Assessing the Occurrence and Mechanisms of Horizontal Gene Transfer during Wine Making

by

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Declaration

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Summary

Saccharomyces cerevisiae is the most commonly used organism in many fermentation-based industries including baking and the production of single cell proteins, biofuel and alcoholic beverages. In the wine industry, a consumer driven demand for new and improved products has focussed yeast research on developing strains with new qualities. Tremendous progress in the understanding of yeast genetics has promoted the development of yeast biotechnology and subsequently of genetically modified (GM) wine yeast strains. The potential benefits of such GM wine yeast are numerous, benefitting both wine makers and consumers. However, the safety considerations require intense evaluation before launching such strains into commercial production. Such assessments consider the possibility of the transfer of newly engineered DNA from the originally modified host to an unrelated organism. This process of horizontal gene transfer (HGT) creates a potential hazard in the use of such organisms. Although HGT has been extensively studied within the prokaryotic domain, there is an urgent need for similar studies on their eukaryotic counterparts. This study was therefore undertaken to help improve our understanding of this issue by investigating HGT in a model eukaryotic organism through a step-by-step approach. In a first step, this study attempted to determine whether large DNA fragments are released from fermenting wine yeast strains and, in a second step, to assess the stability of released DNA within such a fermenting background. The third step investigated in this study was to establish whether "free floating" DNA within this fermenting environment could be accepted and functionally expressed by the fermenting yeast cultures. Finally, whole plasmid transfer was also investigated as a unified event. Biofilms were also incorporated into this study as they constitute a possibly conducive environment for the observation of such HGT events.

The results obtained during this study help to answer most of the above questions. Firstly, during an investigation into the possible release of large DNA fragments (>500 bp) from a GM commercial wine yeast strain (Parental strain: Vin13), no DNA could be detected within the fermenting background, suggesting that such DNA fragments were not released in large numbers. Secondly, the study revealed remarkable stability of free "floating DNA" under these fermentation conditions, identifying intact DNA of up to ~1kb in fermenting media for up to 62 days after it had been added. Thirdly, the data demonstrate the uptake and functional expression of spiked DNA by fermenting Vin13 cultures in grape must. Here, another interesting discovery was made, since it appears that the fermenting natural grape must favours DNA uptake when compared to synthetic must, suggesting the presence of carrier molecules. Additionally, we found that spiked plasmid DNA was not maintained as a circular unit, but that only the antibiotic resistance marker was maintained through genomic integration. Identification of the sites of integration showed the sites varied from one HGT event to the next, indicating that integration occurred through a process known as illegitimate recombination. Finally, we provide evidence for the direct transfer of whole plasmids between Vin13 strains.

The overall outcome of this study is that HGT does indeed occur under the conditions investigated. To our knowledge, this is the first report of direct horizontal DNA transfer between organisms of the same species in eukaryotes. Furthermore, while the occurences of such events appears low in number, it cannot be assumed that HGT will not occur more frequently within an industrial scenario, making industrial scale studies similar to this one paramount before drawing further conclusions.

This dissertation is dedicated to Denise, my parents and my grandparents.
Hierdie proefskrif is aan Denise, my ouers, my ouma en oupa opgedra.

Biographical sketch

Desiré Barnard was born in Port Elizabeth, South Africa on 18 May 1979. She matriculated from Vredenburg High School in 1996. Desiré enrolled at Stellenbosch University in 1997, obtaining a BSc-degree in Microbiology, Biochemistry and Genetics in 2000. The degrees HonsBSc (Medical Biochemistry) and MSc (Medical Biochemistry) were subsequently awarded to her in 2001 and 2004. Her master's thesis was entitled, "*Nucleotide sequence variation and expression levels of TP53 in cancers of the upper gastro-intestinal tract*".

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Preface

This dissertation is presented as a compilation of 4 chapters. Each chapter is introduced separately.

Chapter 1	**General Introduction and project aims**
Chapter 2	**Literature review** Horizontal Gene Transfer and Genetic Modification
Chapter 3	**Materials and Methods Research results**
Chapter 4	**Research Results** Horizontal Gene Transfer in wine fermentation environments
Chapter 5	**General discussion and conclusions**

Contents

Chapter 1

General introduction and project aims

General introduction and project aims

1.1 PREFACE

This dissertation deals with the question of horizontal gene transfers in a eukaryotic system. More specifically, our intention was to investigate the possibility of such transfers during a specific industrial process, namely alcoholic wine fermentation. The study took a systematic approach to this question by assessing each individual step that could be assumed to contribute or be directly involved in such a transfer. As expected, the null hypotheses were mostly proven true, therefore it was decided to present this thesis in a traditional format, with a literature review serving as introduction, followed by Materials and Methods, a Results section with discussions, and a final chapter with General Discussion and Conclusions. Considering the significant interest of the data, this thesis will serve as a basis for one review and two publications, all of which are currently in preparation.

