* versus those that have been cultivated in laboratories (Kuthan *et al.*, 2003; Palkova, 2004). Strains rapidly lose some phenotypes associated with growth in the wild (Palkova, 2004).

One of the main challenges for yeast during fermentation is to maintain cellular parameters within specific restrictions to attain and maintain optimal conditions for metabolic activity. The limiting nutrient in grape juice is most often nitrogen. Numerous factors impact the ability to use nitrogen in the environment: the presence and complexity of nitrogen sources, cellular storage levels at the onset of fermentation, metabolic demands for nitrogen, the presence of ethanol and oxygen, the medium pH, and potassium levels.

It is also important to consider the possible effects of the other microbes present in grape juice that can impact the metabolic activities of yeast (Renouf *et al.*, 2006). Several yeast genera: *Brettanomyces*, *Candida*, *Debaryomyces*, *Hanseniaspora*, *Kloeckera*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Schizosaccharomyces*, *Torulaspora*, and *Zygosaccharomyces*, have all been reported to occur in grape juice (Fleet, 1993; Fleet and Heard, 1993). The levels of yeast found vary quite dramatically, depending on winery practices and the use of antimicrobial agents. Lactic and acetic acid bacteria are also present, the specific genera and species are largely dependent on grape juice pH, the temperature of fermentation, and the sensitivity of the strain to the metabolic activities of *Saccharomyces* (Fleet, 1993; Fleet and Heard, 1993).

Yeast fermentation behavior has been difficult to monitor, given the number of parameters involved and the varying composition of grape juice (Cramer *et al.*, 2002). Glucose is consumed more quickly than fructose, and cell viability is rapidly lost on sugar depletion (Figure 1). Although nitrogen is most often the limiting parameter, the kinetics of carbon utilization during most of the fermentation is not well correlated with nitrogen levels, especially toward the end of fermentation (Insa *et al.*, 1995;

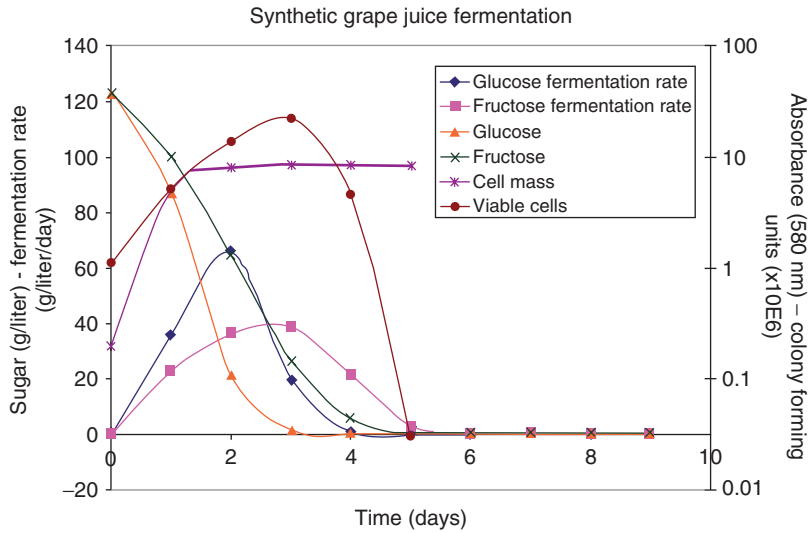

FIGURE 1 Yeast cell growth, viability, sugar consumption, and ethanol production patterns of a typical fermentation. In a synthetic grape juice medium, Triple M (Spiropoulos *et al.*, 2000) was used and inoculated with a commercial strain of *S. cerevisiae*. Glucose and fructose concentrations were determined by enzymatic assay, viable cell counts by plating on YPD medium, and cell mass by absorbance at 580 nm.

Manginot *et al*., 1998). Fermentation rates likewise are not well correlated with cell number as the fermentation capacity of cells can vary. Energy reserves at the point of implementation of stress appear to be a critical factor with higher storage levels of glycogen and trehalose associated with improved survival (Thomsson *et al*., 2005).

Finally, the bulk of the fermentation in grape juice production as well as in beer and during bread production is conducted by nongrowing cells. Our understanding of nonproliferative metabolically active states is limited. In the case of wine, the limitation on growth is caused by the attainment of terminal cell density. We have observed that cells immediately resume growth with no appreciable lag if the cell number in the nonproliferative condition is reduced by centrifugation. Thus, though not growing these cells are primed to grow as soon as biomass levels are reduced providing conditions are still permissive for growth. We have observed growth at ethanol levels considered to be inhibitory based on studies conducted with laboratory yeasts (Kumar, Goyashiki, Karpel, Ramakrishnan, and Bisson, unpublished data).

B. Wine yeast strain diversity

Genomic analyses have already revealed that many of the currently used commercial strains have acquired altered signaling properties (Verstrepen *et al*., 2004). Commercial and native yeast isolates display greater genomic and genetic instability than laboratory strains (Ambrona *et al*., 2005). Furthermore, trisomy and tetrasomy for some chromosomes are common in industrial strains (Bakalinsky and Snow, 1990). Wild strains are generally homothallic and tend to show low sporulation rates, poor spore viability, high levels of heterozygositiy and chromosomal polymorphisms, rearrangements, and karyotype instability (Carro and Pina, 2001; Codon *et al*., 1998; Landry *et al*., 2006a,b; Longo and Vezinhet, 1993; Myers *et al*., 2004). This dynamic instability is the foundation of the ''genome renewal'' hypothesis for *Saccharomyces* (Mortimer *et al*., 1994). Hauser *et al*. (2001) proposed that chromosome imbalances must pose some selective advantage in the environment because of their persistence in wild populations and the frequency with which they arise. These genetic differences give rise to differences in phenotype. Various methods, including molecular and more traditional techniques, have been used to compare the genetic relatedness among wine isolates of *Saccharomyces*.

Initial studies of the differences in the genomes of wine yeast were done using traditional genetic tools. Crosses were conducted with strains that showed characteristics of interest, and spore segregation ratios were determined by tetrad (Cummings and Fogel, 1978; Takahashi, 1978; Thornton and Eschenbruch, 1976) or random spore progeny analysis (Bakalinsky and Snow, 1990; Spencer *et al*., 1980). The initial studies

indicated that there was a high level of diversity in the wine yeast strains. These methods measured only chromosome numbers but gave more information than that provided by measuring total DNA content (Leusch *et al.*, 1985). Methods that require sporulation are limited when sporulation is poor and spore viability is low. Analysis shows that spore viability is highly variable in wine yeast with numbers as low as 0% and as high as 100% (Guijo *et al.*, 1997; Johnston *et al.*, 2000; Thornton, 1986; Thornton and Eschenbruch, 1976). Methods that were not dependant on sporulation ability were necessary to look at genetic diversity in large numbers of wine strains (Schuller *et al.*, 2005). Methods that targeted metabolic products rather than direct genetic analysis, such as fatty acid analysis with gas chromatography (Ockert and Kock, 1989; Tredoux *et al.*, 1987), were developed to investigate strain diversity. This method and the related fatty acid methyl ester (FAME) analysis (Peltrouche-Llacsahuanga *et al.*, 2000) have been used successfully but rely on only a single class of metabolic products from yeast.

The availability of molecular genetic tools for *Saccharomyces* in the 1980s led to a proliferation of techniques applicable to the examination of the genetic diversity of wine yeasts. These methods have the advantage of being more random than fatty acid analysis and are not dependant on sporulation. Currently, restriction fragment length polymorphism (RFLP) of mitochondrial DNA is a popular method, while pulse field gel electrophoresis is often used as the standard of comparison for new methods. Polymerase chain reaction (PCR)-based techniques are also popular. These include interdelta sequence analysis, intron splice analysis, random amplification of polymorphic DNA (RAPD), multi-locus sequence typing (MLST), amplified fragment length polymorphism (AFLP), and simple sequence repeats (SSRs) or microsatellite sequencing. Karyotype analysis using pulse field electrophoresis (Briones *et al.*, 1996; Izquierdo Canas *et al.*, 1997) and DNA fingerprinting using RFLP of various regions as well as microsatellite PCR (Baleiras Couto *et al.*, 1996) were some of the first techniques to be used. More recently, comparative microarray and proteome analyses have also been used to characterize differences in and classify strains of *S. cerevisiae* used in winemaking.

Several comparisons of these techniques were carried out with similar results. Baleiras Couto *et al.* (1996) indicated that a combination of analyses, including RAPD, SSR, and RFLP, were required to give good discrimination between *S. cerevisiae* strains. Schuller *et al.* (2004) concluded that any single method they tried: SSR, interdelta sequence analysis, or RFLP of mitochondrial DNA, gave adequate discrimination between strains. However, the SSR was the most sensitive method. Gallego *et al.* (2005) compared RAPD, AFLP, and SSR and determined that, while all were adequate, SSR again gave the highest level of discrimination. In all of these analyses, the majority of strains were distinguishable and only

a small percentage appeared to be identical strains. In the Baleiras Cuoto study, 13 out of 16 isolates (81%) were determined to be unique strains and the 4 identical strains were isolated from the same location. In the Schuller study, 23 commercially available isolates gave 21 or 22 strain types (91–96% of those tested) depending on the technique used. Of the 27 strains tested by Gallego *et al.* (2005), 81–91% were determined to be unique strains, again depending on the method. Strains that were not unique were isolated from the same must or different musts within the same winery.

It is now possible to rapidly type both commercial and wild wine yeasts to determine if they are unique strains. With the availability of microarray analysis, it may be possible to begin to determine in what genes and characteristics these differences lie. A direct comparison between a laboratory and a wine yeast (Hauser *et al.*, 2001) found more than 40 genes that showed different expression patterns under the same conditions. On careful analysis, these differences were attributable to gene copy number and small variations in promoter regions. A study compared four wine strains with the fully sequenced laboratory strain (S288C) in a microarray karyotype analysis (Dunn *et al.*, 2005). In this study, comparison between the wine strains and the laboratory strains showed differences primarily in transport and permease genes, particularly those involved in drug resistance. There were small but significant differences detected between the four wine strains studied. These differences were enough to distinguish the strains from one another and to give each of the strains a microarray karyotype ''signature.'' While the study looked at relative copy numbers of genes compared to the sequenced laboratory strain (S288C), it was not possible to determine if there were genes that were present in the wine strains and not in the sequenced strain. Determining how the genetic differences in the strains account for the differences observed in fermentation characteristics and variations in finished wines remains a daunting task.

C. The genomic tool chest

The yeast community has benefited from the development of several elegant genomic tools that can be applied to the study of yeast biology (reviewed in Lockhart and Winzeler, 2000). These tools have changed analysis from a focus on single genes and proteins to examination of the comprehensive transcript or protein composition of yeast. Global transcript analysis has been termed transcript profiling or ''transcriptome'' analysis and the companion analysis of total cellular proteins termed ''proteomics.'' In addition, techniques in chemical constituent analysis are also being applied in order to complete the picture and display the

''metabolome'' of yeast. The analysis of the complete set of mutant phenotypes has been termed ''phenomics'' (Bader *et al.*, 2003; Warringer *et al.*, 2003).

Additional experimental tools for genome-wide analysis have been developed and tested on yeast, such as methods allowing global searches for functional elements in promoter regions that can be used by any researcher (Cliften *et al.*, 2003; David *et al.*, 2006; Kellis *et al.*, 2003). Arrays developed for mRNA transcript analysis are also being used to analyze DNA directly (Dunn *et al.*, 2005; Gresham *et al.*, 2006; Winzeler *et al.*, 2003). This provides important information on the global differences between strains and is a critical first step for comparative analyses of different strains (Dunn *et al.*, 2005). Analytical tools have been developed that allow genotyping directly from mRNA arrays (Ronald *et al.*, 2005). Transcript profiling can also be used on populations following adaptive evolution (Ferea *et al.*, 1999). In this case, a population of isogenic cells is exposed to some consistent unique environment for multiple generations. At the end of the adaptive period, both transcriptome and DNA analyses can be performed on DNA chips. This can reveal both the transcriptional changes as well as possible nucleotide polymorphisms that have arisen in this culture as compared to the control culture not exposed to the same environmental conditions. Oligonucleotide arrays have also been used to map the topography of replication (Raghuraman *et al.*, 2001).

In addition to these indispensable technologies, several other tools have been developed to facilitate genome-wide research on *Saccharomyces*. Two types of comprehensive collections of mutants have been generated, based either on insertional or deletional mutagenesis (reviewed in Vidan and Snyder, 2001). Large-scale insertional mutagenesis was used to disrupt open reading frames (ORFs) (Kumar *et al.*, 2002, 2004). This study also used a transposable element gene trap based on detection of expression of a LacZ fusion protein. The appearance of β-galactosidase activity indicated that a fusion to a functional ORF had occurred and that a true expressed gene had been identified or trapped. This analysis resulted in the identification of ORFs that had not been previously annotated in the genome.

The second comprehensive mutant collection was generated directly via systematic and specific disruption of every putative ORF in *S. cerevisiae* (Giaever *et al.*, 2002; Winzeler *et al.*, 1999). Each putative ORF has been deleted, and the set of nonlethal null mutations is commercially available from Open Biosystems (http://www.openbiosystems.com/Geneexpression/Yeast/YKO/). Each deletion also contains a pair of unique sequence tags or ''bar codes'' that allow that mutation to be identified in a population of cells (Figure 2). This important set of mutants allows high-throughput screening for desired phenotypes (Tong *et al.*, 2001; Warringer and Blomberg, 2003). These screens, when used in combination

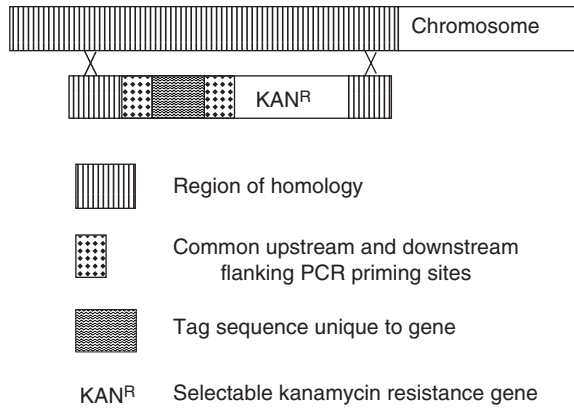

FIGURE 2 Depiction of the strategy used for the creation of the genomic deletion set of strains for *S. cerevisiae*. Each disruption contains a selectable marker, kanamycin (G418) resistance, and a tag or specific sequence unique to each construct. The PCR primers flanking the unique tag or "bar code" can be used to quantify the persistence of that tag and therefore of that specific mutation in a population of cells.

with transcriptome or proteome data, can be used to confirm the functional or regulatory role of a gene. Fitness studies can also be conducted under specific growth conditions, with analysis for each specific tag of the DNA isolated from the final population. Tags that are over- or underrepresented suggest a more or less fit genotype for the conditions under study.

High-throughput microscopy techniques screen for various morphological aberrations in the yeast deletion set (Narayanaswamy *et al.*, 2006). Other tools include a comprehensive set of fusions of fluorescent or affinity tags to each gene in the yeast genome (Andrews *et al.*, 2003; Gelperin *et al.*, 2005). The availability of a set of fluorescently tagged genes has allowed global analysis of protein localization in yeast (Huh *et al.*, 2003). The use of affinity tags has allowed better visualization and quantitation of protein species. The tandem affinity purification (TAP) tag allows immunodetection of tagged proteins as well as immunopurification (Ghaemmaghami *et al.*, 2003). The set of deletion strains is also being used for metabolic footprinting, a comprehensive analysis of the metabolites released into the medium to be correlated with the specific knockout mutation borne by the strain (Allen *et al.*, 2003). The use of excreted metabolites avoids the issues of differential extraction and sample preparation if internal components were being evaluated. Comprehensive studies to define gene function have also been undertaken (Wu *et al.*, 2002).

An issue has arisen with respect to both types of sets of comprehensive mutations. Several studies have found that aneuploidy or other

compensating mutations can arise in these mutant strains on cultivation (Hughes *et al.*, 2000; Oshiro and Winzeler, 2000; Vidan and Snyder, 2001). The appearance of these types of genetic modifiers can arise at surprisingly high frequencies, and can be influenced by experimental design. As a consequence, all genes identified as positively affecting a specific phenotype need to be confirmed, but false negatives, strains with modifiers suppressing the original phenotype, may be more difficult to identify.

Genetic footprinting using Ty1 insertional mutagenesis has also been undertaken (Smith *et al.*, 1995). Genes containing insertions in a population can be identified by PCR screening. After growth or application of selective pressure, the population can be rescreened and those genes that have been enriched or selected against identified. The advantage of this technique is that it can be applied relatively quickly to other yeast strains. This technique can also be used on a new population minimizing the risks of accumulation of compensatory secondary mutations or aneuploidies.

Many studies have also been undertaken to define the complete set of functional and physical interactions among proteins (Bader and Hogue, 2002; Gavin *et al.*, 2002; Hazburn and Fields, 2001; Ho *et al.*, 2002; Ito *et al.*, 2001; Link *et al.*, 1999; Tong *et al.*, 2004; Uetz and Finley, 2005; Uetz *et al.*, 2000; Von Mering *et al.*, 2002), providing an important reference data set for the rest of the research community, even if the different studies have not reached the same conclusions (Grigoriev, 2003). Gene annotation services in *Saccharomyces* (Dwight *et al.*, 2002; Gollub *et al.*, 2003) serve as a model for other organisms and provide a critical database for researchers in the field. The ongoing gene ontology project continues to provide and catalog important molecular function of gene products and their cellular roles. Much of the thrust of this work has been to provide tools or resources for the scientific community. Although this work has been undertaken virtually exclusively in laboratory strains, much of the information gained should be applicable to wine yeast strains.

As beneficial as these technologies are, there are some shortcomings in how data are being analyzed and interpreted. In some cases, the platform chosen for transcriptome or proteome analysis limits the information to be obtained (reviewed in Draghici *et al.*, 2006). A published comparison of mouse transcriptome analyses concluded that there was more cross-platform variation than cross-laboratory variation for the same platform (Kuo *et al.*, 2006). Not surprisingly, they found the greatest disagreement among low expression values than for medium and high expression values. They also found that the variability was greater for noncommercial arrays. To address the variability in microarray data and so that data sets across laboratories and platforms may be compared, several initiatives aimed at improving array reproducibility have been launched: MIAME [Minimum Information About a Microarray Experiment (http://www.mged.org/Workgroups/MIAME/miame.html)], ERCC [the External RNA

Controls Consortium (Baker *et al*., 2005)], and MAQC [Microarray Quality Control Project (http://www.fda.gov/nctr/science/centers/toxicoinformatics/maqc/)]. However, these guidelines tend to focus on documentation of some analytical details or use control nucleotides that do not broadly represent the full dynamic range of the array and fall short of really addressing the technical issues in these types of analyses (Draghici *et al*., 2006; Shields, 2006).

One common use of these technologies is in the analysis of genes of unknown function, and it is a belief among many that coexpression implies coregulation and that the appearance of a transcript under certain stressful conditions means that the gene product is required for tolerance to that condition. Although there is some experimental evidence to support the relationship between coexpression and coregulation, the combination of transcriptome analysis with high-throughput screening of the deletion set of strains indicates that genes that may be highly expressed in response to a specific stress often, when mutated, do not impact stress sensitivity (Birrell *et al*., 2002). Coregulation in some cases is merely circumstantial and gene expression is not functionally linked (Heyer *et al*., 1999).

Finally, it is important to note that the most important gadgets of the yeast tool kit are the excellent gene annotation and database resources that are available such as the *Saccharomyces* Genome Database maintained by Stanford (http://www.yeastgenome.org/)—which includes and maintains up-to-date information on gene annotation, scientific literature, and is a resource for tools and other information of importance to the research community—and the Stanford Microarray Database (http://genome-www5.stanford.edu/). Also important is the companion Comprehensive Yeast Genome Database (http://mips.gsf.de/genre/proj/yeast/). Databases of protein identification from two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (2D SDS–PAGE) also exist in the Yeast Protein Map project (http://www.ibgc.u-bordeaux2.fr/YPM/), SWISS 2D PAGE (http://www.expasy.org/ch2d/), the Yeast Proteome Database (http://www.biobase-international.com/pages/index.php?id=139), and the Yeast Resource Center for analysis of protein interactions and complexes (http://www.yeastrc.org/unknown_orfs/complexes.html). This is only a partial list highlighting the abundant resources available to yeast researchers.

1. Transcriptome analyses

There are four main types of trancriptome analyses utilized in yeast mRNA profiling, each with different benefits and deficiencies (Table 3). The first two methods rely on arraying complementary nucleotide information on a grid with subsequent hybridization of a sample preparation. These methods are based on scaling up Northern blot technology of complementary nucleic acid hybridization to assess multiple transcripts

TABLE 3 Transcript profiling methods

Method	Process	Quantification
Hybridization	Probe: cDNA Labeled mRNA cRNA Target: PCR fragment Oligonucleotide	Intensity equated with relative level of expression
mRNA fragment analysis (SAGE)	Capture PolyA regions DNA sequence analysis of concatemers	Frequency of sequence equated with level of expression
Quantitative reverse transcription PCR	PCR reaction	Intensity of labeling during PCR reaction equated with level of expression

simultaneously. There are two basic types of hybridization methods being employed, those that use arrays based on PCR products specific for each gene or ORF and those using oligonucleotides (reviewed in Draghici *et al.*, 2006). The oligonucleotides may be short (25–30 base pairs) or long (60–70 base pairs), and contact spotted, inkject deposited, or synthesized directly on the array.

In the first of these methods, specific PCR fragments of yeast ORFs are arrayed on a glass slide or grid (DeRisi *et al.*, 1997; Lashkari *et al.*, 1997; Schena *et al.*, 1995), or on a nylon membrane (Alberola *et al.*, 2004). The mRNA is then purified from reference and experimental conditions, labeled with a fluorescent tag directly (biotinylated) or after conversion to cDNA [Cy3 (green) or Cy5 (red)], or during synthesis to cRNA, and the tagged mRNA/cDNA/cRNA is then hybridized against the PCR fragment array (reviewed in Lockhart and Winzeler, 2000). Samples may be hybridized to the array or grid singly, or after mixture of samples labeled with two different dyes. In the double dye-binding method, differential fluorescence is scanned and used to calculate the differences in ratios of expression between the reference and experimental sample. Similar types of strategies have been employed using radioactively labeled cDNA preparations (Rep *et al.*, 2000; Zuzuarregui and del Olmo, 2004). The advantages of this technique are its relatively inexpensive cost, ability to be utilized in individual laboratories, and relative ease of data manipulation. The disadvantages center on the spotting technology and the inability to achieve uniform

spots with the associated difficulty in determining the actual strength of the signal. The comparative analysis of array platforms by Kuo *et al.* (2006) found that these double dye-binding methodologies were not as reproducible as single signal platforms. However, other studies have reached the opposite conclusion, that relative comparisons of expression are more robust than attempts to quantify individual expression patterns (Draghici *et al.*, 2006). Other issues concern the quality of the mRNA preparation and the ability to biologically reproduce the pattern. Replication of the same sample generally yields acceptable levels of reproducibility (Lee *et al.*, 2000). There is greater variation if true biological replication is utilized, that is, a sample is completely replicated in the growth conditions, harvesting and preparation rather than simply analyzing the same mRNA preparation twice (Quackenbush, 2005). The costliness of many commercial array technologies has forced a compromise on replication. Specialized arrays, with a more limited set of genes represented, have also been employed (Rodriguez-Pena *et al.*, 2005).