1.2 INTRODUCTION

Saccharomyces cerevisiae, often referred to as “brewer’s yeast” (9), “baker’s yeast” (12), or “wine yeast” (4), is a yeast species used in modern day fermentation industries. In the wine industry, this species represents almost all commercially available starter cultures, although a recent trend has seen the arrival of several non-*Saccharomyces* yeast on the market (1,11,16). As the worldwide consumer demand for more consistent quality and diversity among wines increases, wine makers and researchers alike are constantly driven to identify or generate new yeast strains that would possess desired qualities such as improved fermentative abilities and/or yield a final product with enhanced sensory qualities and added health benefits. The recent advances in *S. cerevisiae* research has provided an improved understanding of its genetics and phylogenetics, allowing for the development of genetic tools enabling remarkable progress in yeast biotechnology. One such example is the genetically modified (GM) yeast strain, ML01, currently in use on a commercial scale in Moldavia, USA and Canada, capable of both alcoholic and subsequent malolactic fermentation (5).

The potential benefits in using such GM yeast are significant, directly affecting both wine makers and consumers. However, before launching such GM strains into commercial production, the safety of their use requires intense evaluation. Such an assessment takes into account the possibility of a transfer of such genetically engineered DNA from the originally modified host organism to a new host organism. This process is known as lateral gene transfer (LGT) or horizontal gene transfer (HGT) (6,15) and poses a potential risk in the use of any genetically modified organism.

Although the phenomenon of HGT is well understood and characterized within the prokaryotic world (10), there is an urgent need for similar studies within their eukaryotic counterparts. This study was therefore aimed at addressing this issue by investigating such occurrences in a step-by-step fashion, initially determining whether release of large fragments of DNA from wine yeast is a common occurrence during alcoholic wine fermentation and secondly determining the stability of such released DNA within a fermenting background. Considering the technologies applied in practice during wine making today, the combination of mechanical damage and long term exposure of the yeast cultures to an environment high in ethanol

concentration presents the probability of yeast autolysis and subsequent DNA release into the environment (17). The third aspect addressed by this study was whether “free floating” DNA within such an environment could be accepted and functionally expressed by yeast strains during wine making, either through natural transformation, altered recombination or other processes as yet unknown. The conditions experienced by the yeast cultures present during fermentation (high temperature, high ethanol concentrations and direct cell-to-cell contact) are believed to potentially impact on the occurrence of such events (3,7,8,14). Finally, whole plasmid transfer was also investigated as a unified event. In addition, biofilms were incorporated into this study as a theoretically conducive environment for investigating the occurrence of such HGT events. As yet, very little is known regarding the dynamics of *S. cerevisiae* biofilms, however, the characteristics of biofilm organization provide an ideal environment for the occurrence of such events, based on the close physical proximity and direct competition for nutrient availability that are experienced by cells within such biofilm structures (13).

Whilst bearing in mind that yeast are eukaryotic organisms, they are also single celled and thus may not reflect the true complexity involved in eukaryotic HGT. Nevertheless, the data should provide a basis for establishing yeast as a model organism for the investigation of such HGT events on a cellular level, with respect to eukaryotic organisms. Additionally, the results obtained from this study will potentially directly bear on the use of GM yeast on a commercial scale.

1.3 PROJECT AIMS

It is clear that the use of GM organisms holds great potential, on condition that the necessary risk assessment trials have been successfully completed. The focus of this study was to lay the foundation for risk assessment related to GM yeast in the wine environment. We aimed to investigate the occurrence of, and mechanism involved in HGT between a GM *S. cerevisiae* and wild type *S. cerevisiae* present during alcoholic fermentation.

The central questions addressed in this study include:

1. Does *S. cerevisiae* release DNA during alcoholic fermentation?
2. How stable is such released DNA within the fermenting medium?
3. Can released GM DNA be accepted and functionally expressed by *S. cerevisiae* during alcoholic fermentation?

In this study, and using *S. cerevisiae* strains specifically designed to allow for simplified screening, these issues were investigated under standard wine making conditions. Additionally, in an attempt to drive the system to a theoretical threshold were HGT would occur, this study included additional selected stress conditions which were imposed on fermenting cultures.

Results obtained from this study provide the first direct evidence regarding the occurrence and possible mechanism involved in HGT between *S. cerevisiae* strains during alcoholic fermentation as a part of wine production. Not only will essential answers be provided to the wine industry regarding the safety of using GM yeast for wine production, but a basis for establishing yeast as a model eukaryotic system for investigating HGT can be developed.

Biofilms also provide another theoretically advantageous environment for such HGT events to occur (13). The mechanisms of yeast biofilm formation are less well understood than bacterial biofilms, but it is believed that adhesins are responsible for the attachment of cells to various abiotic (including polystyrene plates, polyprophyle and polyvinyl chloride) and organic

surfaces (tissues and cells) (12). This study therefore also aimed at assessing the probability of HGT events occurring within a *S. cerevisiae* biofilm environment, based on the observation that biofilms are naturally established in the winery equipment (2).

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Chapter 2

Literature review

Horizontal Gene Transfer and Genetic Modification

Literature Review

2.1 GENERAL BACKGROUND

By definition, horizontal gene transfer (HGT), also known as lateral gene transfer (LGT), is the transfer of genetic material from one cell to another cell that is not its progeny. This differs from the conventional passing of genetic information from parent to offspring, referred to as vertical gene transfer (70). The best known example of HGT is the endosymbiotic theory, describing the origin of several of the eukaryotic organelles by postulating that a prokaryotic cell which had lost its cell wall and gained the ability to phagocytose other bacteria engulfed a bacterium which gave rise to what is today known as the mitochondria (75). While some may argue that this is not an example of true HGT but rather a fusion of two organisms, the definition of HGT encapsulates this example, since one organism's DNA content has successfully been incorporated into another unrelated organisms' cellular constitution through a non-sexual event.

The first direct observation of HGT, however, was published by a research group in Japan in 1959, showing antibiotic resistance transferred between unrelated bacterial species (6,68). This discovery would not only contribute significantly to our current understanding of evolutionary mechanisms but also form the core of the technologies that underlie modern day genetic engineering (89).

2.2 THE STUDY OF HGT

Natural gene exchange between bacteria has been found to occur frequently, driving evolution by gaining and sharing new metabolic abilities (22). Since the late 1990s, the availability of whole genome data has confirmed the suspected involvement of HGT (90). Continuous advances in molecular tools are constantly providing data implicating HGT to occur more frequently than previously believed, between both related and unrelated species, providing alternative possible solutions to many puzzling phenomena observed in various fields of biology.

HGT has been extensively researched and is well understood within the prokaryotic group where it is known to occur through 3 major mechanisms, i.e. transformation, transduction and bacterial conjugation (16). Briefly explained, transformation involves the uptake and expression of foreign DNA from within the surrounding media, transduction involves the transfer of genetic material facilitated by phages (bacterial viruses) and conjugation involves a transfer mediated by cell-to-cell contact through pili (Figure 2.1) (75).

Transformation refers to free-floating DNA (from any donor organism) within a given medium being accepted by another living cell and subsequently being functionally expressed by this new host (87). Of particular interest in this regard was the discovery of small, circular, auto replicating genetic elements capable of existing independently of the host genome that were found to occur naturally within several organisms (75). Today, these elements are referred to as plasmids and form the base technology of much of modern r. Other cases of transformation can also lead to chromosomal insertion through homologous or non-homologous recombination (17). Furthermore, a phenomenon known as illegitimate recombination has also been identified whereby a single short homologous DNA sequence is required for the insertion of large blocks of DNA (23).