In the second type of hybridization-based analysis, complimentary oligonucleotides are used to identify the specific cDNA species present (Lockhart *et al.*, 1996; Schadt *et al.*, 2000; Wodicka *et al.*, 1997). Each transcript is represented by a few to several oligomers providing independent signals for each gene. As a control for nonspecific hybridization, the Affymetrix design also includes a mismatch of each oligonucleotide. The strength of the signal is then estimated both on the absolute values as well as on the difference between the perfect match and mismatch signals across the gene. The Affymetrix version also includes control chips, which we have found to be invaluable in the analysis of replication error. These control chips contain 3′ and 5′ regions of three genes. A 3′ to 5′ ratio of 1 indicates that the 3′ and 5′ regions are equally represented in the population of labeled cDNA. We have found that if the Affymetrix platform is used and the test chips are employed, the differences between biological replications is greatly diminished if high-quality standards (nearness to a 3′ to 5′ ratio of 1 for all three marker genes) are utilized.

In yeast, the detection limit or dynamic range of mRNA for these technologies is on the order of 1–10 copies of mRNA per cell (Draghici *et al.*, 2006; Holland, 2002). Other studies have shown that the majority of mRNA species in *S. cerevisiae* are at or below this limit (Varela *et al.*, 2005) and are therefore below the sensitivity of current array technologies (Shields, 2006). A single mRNA molecule on average can produce ~4800 protein molecules, so it is not surprising that the majority of genes would be expressed in this low range (Wohlschlegel and Yates, 2003). In addition to obstacles imposed by low transcript abundance, accurate quantitation of absolute expression levels can be difficult to achieve (Draghici *et al.*, 2006). At issue is the need to select single-hybridization conditions for these technologies at the same time that specificity of probe design is

maintained. Cross-hybridization can also be a major impediment in accurate quantitation and has been shown to lead to compression of expression ratio differences where the observed ratio is less than the true ratio due to the high background of nonspecific binding (Draghici *et al.*, 2006). Probe selection influences signal strength, and it is important that imaging software, signal detection threshold, and quantitation take this into account (Wang *et al.*, 2006).

The third class of transcript analysis is serial analysis of gene expression or SAGE (Kal *et al.*, 1999; Velculescu *et al.*, 1995). In this technology, the ends of the mRNA transcript are harvested, formed into concatemers, and then the concatenated molecules are sequenced. The higher the number of times a particular sequence appears, the higher the level of that transcript in the population. SAGE analysis works well for highly expressed genes, but has limitations for genes that are not that highly expressed, as they may be represented only once or twice in the sequenced pool. The limitation here, as with the other array platforms, is the expense both in cost and in time, of sequencing a much larger set of concatemers to have statistically valid information for the messages with low expression levels.

The final type of transcript analysis is quantitative reverse transcription PCR (QRT-PCR) (Holland, 2002). This technology can be scaled to be genome wide, although its major use seems to be in confirming array data generated by other means. QRT-PCR has a broader dynamic range than array technologies but is subject to other types of limitations, such as the nature of the primers used (Freeman *et al.*, 1999). The comparative analysis by Kuo *et al.* (2006) indicated good agreement among the array and QRT-PCR methodologies for highly expressed genes. Although these study centered on mouse genomics, the conclusions reached are likely broadly applicable to genomic analyses in general.

2. Proteome analyses

The term ''proteome'' was coined as a companion to transcriptome to define tools and analyses directed at dynamically profiling the protein complement of a cell (reviewed in Graves and Haystead, 2002). The aim of proteomic analyses is the separation, identification, and quantitation of all proteins in a mixture. Proteomic analyses have been as valuable as transcript profiling in the analysis of yeast physiology. These technologies are not as robust as transcript profiling since proteins are far more heterogeneous than RNA. Currently, it is not possible to know with any degree of certainty that all proteins are being visualized and that relative ratios of proteins in the cytoplasm are being maintained through sample preparation and protein separation (Gygi *et al.*, 1999a,b, 2000; Moseley, 2001; Peng *et al.*, 2003; Ravichandran and Sriram, 2005). However, as with proteomics, some important observations have been made using subsets of components that could be accurately quantified. When these limitations

are taken into account, these technologies can provide significant insights into yeast biology, especially when used in combination with other types of genomic analysis. There are several techniques currently being employed for proteome analysis of yeast, ranging from traditional 2D SDS-PAGE to more sophisticated separations technologies coupled to mass spectrometry.

Proteome analysis in the form of electrophoresis (2D SDS-PAGE) (Garrels, 1983) actually preceded mRNA profiling. In this technology a protein mixture is separated in two dimensions electrophoretically. In the first dimension, a pH gradient is employed to separate proteins according to their isoelectric point. The second dimension separates proteins according to molecular weight as influenced by the Stoke's radius of the protein. Different staining techniques are employed to visualize proteins. 2D SDS-PAGE allows visualization of proteins modified either by charge (change in isoelectric point) or by size and therefore has the potential to readily detect key protein modifications.

Individual proteins can then be identified following in-gel digestion and analysis of peptide fragments by mass spectrometry (Muddiman *et al.*, 1997) or by Edman degradation and peptide sequencing (Edman and Begg, 1967). Tandem mass spectrometry (MS/MS) allows the initial peptides and accurate mass determination followed by selection of peptide masses of interest and further fractionation (Shevchenko *et al.*, 1996a,b; reviewed in Chalmers and Gaskell, 2000). Mass identification of the peptides obtained is then used to determine the sequence of the original peptide and ultimately of the protein (Figure 3). The completion of a genome of an organism greatly enhances this technology in providing a database of possible protein sequences allowing more rapid identification of the protein. Peptide mass fingerprinting, the enzymatic fractionation of a protein followed by accurate mass determination of the peptides produced, provides a robust mechanism of protein identification without the need for sequencing (Shevchenko *et al.*, 1996a,b). Two analytical methods are utilized to ionize proteins or peptides without damage: matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Larsson *et al.*, 1997; Sagliocco *et al.*, 1996) and electrospray ionization tandem mass spectrometry (ESI MS/MS) (Shevchenko *et al.*, 1996a,b). Both methods are ideal for analysis of complex mixtures of proteins or peptides, but suffer from interferences from the protein matrix that may require sample manipulations that could affect the proteins obtained.

Although the most commonly used method for proteome analysis remains 2D gel electrophoresis, there are several important limitations of this technology (Gygi *et al.*, 2000; Olineka *et al.*, 2005). First, large proteins and hydrophobic (membrane) proteins have difficulty entering the gel and are typically lost from the protein profile as are small proteins that may migrate too rapidly. Proteins that are highly basic or acidic are

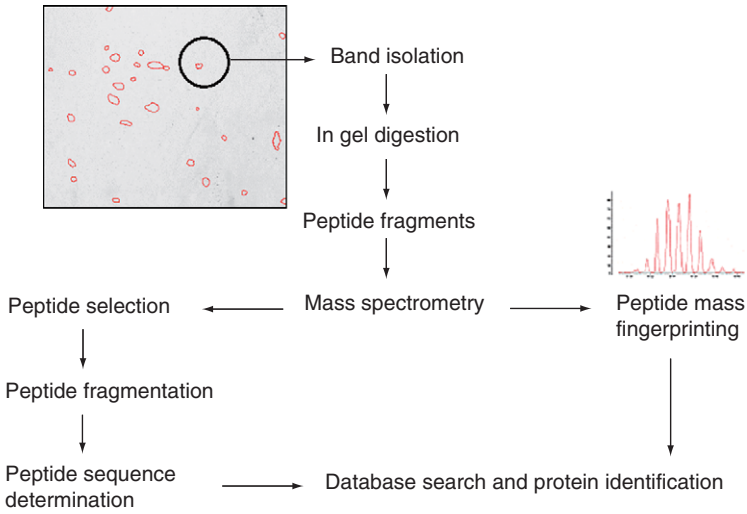

FIGURE 3 Protein separation and identification using 2D SDS-PAGE electrophoresis. Spots of interest are identified, excised from the gel, and subjected to in-gel digestion with a protease with well-characterized cleavage sites such as trypsin. The peptide fragments generated are then subjected to separation and analysis by mass spectrometry. The array of peptide fragments produced, or peptide mass fingerprint, may be used to identify the protein directly by searching databases consisting of predicted cleavage sites of proteins predicted from genomic sequencing. Alternately, tandem mass spectrometry may be used to directly determine the sequence of the peptides. The peptide sequence can then be used to identify the protein.

poorly represented in the gel. A third problem is the limited dynamic range of protein detection. Protein concentrations are estimated to vary from a few copies per cell to over a million of a single protein species. Highly expressed proteins can be readily visualized but there is no technique equivalent to PCR that allows amplification of minor protein species. Simply loading more protein is not an option as trailing on the part of the major species would obscure detection. A fourth problem is the inherent variability in the separations of proteins in replicate samples. This is mitigated somewhat by using commercially prepared gels, but the actual running conditions of the gel can lead to differences in temperature gradients across the gel as a function of how many gels are run concurrently, ambient room temperature, and other factors. Comparison of replicate gels requires use of sophisticated morphing or warping software that can be time consuming. An alternate approach is to use a double dye-binding method. In this methodology, proteins in two samples of interest to be compared are each labeled with a different fluorescent dye or isotope (Jiang and English, 2002; Smith *et al.*, 2002; Zhou *et al.*, 2002);

the protein samples are then mixed and run on the same gel. Direct comparisons of differences in fluorescence can be used to determine the ratio of expression of the protein between the two samples following the same logic as the double dye-binding transcriptome technologies (reviewed in Graves and Haystead 2002). The final major problem with 2D SDS-PAGE-based methodologies is that they are labor intensive and time consuming and challenging to automate.

To address some of these limitations, nongel technologies are being developed and utilized for protein analysis. These methods rely on other types of separation protocols, liquid chromatography (LC) or capillary electrophoresis (CE). These methods can also be adapted to a multidimensional format. Multidimensional liquid chromatography tandem mass spectrometry (MudPIT) employs a strong cation exchange and reverse phase separation technology and has been successfully applied to yeast proteomics (Link *et al*., 1999; Washburn *et al*., 2001) Capillary isoelectric focusing coupled to capillary reversed phase electrophoresis has been used to separate yeast proteins and peptides (Chen *et al*., 2003; Haynes *et al*., 1998). In this case, proteins can be focused or concentrated in the first phase, then separated in the second phase, allowing detection of a much wider set of proteins from the mixture. Breci *et al*. (2005) compared several methods of protein separation and detection using the same yeast extract: nanoflow liquid chromatography-liquid chromatography tandem mass spectrometry [nano LC/LC MS/MS (MudPIT)], nano LC MS/MS with gas phase fractionation by mass range selection (the same technique but with gas-phase fractionation by ion abundance selection), 2D SDS-PAGE coupled to in-gel digestion and nano LC MS/MS of gel slices, and isoelectric focusing followed by nano LC MS/MS of gel slices. The 2D SDS-PAGE method led to the identification of the greatest number of proteins and allowed higher sequence coverage of individual proteins (more peptides visualized per protein), making protein identification more robust. It is also possible to digest a complex mixture of protein prior to separation and identification of peptides using a tandem MS/MS protocol. This allows identification of proteins in a specific mixture but is not directly quantitative (Patterson and Aebersold, 2003).