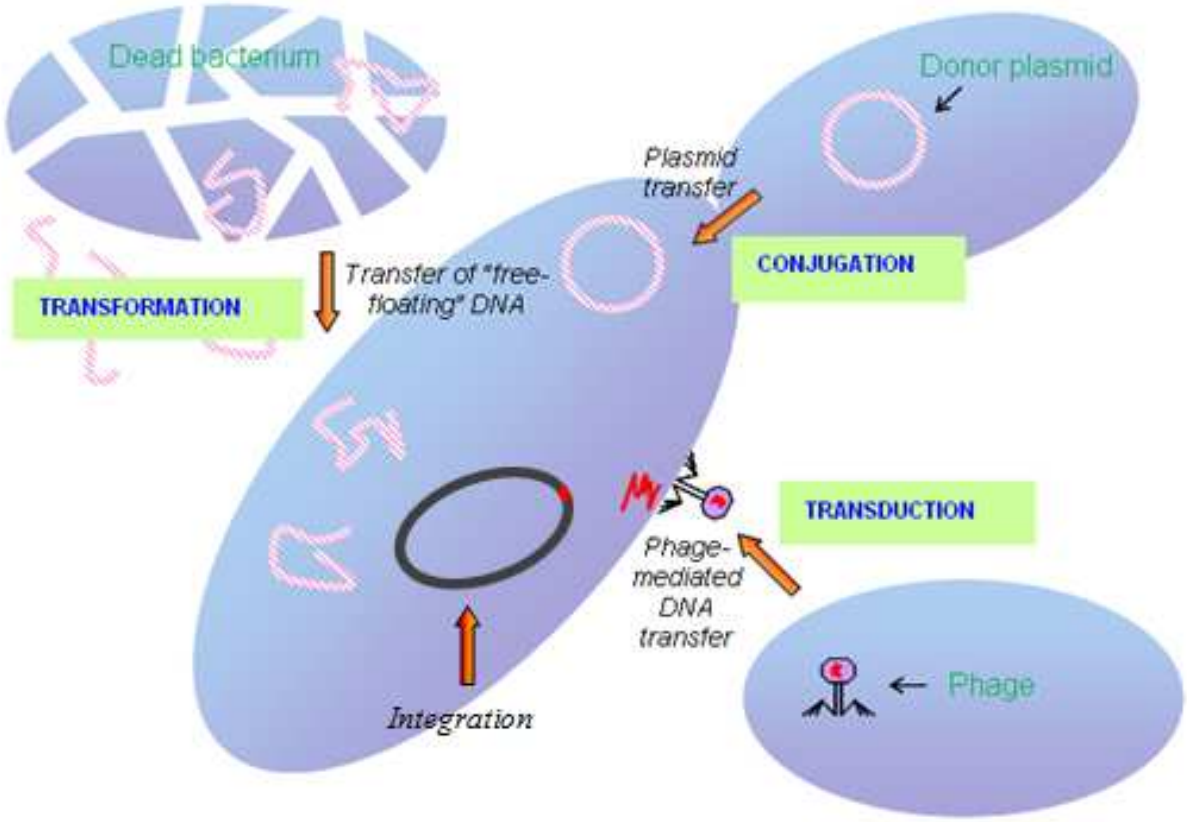

Figure 2.1 Horizontal gene transfer as observed in bacteria; demonstrating transformation, conjugation and transduction. *(Adapted from http://www.scq.ubc.ca/attack-of-the-superbugs-antibiotic-resistance/).*

Transduction is defined as the transfer of bacterial genetic information through the involvement of bacterial viruses, known as bacteriophages. The bacteriophage structure encapsulates viral DNA with an outer protein coat. Upon infection of the host bacterium, the phage hijacks the host by injecting its nuclear content into the bacterium and utilizing the bacterial machinery for its own viral DNA replication. Once the bacterium lyses, newly packaged phages are released into the environment. However, during the packaging process, errors are known to occur, often leading to the incorporation of bacterial DNA. The virus is then capable of injecting this erroneous DNA into a new bacterial host, successfully completing a HGT event (75).

Conjugation requires physical cell-to-cell contact, involving donor (F^+) and recipient (F^-) strains. The F^+ strain carries an additional chromosomal F factor, bearing the genes necessary for pilus formation. The pilus then joins the donor and recipient strain and often contracts to bring the two individual cells closer together, allowing for direct plasmid transfer. This process also frequently involves the transfer of the F factor, altering the F^- strain to an F^+ status (75).

Considering the above diversity of mechanisms, and the fact that large numbers of microbial species co-exist in every imaginable environment in close physical proximity to one another (45), it becomes clear that the possibility of gene exchange is a very relevant issue in the broader scope of past and present evolution (87).

While there is significant evidence for HGT in prokaryotes, the occurrence and the mechanisms involved in eukaryotic HGT are less well understood. Various hypotheses suggest the involvement of transfection (viral mediated transduction in eukaryotes, although no viruses are yet know to infect this organism), transposable elements (segments of DNA containing

genes allowing for homology-independent movement between different chromosomal locations), anastomosis of fungal mycelia (branched connecting network of fungal hyphae) or a native phagotrophic (a mechanism of engulfing particles through the cell membrane into internalized phagosomes) feeding ability of some eukaryotes (75). Although to date, no data exists in favour thereof, one has to consider the possibility of eukaryotic HGT to involve mechanisms similar to the transduction process described for prokaryotes.

2.3 EXAMPLES OF HGT

Several studies involving many groups of organisms have provided direct evidence for HGT. Examples include the intra-species transfer of ampicillin resistance between two *Escherichia coli* strains isolated from infant bowel microbiota following antibiotic treatment (40); the inter-genera transfer of vancomycin resistance from enterococci to a commercial strain of *Lactobacillus acidophilus* within the gut of mice (62) and the trans-kingdom transfers between *Agrobacterium tumefaciens* carrying a tumor-inducing plasmid which, once transferred, results in crown gall disease in affected plants (75,80). Further examples are listed in Table 2.1.