The intensity of a peptide does not reflect its relative concentration as different peptides may produce different signal intensities depending on their composition or the matrix in which they are separated. Various technologies to allow comparative analysis of peptide abundance in two samples have been developed. These methods rely on the use of different isotopes used as label for each sample, and then peptides from the same protein can be identified from the discrete change in mass resulting from the particular label used for that mixture. In one specific example, termed ICAT analysis, a cysteine reactive molecule, labeled with either light or heavy deuterium, is used creating a mass tag for all cysteine-containing

peptides (Gygi *et al.*, 1999a). Peptides containing the tag can be isolated by avidin affinity chromatography, reducing the number of peptides analyzed.

These newer methodologies are not ideal either and suffer from their own limitations. For many of these techniques, some fractionation or reduction in the number of proteins or peptides identified is necessary prior to or during analysis. These methods also may not detect all species in a mixture, depending on the nature of the separation and the biochemical properties of the protein.

Another method using protein ''chips'' has also been developed (Haab *et al.*, 2001; Zhao *et al.*, 2001; Zhu *et al.*, 2001). These chips contain ordered arrays of protein-binding molecules with the proteins then bound or enriched on a segment of the chip to be analyzed via various technologies. Antibodies recognizing specific protein sequences or modifications can be used, for example, to separate proteins with a specific activity from a complex mixture. A different version of a protein chip has been created by Zhu *et al.* (2001) and by Martzen *et al.* (1999). In these cases, the proteins are arrayed on the chip and the proteome can then be screened for interaction with particular substrates and ligands, and for certain classes of biochemical properties and activities.

Often it is of interest to compare the relative levels of proteins across strains or conditions of growth. There are several methods available for these types of analyses (Table 4) (reviewed in Graves and Haystead, 2002). Individual 2D SDS-PAGE gels can be run with the gels and then directly compared using spot identification software and gel morphing or

TABLE 4 Comparative analyses of proteome profiles

Method	Process	Quantification
2D SDS-PAGE gel	Morph/warp-stained gels for constellation identification and spot matching	Relative intensities of spots (stained or labeled proteins)
Protein chip	Proteins labeled and blotted against chip	Relative intensity at a specific chip locale correlates with relative expression level
Differential protein targeting	Protein species tagged using tags of differing molecular weight; protein samples then mixed	Mass spectrometry analysis of peaks separated by the precise molecular weight of the tag

warping tools based on constellation (characteristic subclusters of proteins) matching. Alternatively, protein samples may be differentially labeled in two extracts, the two extracts mixed, and the proteins blotted against a protein chip. The relative intensities of the two labels are correlated with the relative concentrations of the two proteins. Mixed extracts from two differently labeled samples can also be subjected to protein digestion and their peptide fragments analyzed directly by mass spectrometry. Peptides derived from the identical proteins in the two extracts will be separated from each other by the precise molecular weight of the tag. The sequence of peptides of interest can then be determined if quadrupole mass spectrometry is used.

An obvious question concerns the degree of correlation between transcriptome and proteome data. Several yeast studies have addressed this topic with different conclusions having been reached. Gygi *et al.* (1999b) and Griffin *et al.* (2002) found insufficient correlation between 2D SDS-PAGE and SAGE data (both of which are biased toward abundant proteins and mRNA species) to allow prediction of protein content from transcriptome data. Other studies have found a statistically significant correlation between protein and mRNA abundance (Futcher *et al.*, 1999). These studies are not actually in conflict. There does seem to be a correlation between protein and mRNA abundance, just not one that allows prediction of protein level from mRNA level. Belle *et al.* (2006) explored this issue from the perspective of including changes in protein turnover rates. These researchers took advantage of an epitope-tagged library for quantification of protein half-lives and found that proteins generally fell into one of two categories: optimized for efficient production or optimized for efficient regulation. They found a correlation between half-life and transcriptional regulation in some sets of coregulated genes. Transcriptional regulation may be used to buffer the consequences of changes in protein half-life so that overall protein content remains the same (Belle *et al.*, 2006). Changes in levels of highly abundant proteins may be difficult to detect over a short time course, so there is a dynamic interaction between mRNA and protein stability. Obviously, how a study is conducted will influence the conclusion regarding the relationship between protein and transcript abundance. If cell growth has been arrested, for example, both processes may be in a state of flux as the cell adapts to the new environmental conditions and correlations may be difficult to detect.

3. Metabolome analyses

The third level of genome-wide analyses of cells following the transcriptome and proteome is that of the metabolome. The ''metabolome'' refers to the entire complement of all the low- and intermediate–molecular-weight metabolites inside a cell suspension of interest, but for practical reasons, subsets of metabolites (metabolic profiles) are more typically

obtained with each screening technique. There is a growing consensus that knowledge of transcript and protein profiles is insufficient to predict actual metabolite levels, and without this information, whole cell metabolic reconstruction and modeling will not be possible. Similar to proteomics, issues of analyte complexity confront the field of metabolomics (Fiehn, 2002). The chemical diversity of components to be evaluated makes selection of a single profiling technology difficult if not impossible. Differential extraction, chemical reactivity, and sample preparation impact the relative levels of compounds in a given sample; so with current techniques, it is not possible to display all cellular components. Several technologies, as described below, are being developed for metabolomic investigations.

Metabolites produced in microorganisms (referred to as secondary metabolites) are also an invaluable source of useful compounds, including pharmaceuticals, toxins, and other chemicals (Peric-Concha and Long, 2003). Only recently have analytical techniques progressed to a level that broad metabolic profiling has become a reality and has evolved into the science of metabolomics. For instance, over 700 different biochemical compounds have been identified within a single bacterial species alone (Nobeli *et al.*, 2003). How to measure these compounds, how to identify the molecular structure of each individual compound, how to place each individual compound in the relevant biosynthesis pathway, and how each compound relates to functional properties within an organism or for human/animal health and nutrition are all different aspects of metabolomics.

To monitor, in parallel, hundreds or even thousands of metabolites, high-throughput techniques are required that enable screening for relative changes rather than absolute concentrations of compounds. Most analytical techniques for profiling small molecules consist of a high-performance liquid chromatograph (HPLC) or a gas chromatograph (GC) coupled to a mass spectrometer (MS). These techniques include GC-time-of-flight-MS (GC-TOF MS) (Fiehn, 2002), LC-MS (Lenz *et al.*, 2004), direct-electrospray MS (Allen *et al.*, 2003), CE-MS (Soga *et al.*, 2003), multidimensional chromatographies linked to MS (Blumberg, 2003), and the newly emerging Fourier transform ion cyclotron resonance MS (FTICR-MS) (Aharoni *et al.*, 2002). Other techniques such as Fourier transform infrared (FTIR) (Oliver *et al.*, 1998) and nuclear magnetic resonance (NMR) spectrometries (Raamsdonk *et al.*, 2001) are also popular. Somewhat in contrast to transcriptomics and proteomics, the great chemical heterogeneity of the metabolome means that no single method will realistically capture all metabolites.

Mass spectrometers are generally more sensitive and more selective than any other types of detectors. When coupled with the appropriate sample-introduction and ionization techniques, mass spectrometers can selectively analyze both organic and inorganic compounds (Baldwin, 2005).

Nevertheless, prior to detection, the metabolites have to be separated by chromatographic techniques that are coupled to the mass detector. GC is used to separate compounds on the basis of their relative vapor pressures and affinities for the material in the chromatography column, but is restricted to compounds that are volatile and heat stable (Littlewood, 1970). Most biological compounds, such as sugars, amino acids, and organic acids, are not sufficiently volatile to be separated by GC in their native state and must therefore be derivatized prior to GC separations (Gehrke *et al.*, 1987). This can be time consuming and adds to the difficulty of analyzing these compounds. HPLC separations are better suited for the analysis of labile and high-molecular-weight compounds and for the analysis of nonvolatile polar compounds in their natural form (Unger, 2002). Although GC- and HPLC-based profiling techniques are not truly quantitative, the compounds detected and their relative amounts may be compared between studies by employing the proper standards. This is in contrast to NMR techniques that can provide truly quantitative measurements, yet NMR cannot provide the sensitivity offered by MS. The high-throughput screening with GC- and HPLC-MS techniques also generates large volumes of analytical data that require advanced informatics technologies to organize vast amounts of information.

NMR techniques offer several advantages for metabolomics data acquisition. This technique requires minimal or no sample preparation, is nondestructive, and can be implemented in a noninvasive manner. Therefore, NMR is useful for studies of biofluids, for cell extracts, and for cell cultures and tissues *in vitro* or *in vivo* (Serber *et al.*, 2005). The multinuclear capabilities of NMR provide various means to observe different chemicals. Metabolomics work in NMR has mainly used ^{1}H NMR since no labeling is necessary, but other nuclides (e.g., ^{13}C, ^{31}P, ^{15}N, ^{19}F, ^{23}Na, and ^{2}H) may provide additional information about various metabolite pools in microbiology (Grivet *et al.*, 2003). The limited availability and high cost of labeled compounds can offset the usefulness of some of these nuclides. The other major limitations of NMR relate to spectral resolution and sensitivity, both of which are improved by experimentation at high magnetic field strengths. Broadened NMR spectral lines can degrade resolution and the ability to differentiate metabolite signals (Wang *et al.*, 2003). Factors influencing the NMR line widths are due to molecular dynamics and include sample viscosity, macromolecules, binding of small molecules, compartmentalization, and sample heterogeneity, which are inherent problems with cells and tissues.

Finally, metabolic analysis results in large collections of data. Data-mining techniques reduce complexity by focusing on the information content of a given data set (Fiehn *et al.*, 2000). Clustering or principal component analyses are among the main approaches that are used for data analysis (Glassbrook *et al.*, 2000). The integration of data from

metabolomics to data from transcriptomics and proteomics still represents an intriguing challenge. Private companies like Beyond Genomics and Paradigm Genetics are trying to develop comprehensive bioinformatics systems to analyze and interpret these large sets of data (Harrigan, 2002).

4. Systems biology

Systems biology endeavors to integrate diverse types of genomic, proteomic, phenomic, metabolomic, and biochemical data to create a comprehensive description of a biological system (reviewed in Alberghina *et al.*, 2005; Förster *et al.*, 2003; Patterson and Aebersold, 2003; Van Speybroeck *et al.*, 2005). The ultimate goal of systems biology is the accurate prediction of cellular behavior using computational or noncomputational methodologies. When system simulations match system observations, it can be concluded that the existing data set accurately explains the operations of the organism. Of course, attaining this perfect match is a futuristic goal (reviewed in Alberghina *et al.*, 2005; Patterson and Aebersold, 2003; Van Speybroeck *et al.*, 2005). Some success has been attained as in the detailed description of the regulatory and metabolic response to saline stress from a systems approach (Warringer *et al.*, 2003). The limitations of existing data sets and technologies, such as the persistence of genes of unknown function, are only now being realized and impede accurate simulation (Wu *et al.*, 2002). Even more comprehensive studies, such as the systematic evaluation of multiple deletants, are being undertaken (Tong *et al.*, 2001), but these endeavors are quite tedious. Depending on the regulatory mechanisms in play, there are cases with good correlation between transcriptome, proteome, and metabolome data; while in other growth conditions, this is not the case (Lafaye *et al.*, 2005).

The importance of noise in gene expression and population variation is becoming more appreciated. General principles of metabolic gene regulation are emerging from the integration of global transcript data with transcriptional regulatory mechanisms (Ihmels *et al.*, 2004). Chromatin remodeling may be a slow process (Paulsson, 2004) leading to variation in mRNA levels across a population. Increased translational efficiency, as would occur under active growth conditions, when coupled to variation in timing of transcriptional initiation, leads to an amplification of the noise in protein production patterns (Blake *et al.*, 2003). Similarly, conditions that negatively impact translational capacity, as occurs during glucose depletion in yeast (Ashe *et al.*, 2000), would also impact the influence of noise in transcript data. Often, these factors were not taken into account in experimental design. Nonetheless, this integrative systems approach is vital to understanding the deficiencies of existing analytical tools and will spur their improvement or further development.

III. FUNCTIONAL GENOMIC ANALYSIS OF WINE YEAST

Functional genomic technologies developed with laboratory strains have been applied to both commercial and native isolates of *S. cerevisiae*. In addition, these tools are being employed to better understand yeast in the baking and brewing process, and for the production of biofuels. Although in most cases no analysis of the differences in genomic structure of the analyzed strain versus the popular laboratory strain (S288C and its derivatives) has been conducted, some useful information can be culled from these studies. This is especially true if the analysis is focused on overall changes in pathway or process behavior and physiological profiling, and not on a comparative quantitative assessment of single proteins or the expression levels of individual genes. It is also important to have a solid understanding of the limits of current technologies as discussed above, in the interpretation of data across platforms and strains.

A. Transcript profiling

Transcript profiling studies of wine strains of *S. cerevisiae* have largely focused on three areas: the comparison of wine strains to laboratory strains and to each other under various growth and environmental conditions, the profiling of a ''normal'' grape juice fermentation in both synthetic media and actual juices, and the analysis of the impact of normally occurring stress conditions on the wine yeast transcriptome. In the natural environment, *S. cerevisiae* encounters several types of stress but the most common and universal are high osmolarity, nitrogen limitation, and high ethanol concentrations.

1. Comparisons of laboratory and wine strains

Functional genomic technologies have been utilized to profile genetic variation in domesticated and wild populations of *S. cerevisiae* (Fay and Benavides, 2005a,b). The oldest lineages and the greater variation were found among strains from sources not related to grapes or wine. There was surprisingly little variation among grape isolates, suggesting that these yeasts are highly related evolutionary in spite of being found at great geographical distances. Genomic analysis suggests that the wine strains are derived from wild populations of yeast (Fay and Benavides, 2005a). Variation in gene expression was also evaluated in a subset of these strains by Fay *et al.* (2004). In this study, the expression profile of nine strains grown in the presence of copper sulfate was investigated. Copper is commonly used in winemaking to remove sulfides that have been formed as a consequence of yeast metabolic activity. Over 600 genes showed variation in gene expression among these strains, with only a small subset varying in response to copper addition (Fay *et al.*, 2004).

A detailed analysis of a genetic cross between a laboratory strain and a vineyard isolate has revealed crucial information on the role of genetic architecture in naturally arising variation in gene expression (Brem *et al.*, 2002, 2005; Ronald *et al.*, 2005; Storey *et al.*, 2005). This series of studies used oligoarray transcript profiling to identify pairs of alleles displaying allele-specific differences in expression. Allelic differences were detectable by changes in the coding sequence. By using oligonucleotide arrays, it was possible to differentiate changes in expression due to regulatory effects by using tools developed to model the energy of probe-target sequence duplex formation (Zhang *et al.*, 2003). Both *cis*- and *trans*-factors were responsible for the observed variation, with cis-regulatory variation being the most common facet (Brem *et al.*, 2002). The occurrence of *trans*-regulatory differences was rarer, but with more widespread consequences for the organism (Brem *et al.*, 2002). The differential level of gene expression can then be used as a heritable trait in genetic analyses. This subsequent segregation analysis revealed that many gene expression traits are linked to two or more genes (Storey *et al.*, 2005). It was also found that the progeny of a cross of two parents may display a wider range of expression profiles than that of either parent, termed transgressive segregation (Brem *et al.*, 2002). Thus, the underlying cause of an observed difference in expression between laboratory and wine yeast, or among wine yeasts, may be complex in nature.

A variety of strains have been used in the comparative analyses of wine strains of *S. cerevisiae* to laboratory strains, most commonly to S288C as the genome of this particular strain was sequenced. In addition to strain variation, the media and growth conditions used as well as the platform differ, making comparisons across studies challenging. Only rarely have differences been confirmed using a technique such as QRT-PCR. In one study, Northern blot analysis of 49 genes found on Chromosome III was conducted comparing the common laboratory strain S288C to a commercial strain, V 5 (Rachidi *et al.*, 2000). In standard laboratory cultivation conditions, the strains were very similar in expression profiles, but in synthetic grape juice media, the commercial strain altered expression of several genes, particularly the *PAU* stress response genes, not altered in expression in the laboratory strain. Hauser *et al.* (2001) reported that sequence homology between laboratory and wine strains is extensive, but that the differences seem to have profound effects on gene expression. They found that over 40 genes were consistently changed in expression pattern in standard complex laboratory growth media, and that changes in promoter regions, number, and location of transposable elements and gene copy number were likely responsible. Another study focused on a comparison of ''flor'' strains of *S. cerevisiae*. These strains have adapted to a distinct environment and form a film or ''flor'' on the air interface of wine during sherry production (Infante *et al.*, 2003).

Multiple differences in gene copy number were found, affecting ~38% of the genome. Dunn *et al*. (2005) compared four commercial wine yeast strains to S288C. The four commercial strains displayed common as well as unique differences in expression profiles as compared to S288C. Some of these differences were again attributable to differences in gene copy number. The wine strains were very similar to each other, in spite of having been originally isolated from very different regions. The similarity may reflect the fact that commercial strains are selected for similar attributes or that wine strains are indeed quite similar to each other genomically. Many of the differences observed between the commercial strains were for expression of transporter genes. This family of genes may be particularly important in adaptive evolution to specific microenvironments.

Zuzuarregui and del Olmo (2004) examined transcript profiles in strains displaying differences in fermentative ability. Those strains with more severe fermentation problems had both higher and maintained levels of mRNA as compared to strains that were able to completely consume available sugar. The strains capable of total sugar metabolism showed an intermediate consumption of nitrogen. Additionally, strains that consumed nitrogen more quickly or more slowly had reduced expression of stress genes. Two strains were not able to adapt to the high osmolarity of the synthetic grape juice media. In this study, strains were not nutrient limited, and presumably entered stationary phase because maximal cell density was attained. The expression profile on entry into nonpermissible growth conditions showed some similarity to nutrient arrest, but represents a distinct physiological state. Another observation in this study (Zuzuarregui and del Olmo, 2004) was that the appearance of aneuploidy or polyploidy may lead to altered basal or expressed levels of gene expression that prevent certain adaptive responses from occurring.

These studies all indicate that, although there are differences between wine and laboratory strains of *S. cerevisiae*, those differences are explainable by normal processes of genetic modification and chromosomal rearrangements. It appears that the basic elements of the genome are highly conserved among *S. cerevisiae* strains with the differences largely explainable by known adaptive and evolutionary mechanisms. One study that analyzed genetic variation in a native vineyard strain of *S. cerevisiae* found far more differences among spores arising from a single isolate (Cavalieri *et al*., 2000). The strain was found to contain multiple heterozygosities, and over 6% of the genome showed a significant change in expression pattern across the different spore types obtained. The major differences occurred in protein degradation and amino acid and sulfur metabolism (Townsend *et al*., 2003). These observations suggest that genetic variation in strains in the wild may occur to a greater extent than in strains in a controlled environment, as would occur in a laboratory or in a production facility for commercial yeast products. Higher

spontaneous mutagenic rates or genome tweaking may be advantageous for the natural environment by allowing more rapid rates of adaptive evolution.

Investigation of the impact of change in growth environments among wine strains on global transcript patterns has also been analyzed. Rossignol *et al*. (2006) explored changes in the transcript profile on rehydration of commercial strains and inoculation into synthetic grape juice media. The changes observed were all predictable from studies of laboratory strain adaptations to these two types of growth environments. The rehydrated strain displays transcript profiles consistent with limitation for nitrogen and carbon under aerobic conditions (the conditions of commercial strain preparation) with a shift to a fermentative mode of metabolism on introduction into grape juice. Similarly, Roberts and Hudson (2006) examined the switch from aerobic growth on glycerol to fermentative conditions and observed similar kinds of adaptations. The conclusions reached in these studies and patterns of regulation and expression observed are in concert with known physiological adaptations observed in laboratory strains. In some cases, the details of expression of individual genes may vary, but the overall depiction of physiological activity is predictable. Of course, the danger here is that there is too strong of a reliance on what is already known about yeast biology in the interpretation of the data. Models of metabolic and regulatory behavior generated from analysis of laboratory strains appear remarkably consistent with the behavior of native and commercial isolates.

2. Assessing gene expression in the wine environment

Since *S. cerevisiae* is a domesticated microbe, grape juice fermentation represents an important adaptive environment for this yeast. In order to understand the physiological responses of this yeast to native conditions, several investigators have profiled yeast expression patterns in natural grape juices or in synthetic juice media with the goal of defining a ''typical'' profile (Backhus *et al*., 2001; Marks *et al*., 2003; Puig and Perez-Ortin, 2000; Riou *et al*., 1997; Rossignol *et al*., 2003; Varela *et al*., 2005; Zuzuarregui *et al*., 2006). Global transcript profiling has also been undertaken for brewing strains under brewing conditions (James *et al*., 2003).

In spite of the fact that different transcript profiling platforms and a range of commercial and native isolates were used, a consistent picture of the typical gene expression changes during fermentation of synthetic or actual grape juices has emerged. A typical fermentation is defined as one in which the majority of yeast growth occurs within the first 48–72 hours, following a variable lag phase. At this point, the cells are at terminal cell density and have consumed most of the available nutrients. The cells enter a nonproliferative phase, but one during which high levels of metabolic activity are maintained. As ethanol accumulates in the environment,

metabolic levels are reduced presumably due to the inhibitory effects of high ethanol. Metabolic activity continues albeit at a reduced rate until all sugar has been consumed. After exhaustion of sugar, significant loss of culture viability occurs, again most likely due to the need for sustained ATP production to counter the inhibitory effects of ethanol. Transcript profiling of each of these stages has been undertaken in order to identify key physiological markers of normal progression through fermentation.

Transcript profiling has revealed that on entry into the nonproliferative metabolically active state, there is a global remodeling of ribosomal composition, translation, and mRNA processing to adapt to the new conditions. These responses likely signal exit from active growth and occur regardless of the cause of growth cessation. As fermentation progresses, ethanol stress increases, activating a stress response. This response appears to be a graded response with a gradual decrease in the expression of genes involved in biosynthesis, and global changes in transport proteins. It is clear that the cells are undergoing a gradual and continual adaptation to the disruptive effects of ethanol. There is also an increased expression of genes involved in oxidative stress response. This may appear paradoxical, given that these fermentations are largely anaerobic. However, acetaldehyde, an oxidizing agent, is an intermediate in ethanol production and may be responsible for the need to induce these pathways. The phenolic compounds found normally in grape juice can react with oxygen to produce hydrogen peroxide. Thus, even in the absence of respiration, reactive oxygen species may be present. It is also interesting that genes in the multidrug resistance pathway are also expressed late in fermentation, perhaps the true substrate of these proteins is the phenolic constituents of grape juice. Increases in expression of genes known to be involved in ethanol tolerance are observed, as is a change in the isoforms of many of the proteins of glycolysis. Genes involved in glycogen, trehalose, and glycerol metabolism also increase in expression, and these components have been shown to be important in survival of ethanol stress. It has also been suggested that they form a futile cycle with glucose degradation to allow fine-tuning of the ATP status of the cells (Rossignol *et al*., 2003; Varela *et al*., 2005). The similarities observed in these studies establish a consistent profile of changing expression patterns throughout fermentation and provide an excellent base on which to further compare strains to associate strain behavior with strain expression profile.

Although different transcriptome platforms were used, many of these studies used the same commercial yeast, EC1118. Rossignol *et al*. (2003) examined transcript profiles for EC1118 during growth and fermentation in a synthetic grape juice media. Over 2000 genes showed a significant change in expression. The authors observed that genes in specific pathways behaved in a highly coordinated manner. Entry into stationary phase in this case was caused by nitrogen depletion of the medium.

Significant changes in the transcriptome accompanied arrest of growth; however, only 30% of the induced genes corresponded to genes reported to be induced in previous reports of the stationary phase response (Rossignol *et al*., 2003). Of the 367 genes found in common in analyses of the common stress response genes (Causton *et al*., 2001; Gasch *et al*., 2000; Gasch and Werner-Washburne, 2002), 213 were expressed during fermentation of the synthetic grape juice. This difference may be due to the simultaneous presence of ethanol stress in addition to arrest of growth. The yeasts were still metabolically active and continued to ferment, increasing ethanol levels. Thus although the initial stress factor was nutrient limitation, as fermentation continues, ethanol stress became an equally critical factor.

Although the nitrogen level used in this study was considered low by the authors, it is in fact sufficient to allow complete utilization of substrate within 5 days (Rossignol *et al*., 2003). All of the nitrogen was consumed within 48 hours in this study. It is not clear that the yeasts were in fact starving as this pattern of nitrogen consumption may be normal for these conditions. Studies with laboratory yeast under laboratory conditions of high nitrogen levels and low energy source concentrations suggest that in the typical cell cycle, sufficient nitrogen is consumed during the cell cycle to meet the biosynthetic needs for production of a daughter cell. When sufficient nitrogen is present within the cell, a new cell cycle is initiated. At the end of this cell cycle, nutrients must then be accumulated for a succeeding cell cycle to occur. Our observations of yeast in juice and juice-like conditions are inconsistent with this view. We have found similar kinetics of nitrogen consumption (Monteiro and Bisson, 1991a,b, 1992a,b); however, if the cell number is reduced via mild centrifugation, the cells that remain regrow to the same terminal cell density without further addition of nitrogen. This observation suggests that when an energy source is plentiful, indeed in great excess over the metabolic needs of the cell for growth, nitrogen accumulation may be uncoupled from initiation of a new cell cycle. Instead, nitrogen is consumed until depleted from the medium and stored inside of the cells, allowing subsequent multiple generations until cellular pools are depleted. These observations are consistent with earlier reports that nitrogen limitation alone does not lead to a quiescent state in *S. cerevisiae* (Granot and Snyder, 1993). Our conclusions regarding laboratory yeast physiology under laboratory conditions may be overly biasing interpretation of the results obtained from studies of wine yeast in juice environments.

A similar study, profiling transcripts of EC1118 using the same synthetic medium as used by Rossignol *et al*. (2003) but a different platform, SAGE, for transcript quantification was conducted by Varela *et al*. (2005). Since SAGE was used, this study was able to quantify messages not represented on commercial yeast arrays. The authors found expressed

sequences from intragenic regions as well as messages that did not match any known sequence in the S288C genomic sequence, particularly in stationary phase. However, they did not compare their analysis to the study by Kumar *et al*. (2002) that identified several similar types of non-annotated ORFs in a laboratory strain. Two other possibilities exist. Either the genome of EC1118 is not pure *S. cerevisiae* and instead represents a hybrid between species, or the laboratory strain S288C has lost a significant component of its genome, perhaps as a consequence of laboratory cultivation. Three independent commercial preparations of EC1118 were compared to S288C by Dunn *et al*. (2005), and these authors concluded that although they could not completely rule out the presence of additional genomic DNA in this strain, their analysis suggests that it is unlikely. Resolution of this issue will require sequencing of the wine yeast genome. For the genes that were represented in the S288C genome, the authors found that the majority (88.6%) were expressed at 10 copies per cell or less. This value may be below the sensitivity of array technologies, depending on how they are conducted. Many of the same gene families were identified in these two studies. Both Rossignol *et al*. (2003) and Varela *et al*. (2005) used the identical synthetic grape juice medium MS300 in addition to using the same commercial yeast strain. Varela *et al*. (2005) modified the medium by increasing the sugar concentration from 200 to 240 g/liter. Rossignol *et al*. (2003) used microarray analysis for studying the transcription profiles at six different stages during the fermentation, and Varela *et al*. (2005) used SAGE to profile transcript levels at mid-log phase, early-stationary phase, and late-stationary phase during the yeast growth and fermentation. SAGE analysis developed by Velculescu *et al*. (1995) allows identification and quantification of both known and novel gene transcripts since it is independent of the genome sequence. Both groups observed growth arrest coinciding with the depletion of assimilable nitrogen, and an increase in the expression of stress-responsive genes was observed by both methods. Similar profiles were also observed for genes involved in carbohydrate metabolism.

On comparing the data, one noticeable observation is that many of the transcripts detected as significantly different by microarray were not detected using SAGE analysis. Rossignol *et al*. (2003) found a decrease in expression of genes involved in protein, nucleotide, and amino acid biosynthesis whereas Varela *et al*. (2005) saw a decrease in transcript levels of biosynthetic pathways as fermentation progressed, but were not able to detect most of the transcripts for genes involved in amino acid biosynthesis. Similarly, genes involved in putative cell wall proteins were found to be induced over time in the microarray study but were undetected by SAGE analysis. *MET30*, a key regulator in the methionine biosynthesis pathway, was upregulated during late-stationary phase by both the methods.

The expression profiles of some of the hexose transporters were found to be inverse by the two papers. While the Rossignol group found an increase in expression of *HXT3* and *HXT7* over the time course of the fermentation, Varela *et al*. (2005) found the transcript levels of *HXT3*, *HXT6*, and *HXT7* to decrease over time. Expression of genes involved in the reserve carbohydrate biosynthetic pathways also differed in the two methods. Many of these differences reflect differences in the dynamic range of the two methods. SAGE is better able to detect abundant mRNA species. Other differences are more difficult to explain, and an independent method would need to be employed to validate the observations. However, these two methods do provide similar conclusions on the physiology of yeast at different stages of fermentation.

It is clear that a native yeast strain spends most of its life in a nonproliferative stage, as is thought to be true for most microbes (Palkova, 2004). In addition, environmental conditions rarely are conducive to growth at maximal rates as can be created in the laboratory environment. It is a common concern that analyses of growth and metabolic behavior in laboratory growth conditions have little to no bearing on understanding the physiological status of cells in their native environments. This is only true if one takes a restricted view of laboratory growth. As noted above, laboratory growth is typically energy source limited but macronutrient rich, in contrast to the natural environment of grape juice. As long as this difference is appreciated and its impact on the physiology of the cell understood, laboratory studies do indeed provide a valid framework for the analysis of strains in native environments. The nonproliferative phase of fermentation has been studied in detail by several authors. It has been compared to energy source depletion in laboratory strains, and many of the same genes are observed to be expressed. The nonproliferative phase of juice fermentation has been likened to stationary phase in laboratory strains. Indeed, many of the same genes are expressed under both conditions. Conventional wisdom holds that the absence of growth or limitation of the ability to grow is defined as stress.

Transcript profiling has revealed many features of the nonproliferative nonquiescent fermentation stage of *S. cerevisiae*. On attainment of maximal cell density, further growth ceases and fermentation rate is at its maximum (Rossignol *et al*., 2003). As fermentation continues, the fermentation rate gradually decreases. Genes associated with cell growth and amino acid biosynthesis also are increasingly downregulated as fermentation progresses, with the exception of the methionine pathway. Since this pathway is required for the synthesis of pathway intermediates, factors needed for stress tolerance, S-adenosyl methionine, and cysteine needed for glutathione, it is not surprising that expression of these proteins is maintained. Interestingly, the expression of genes required for sterol biosynthesis also gradually decreases, explaining the failure of late

oxygen additions to enhance ethanol tolerance as this is largely mediated by sterol production. There is a shift in the isoforms expressed for enzymes of glycolysis. The different isoforms may have altered function or substrate specificity, as is the case in the change of hexokinase P2 for the more fructophilic hexokinase P1, or may reflect the need for a different subcellular localization or complex. Alternately the isoforms may be more resistant to the denaturing effects of ethanol or the oxidative damage from acetaldehyde. Both Rossignol *et al*. (2003) and Backhus *et al*. (2001) found that genes involved in vitamin biosynthesis show an increased level of expression suggesting that these compounds also play a role in stress tolerance and increased expression of genes involved in nitrogen recycling. Certain heat shock proteins were dramatically induced. Interestingly, in the nitrogen-limited synthetic juice conditions (Backhus *et al*., 2001); a decrease in expression of genes involved in growth was not seen. This is again consistent with the observation that nitrogen limitation does not lead to a quiescent state (Granot and Snyder, 1993).

Several gene candidates as markers for normal fermentation progression have been proposed from these studies. The heat shock protein encoding genes: *HSP12*, *HSP26*, *HSP30*, and *HSP82*, show specific increases in expression at specific times during the fermentation as ethanol increases. *HSP12* and *HSP26* are expressed late in a normal fermentation that is accumulating ethanol (Backhus *et al*., 2001). Strains with higher basal and induced levels of *HSP12* were found to resist stress more effectively (Ivorra *et al*., 1999). *HSP12* and *HSP26* expression increased during low-temperature stress, in contrast to many other heat shock genes, and may therefore represent generic markers of cellular stress response (Sahara *et al*., 2002). *HSP30* appears to be expressed to a greater extent in nitrogen limited than in nitrogen sufficient fermentations (Backhus *et al*., 2001). It may be challenging however to develop absolute measures of expression of these genes that would indicate either normal or aberrant stress response was occurring due to differences in basal levels of expression.

3. Analysis of stress responses in wine yeast

One of the major areas of research interest in wine strains of *S. cerevisiae* is the analysis of response to stress. This focus is motivated by both practical and fundamental interests. The production of wine imposes both biotic and abiotic stresses on the yeast. The principal stresses encountered are high osmolarity, high ethanol, extremes of temperature, nutrient limitation, and presence of inhibitory metabolites (Bisson, 1999). Genomic analysis of the response to each of these types of stress has been conducted (Alexandre *et al*., 2001; Aranda and del Olmo, 2004; Backhus *et al*., 2001; Erasmus *et al*., 2003; Kuhn *et al*., 2001; Marks *et al*., 2003; Rep *et al*., 2000; Rossignol *et al*., 2006; Sahara *et al*., 2002). Several

excellent reviews on the yeast stress response have been published (Gasch, 2003; Gasch and Werner-Washburne, 2002; Gray *et al.*, 2004; Siderius and Mager, 2003).

The existing environmental growth conditions can profoundly influence the stress response (Siderius and Mager, 2003). Plasma membrane composition at the time of stress can impact detection of stress and signal transduction, and the availability of nutrients can be important for the synthesis of stress response factors. Even under permissive growth conditions, the stress response on rich media (YPD) varies from that on minimal media (YNB) (Siderius and Mager, 2003). Environmental conditions may also mitigate a stress response. Davidson and Schiestl (2001) found that cells were more heat tolerant under anaerobic conditions than under aerobiosis. This observation suggests that a primary consequence of heat exposure is the release of a reactive oxygen species that accompanies disruption of metabolically active mitochondrial membranes.

Studies of arrest of growth in laboratory strains, typically due to energy source depletion, has led to the identification of a set of genes expressed in stationary phase. Many of these genes are expressed on exposure to stress as well as a DNA element, the stress response element (STRE), which has been identified in the coding regions of many of these genes (Kobayashi and McEntee, 1990, 1993; Marchler *et al.*, 1993). Many, but not all, of the genes with STREs are expressed in the later stages of fermentation (Puig and Perez-Ortin, 2000). Gasch *et al.* (2000) in a detailed study of responses to a series of stress situations, including temperature shock, osmotic shock, nitrogen depletion, and the presence of various drugs and inhibitors, defined elements of the ''environmental stress response'' (ESR). Approximately 900 genes displayed a similar response, either increase or decrease in expression, across all stress conditions. Approximately 600 genes decreased in transcript level, either due to repression or increased message turnover rates. The majority of these genes (70%) are involved in protein synthesis (Gasch, 2003). The remaining genes are associated with cell growth. Of the roughly 300 genes showing an increase in transcript level, either due to increased expression or message stabilization, 45% are genes of unknown function (Gasch, 2003). The remaining genes impact carbohydrate metabolism (primarily in the change of isozyme species produced), detoxification of reactive oxygen species and mitigation of oxidative damage, metabolite transport, protein folding and degradation, DNA repair, and cytoskeletal reorganization. In addition, there were specific patterns of expression unique to each type of stress. Other studies of stress responses in laboratory and wine strains have largely found identical results (Causton *et al.*, 2001; reviewed in Gasch, 2003; Gasch and Werner-Washburne, 2002). In general, the imposition of stress results in a transient adaptive phase to the new growth conditions or results in transit of the yeast from growth to

a nonproliferative state. When the cells are returned to permissive conditions, the transcript profile returns to the nonstressed state (Gasch and Werner-Washburne, 2002). The principal transcriptional regulators involved in the general ESR are Msn2p and Msn4p and the heat shock factor Hsf1p (Boy-Marcotte *et al.*, 1998, 1999).

The ESR is a graded response. The primary goal of the response is to allow adaptation to the new growth conditions to maintain optimal cellular performance or, failing that, to equip the cell for entry into a true resting stationary phase (Figure 4). Thus, the overlap with changes observed during the progression of fermentation is to be expected. In this case, there is a gradual, yet steady, increase in the level of stress and the cell's ability to acquire tolerance and continue growth or, at later stages, metabolism (Figure 4).

Regardless of the nature of the stress, some of the cellular consequences are identical, so it is not surprising that there is a common set of gene expression changes in the response to stress. The imposition of stress results in the inability to continue to function in the existing physiological state. The first phase of this response would then be to mitigate the effects of the stress by ceasing transcription and translation, DNA synthesis, arresting protein localization and organelle biogenesis, and redirecting available resources. The initial aim of the stress response is to repair damage; to restabilize cellular structures, membranes, metabolite gradients, and pools; and to acquire tolerance of the new condition in

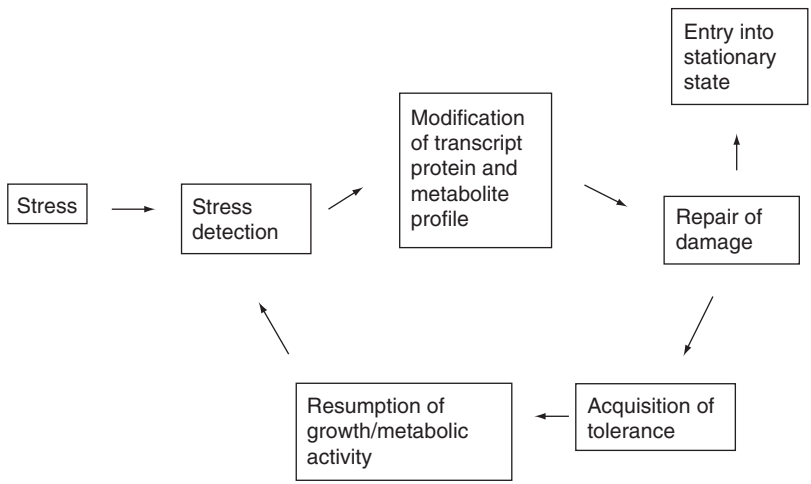

FIGURE 4 Cyclical nature of the adaptation to stress during wine fermentation. Cells continually monitor and adapt to changing environmental conditions with the goal of restoring a permissive growth or metabolic state. When acquisition of tolerance is no longer possible, cells will enter a resting or quiescent state.

order to recommence growth and/or metabolism. Alternately if the stress is severe, the aim is to attain a resting or hibernating state that protects cellular functionality and viability at the expense of growth.

There appears to be a delicate balance between loss of ability to proliferate and expression of ESR genes with varying consequences. For example, one key component of the ESR is to express genes involved in synthesis of glycogen, glycerol, and trehalose. These components may serve as energy reserves or alternately fine-tune ATP levels based on a dynamic fluctuation between glycolysis (ATP generation) and energy reserve compound synthesis (ATP consumption). These compounds, however, have also been shown to play roles in stabilization of cellular structures due to the change in temperature (stabilize membranes), loss of water (osmotolerance), or loss of water of hydration (ethanol tolerance). Trehalose binding to proteins has been shown to protect these molecules against oxidative damage (Benaroudj *et al.*, 2001). Such interactions would also be predicted to limit function of the macromolecule affected. Thus, restoration of growth may require elimination of these protective factors in order to increase rates of cellular activity. The observation that both enzymes of trehalose and glycogen synthesis and degradation are induced simultaneously may not indicate the need for fine-tuning of the ATP pool, but for maintenance of a dynamic state between a protected (sugar bound) and fully active (no sugar) state of proteins and macromolecular structures. Conditions that lead to enhanced tolerance of stress factors tend to reduce growth capacity. Different types of stationary phase have been identified in yeast (Drebot *et al.*, 1990). These authors found, by using a specific mutation that resulted in death in true stationary phase, that there is a distinct difference between arrest of growth and entry into the classically described stationary phase. The physiological properties of arrested cells are a function of the conditions leading to arrest.

The genotype may influence the type of response that can be mounted by the cell. How the cell responds to stress is also dependent on previous growth conditions and available nutrients and substrates. For example, in the presence of oxygen, nitrogen limitation leads to the expression of genes involved in respiration, suggesting that nitrogen limitation places a greater demand on the cell for ATP or limits glucose fermentation such that respiration is needed to provide for cellular energy needs. Our analysis of nitrogen limitation in a synthetic grape juice medium also demonstrated an increase in expression of genes involved in respiration in spite of the very high glucose concentrations remaining in the medium, but there was no general relief of glucose repression (Backhus *et al.*, 2001). Stress response genes were elevated in both the nitrogen-sufficient and nitrogen-limited cultures, in the former perhaps because of ethanol stress and the latter due to nutrient limitation. In general, the response to ethanol observed in the nitrogen-sufficient culture was not seen under

nitrogen-limitation. This may be because the cells had not attained a high enough ethanol concentration, or that the absence of nitrogen prevented adaptation to ethanol. In any event, respiration appears to be more nitrogen conserving than fermentation.

In a parallel study, nitrogen in the form of diammonium phosphate was added during fermentation of white grape juice at the point of entry into stationary phase and the transcript profile changes evaluated (Marks *et al.*, 2003). Approximately 350 genes changed in expression with roughly half increasing and half decreasing in expression. Not surprisingly, many of the genes increasing in expression were associated with active growth while those that decreased were associated with alternate nitrogen source use and the stress response.

Two critical stressors that have been examined in industrial yeasts are the response to osmotic stress and to ethanol. Osmotic stress leads to an increase in expression of the glycolytic and pentose phosphate pathways and a decrease in expression of genes involved in biosynthesis (Erasmus *et al.*, 2003; Rep *et al.*, 2000; Zuzuarregui *et al.*, 2005). In one study, osmostress tolerance was shown to be affected by mutations in the genes required for adenine biosynthesis (Ando *et al.*, 2005). However, expression of these genes decreases in the transcriptome analysis. This discrepancy is likely due to the need to make ATP and assure sufficient energy balance for the cell under these conditions such that inability to synthesize adenine would be problematic. In contrast, since the growth rate of the cell was reduced, high levels of expression of these genes were not required. Another study involving the comparison of transcriptional response to saline stress between laboratory (FY834) and brewing yeast (IFO2347) strains found that an addition of up to 1M NaCl to complete rich media (YPD) led to an increased expression of genes involved in stress response, carbohydrate metabolism, and energy metabolism (Hirasawa *et al.*, 2006). In addition, the brewing strain displayed increased expression of genes in vitamin metabolism, glycerol synthesis, sodium ion efflux pump, and copper binding as compared to the laboratory strain. These genes are all speculated to be responsible for the quick adaptation of the brewing strain to high concentrations of NaCl as compared to the laboratory strain (Hirasawa *et al.*, 2006).

In wine strains, the response to increasing ethanol is quite complex. This is because ethanol rarely increases in the absence of other stress factors, such as nutrient limitation, and is accompanied by acetaldehyde production which is itself a stress factor (Aranda and del Olmo, 2004). Short-term ethanol stress has been evaluated in an attempt to dissect the responses specific to this compound (Alexandre *et al.*, 2001). Approximately 3.1% of the transcripts analyzed increased in expression in response to exposure to ethanol. Of these genes, 49.4% belong to the ESR genes; however, this represents only 73 of the 300 known ESR genes.

An additional 14.4% of the genes increasing in expression have other known roles in response to stress. Genes involved in energy production, protein localization, and ion homeostasis also increase in expression. Genes decreasing in expression are associated with growth and biosynthesis.

We have examined the effect of simultaneous imposition of heat and ethanol stress, as this is a common occurrence in commercial wine production, and yeast strains differ in their ability to tolerate both stressors simultaneously. In spite of crossover tolerance seen for ethanol and heat (Gasch, 2003), the presence of ethanol adversely affects temperature tolerance under winemaking conditions (reviewed in Bisson, 1999). We evaluated the effect of a moderate temperature shift, from 25 to 30 °C, at 8% and 11% ethanol for three commercial strains, UCD904, UCD522, and UCD2032 (Nugent, 2004). Two strains, UCD522 and UCD2032, completed fermentation regardless of the shift in temperature. UCD904 was able to complete fermentation in the absence of a temperature shift, but arrested sugar utilization at the higher temperature. Interestingly, nearly 70% of the differences that we observed between strains were in genes of unknown function. All three strains were less transcriptionally active at 11% ethanol. In a parallel study (Mangahas, 2003) using two different strains, UCD905 and UCD2031, there were clearer differences between the temperature-tolerant and less-tolerant strains. The more tolerant strain had both higher basal levels of expression of stress genes and a higher level of a stress response.

One of the important issues in interpretation of these types of studies is to understand the reason for the observed changes. Strains with high basal levels of expression of some stress genes show a stronger stress tolerance than strains with lower basal levels; yet if stress is imposed, the strains with the low basal levels will show the strongest induction, but not necessarily a stronger tolerance. It is likewise important to consider if the change in expression is needed to maintain a steady state of protein rather than relative protein concentration changing. In this case, the change in expression may appear to be coregulated with the stress response, but this is coincidental. Protein turnover rates and efficiencies of translation may also differ between environmental conditions, and changes in expression may occur simply to counter these effects (Kuhn *et al*., 2001; Sahara *et al*., 2002; Stahl *et al*., 2004).

B. Proteomics

Proteomic analysis has been applied to laboratory, commercial, and native wine yeast strains predating completion of the sequence of the yeast genome, but not to the same extent as transcriptome profiling (Norbeck and Blomberg, 1997; Pardo *et al*., 1999). The studies conducted

can be divided similar to transcript profiling: comparison of strains, analysis of the normal proteome of fermentation, and the presence of stressors in the environment. In this latter case, one major goal has been to identify key proteins that could then be used in a rapid assay, such as an immunochemical assay, to monitor the status of fermentation. An alternate goal is to use protein or peptide profiles to monitor the microbial complexity of fermentation, although this has been more strongly pursued in brewing strains (Dowhanick *et al*., 1990). The lack of studies on wine proteomics is likely due to the fact that the majority of proteins detectable on the gels are involved in glycolysis and that the major focus in yeast proteomics remains technique development and optimization (Joubert *et al*., 2001; Kobi *et al*., 2004).

Comparisons of wine and laboratory yeast strains has identified electrophoretic protein variants unique to wine strains (Brousse *et al*., 1985). Comparative profiling of two wine strains with different fermentation properties has also been undertaken (Zuzuarregui *et al*., 2006). This study included transcript profiling as well. The two strains selected had similar nitrogen consumption profiles, growth rates, and attained similar levels of biomass. However, one strain, ICV27, was unable to complete the fermentation leaving high residual levels of sugar while the other, ICV16, had no difficulty finishing fermentation. At the point of entry into stationary phase, ICV27 displayed a decrease in fermentation capacity and glucose consumption eventually leading to arrest of sugar uptake. Although different sets of genes and proteins were observed in the transcript and protein profiling, the overall conclusions reached from each analysis were similar. The ICV16 strain showed higher expression of genes and proteins involved in carbohydrate metabolism, telomere maintenance, cell wall and sterol metabolism, and increased expression of stress factors associated with protein degradation. These changes are all predicted from a strain that is normally progressing in fermentation. In contrast, the ICV27 strain showed greater levels of expression of genes involved in gluconeogenesis, ATPase activity, oxidative and osmotic stress, and transporter proteins, particularly those for amino acids. This profile is quite informative. It suggests that this strain is struggling to deal with excess proton entry into the cell, a hallmark of ethanol intolerance, and increased oxidative damage likely due to increased levels of acetaldehyde. Although there was no congruence between the transcripts showing the most dramatic effects and the proteins, independent analysis of both sets of data would lead to the same physiological conclusions. Trabalzini *et al*. (2003) also profiled changes in the proteome as cells entered stationary phase under enological conditions. They observed three classes of proteins: repressed, induced, and autolyzed. They most frequently observed that protein spots were either present or absent, not reduced or increased in expression. The largest changes in expression were for proteins of glycolysis. In our studies

of wine strain proteomics (Cooney, 2003; Olineka *et al.*, 2005; Weiss and Bisson, unpublished observations), we have observed a similar trend. As fermentation progressed, there was a change between isoforms of glycolytic enzymes as well as the appearance of specific degradation products. Our analysis suggested that these degradation products can be quite stable over several days of the fermentation, suggesting that continued protein degradation has been impaired or that these protein fragments play some other role in the cell, perhaps as a storage form of nitrogen to augment the vacuole. We found specific degradation fragments from the C-terminal end, the N-terminal end, and both ends using MALDI-TOF MS (Weiss and Bisson, unpublished observations). Larsen *et al.* (2001) investigated the appearance of multiple forms of enolase during fermentation and found identical results to that of our own. They reported 11 forms of enolase appearing during fermentation with modifications other than common degradation.

Imposition of sulfur stress through the inclusion of cadmium in the medium has also been explored (Fauchon *et al.*, 2002). In this case, the proteome was modified to switch between glycolytic protein isozymes to produce versions with less S-containing amino acids. This study suggests that one role of isozymes may be to maintain metabolic rates in the presence of nutrient limitation. Proteome analysis has also been used to evaluate the response to sorbic acid stress (De Nobel *et al.*, 2001), a compound used to stabilize wine against further yeast growth postfermentation. These authors also conducted transcript profiling and concluded that both analyses provided unique insights into the physiological processes disrupted in the yeast strains.

C. Metabolomics

One of the most seminal metabolomics papers concerning laboratory yeast was published a few years ago in *Nature Biotechnology*. Raamsdonk *et al.* (2001) established a technique to reveal the phenotype of silent mutations in the yeast genome. They have developed functional analysis by coresponses in yeast (FANCY), which is based on the measurement of the steady-state response of two variables to the change of a system parameter, in this case, the change in concentrations of two metabolites in response to a mutation. The lack of an observable phenotype, such as a change in growth rate, due to a mutation can be explained by the presence of another gene (or genes) in the genome that can substitute for its function. The premise for this approach is that similar responses can be produced due to mutations in the cell that have the same functional response. Therefore, matching the metabolic profile of known gene mutations with those associated with genes of unknown function can reveal the function of unknown genes.

The authors used two deletion mutants in the glycolysis pathway to demonstrate this powerful technique. The two mutants without growth-rate phenotypes showed metabolic phenotypes when concentrations of six metabolites were measured (Raamsdonk *et al.*, 2001). To analyze all the metabolites in the cell at the same time, they used NMR spectroscopy and found that changes in the concentrations of metabolites were similar in these two mutants. The authors concluded that mutant strains containing defective genes involved in similar pathways displayed metabolite profiles that could be clustered together using principal component analysis.

Another approach for the identification of individual metabolites in yeast was undertaken by Mashego *et al.* (2006). The authors used specialized perturbation experiments to elaborate the kinetics of metabolism and identify targets for metabolic engineering in laboratory yeast, but used classic techniques to differentiate between intracellular and extracellular metabolites. The authors found that when the chemostat conditions were varied from aerobic to anaerobic, there was a large effect on the glycolytic flux. In addition, the authors critically evaluated several different sampling techniques in a previous paper (Mashego *et al.*, 2003) and found that quenching metabolism by exposing the cells to precooled stainless steel beads worked the best.

There has been a growing application of metabolomics to wine yeast and fermentation as more people have recognized the power of these techniques. One application of metabolomics has been to study the interactions of yeast during fermentation. Howell *et al.* (2006) used metabolic profiling to study how multiple strains of *Saccharomyces* spp. grown together in grape juice affect the flavor and aroma compounds in the fermented wine.

Another interesting application of metabolomics to wine yeast was the investigation of the central carbon metabolism in yeast during fermentation (Camarasa *et al.*, 2003). Alcoholic fermentation takes place in almost complete absence of oxygen and in the presence of a large glucose concentration. As a consequence, many enzymes are repressed and the carbon flux through the tricarboxylic acid (TCA) pathway is severely reduced. Using [3–^{13}C]-aspartate as the sole nitrogen source, these authors showed that the TCA cycle is in fact interrupted. Malate and succinate were both labeled on C2 and C3, consistent with a synthesis by the reductive branch of the TCA cycle (oxaloacetate to malate and succinate). When [3–^{13}C]-glutamate is used as a substrate, only labeled succinate is produced by the oxidative branch of the cycle (through ketoglutarate). NMR evidence alone did not pinpoint the site of interruption, but comparison with mutants proved that the succinate dehydrogenase complex is in fact inactive (Camarasa *et al.*, 2003).

The metabolomic techniques described so far are even easier when applied to extracellular or secondary metabolites to gain information on

yeast from the medium. Allen *et al*. (2003) employed noninvasive, mass spectrometric monitoring to measure metabolites in spent culture medium. The authors found that this ''metabolic footprinting'' allowed them to distinguish between different physiological states of yeast growth and even between different yeast single-gene deletion mutants.

This line of research has also been applied to the analysis of musts for brewing and wine yeast fermentations. Secondary metabolites play a large role in the quality of the final fermented product. For example, bitter acids are ubiquitous in beer and contribute to astringency, while esters and higher alcohols are desirable metabolites in wine that markedly influence flavor and depend on the presence of precursor compounds in the grape must. Kaukovirta-Norja *et al*. (2004) studied the effects of germination of oat and barley on the metabolite content of grains used in beer fermentation. One of the main goals of germinating, or ''malting,'' these grains is to produce nutrients for brewing yeast, but this process also releases secondary metabolites that have flavor- and color-enhancing effects. Metabolomics can be used in this capacity to analyze beer worts in preparation of brewing and to glean information on how the wort affects the final product.

IV. CONCLUSIONS

Functional genomic technologies have been productively applied to the investigation of both commercial and native wine yeasts under a variety of conditions. Although the specific details of which genes or proteins may be changing in concentration and relative levels may differ, the physiological depiction that emerges is consistent. Wine strains are similar in gene regulation and protein expression patterns to laboratory strains, as would be predicted from the similarity of their genomes. Differences that have been observed are due to the natural processes of mutation and genome rearrangement, phenomena that underlie adaptive evolution to restricted microenvironments. The importance of *cis*- and *trans*-regulatory variation in gene expression has been documented suggesting that a ''typical'' expression profile will vary depending on the specific changes in genetic architecture inherited by a given strain.

The behavior of yeast in commercial environments is largely predictable from the vast data available for laboratory strains, a fact that is really not all that surprising. The physiological profile during fermentation of grape juice changes from an initial adaptive response to high-sugar conditions, active growth, arrest of cell proliferation, and a graded stress response as nutrients become limiting and ethanol accumulates in the environment. Strains that show a deficiency in growth or fermentation in the native environment largely fail to undergo this adaptive regime. In general, strains with higher basal levels of expression of genes associated

with the stress response survive and tolerate stress in the environment more effectively than strains with lower levels of expression. However, strains with high basal levels of stress gene expression do not initiate growth quickly, thus explaining the persistence of such genetic diversity in the wild.

Functional genomic technologies will greatly aid in further study of the biological properties of wine yeast. Of the 6604 ORFs in the *Saccharomyces* genome, about 20% of the gene products are of uncharacterized function (www.yeastgenome.org). Many of these genes of unknown function appear to be expressed during growth and metabolism of wine strains, and wine strains may therefore be exploited to define the role of these genes in the biology of the organism. Genes important to flavorant production will be able to be identified, and metabolism will be understood at a more detailed level. Equally important will be the understanding of the ecology and evolution of *Saccharomyces*, and the forces that drive genetic change and exchange.

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