These naturally occurring mechanisms of DNA transfer are so versatile that they have been adopted and adapted by molecular researchers as commonly used tools in the biological sciences. The first laboratory replicate of such HGT was described in 1976, were yeast DNA containing a histidine biosynthesis gene was placed into a histidine deficient mutant strain of *E.coli*. The result was the restoration of histidine biosynthesis, demonstrating for the first time that eukaryotic DNA could be expressed in a bacterium (85). The inverse was demonstrated in 1980 when a bacterial neomycin phosphotransferase gene was expressed in yeast, resulting in aminoglycosidase resistance (15). Only 3 years later, the first transgenic mouse was generated, expressing the human growth hormone (72). All these results suggested the possibility that HGT across species boundaries was more likely than previously imagined, and may not be limited to prokaryotes alone.

Several studies focusing on pathogenic organisms have provided evidence of gene exchange. The genome sequences of pathogenic *Salmonella* strains, for example, indicate the presence of common surface antigens H and O, used for serotyping, to be present among distantly related strains (9). Similarly, the sialidase gene, *nanH*, has also been found to move between strains of *Salmonella* (35). Pathogenic strains of *Neisseria lactamica, Neisseria meningitides* and *Neiserria gonorrhoeae* were originally susceptible to antibiotic treatment, but have recently acquired resistance through HGT events involving the generation of mosaic constructs of the *penA* gene confering penicillin resistance *(*from the *penA* genes of *Neisseria flavescens* and *Neisseria cinerea*) (10,59,83,88). In addition, large chromosomal segments containing sets of virulence genes, known as pathogenicity islands (PAIs) have also been suspected of HGT, as observed in a study of the PAPI-1 PAI between two *Pseudomonas aeruginosa* strains. Interestingly, this PAI is capable of existing both as an integrated cassette and as an extrachromosomal plasmid (76).

Today, the versatility of these naturally occurring gene transfer mechanisms has been exploited to such an extent that several gene transfer protocols are routinely applied in microbiological research studies, including eukaryotic transfection (viral mediated), chemical transformation (e.g. Li-OAc yeast protocol), electroporation and heat shock to artificially rendered competent bacterial cultures (cultures with altered cell walls to increase permeability allowing for easy penetration of DNA) (See http://www.currentprotocols.com for an assortment of such protocols).

Table 2.1 Direct evidence of horizontal gene transfer events

Protein	Donor	Recipient	Reference
Erythromycin resistance	*Treponema denticola*	*Streptococcus gordonii*	(47)
Kanamycin resistance	Transgenic *Beta vulgaris*	*Acinetobacter* sp. strain BD413(pFG4)	(66)
Mercury resistance proteins	*Alcaligenes eutrophus* JMP134	*Pseudomonas glathei,* *Burkholderia caryophyllii* and *Burkholderia cepacia*	(18)
Dichlorophenoxy-acetic acid degrading proteins	*Ralstonia eutropha* JMP134	*Burkholderia* and *Ralstonia* members	(8,65)
Vancomycin resistance	*Enterococcus faecium* HC-VI2	*E. faecium* 64/3 and *E. faecalis* JH2-2	(61)
Tetracyclin resistance	*Campylobacter jejuni*	*Campylobacter jejuni*	(6)
TGF-β	Intranasal administration of an eukaryotic expression vector encoding TGF-beta1	Expression in lung and lymphoid tissue	(46)

2.4 PHYLOGENETIC EVIDENCE

The hypothesis that horizontal gene transfer may be a rather common event in biological systems has found strong support through the phylogenetic analysis of genome sequences. Phylogenetic analyses investigate the evolutionary relationship between organisms. These evolutionary relationships are determined by comparing nucleotide sequences and establishing the degree of homology. The degree of heterogeneity between two organisms is often expressed as the evolutionary distance and such data are graphically demonstrated by constructing phylogenetic trees (75). The vast amounts of data generated through these phylogenetic analyses of genome sequences indeed suggest HGT to be a role player in the evolution of both the prokaryotic and eukaryotic worlds.

A HGT event is usually suspected when protein or nucleic acid sequences from one organism show strong homology to sequences observed in other taxa, while the general and molecular structure of other parts of the genomes is very different (45). Several methods have been used to identify suspected HGT events. Such methods include:

1) Analysing the %G+C content as an indication of unique mutational patterns within organisms (69). The G+C content of any genome is defined as $\frac{G+C}{G+C+A+T}\times 100$ and is usually fairly constant, both within individual genomes and between strains of the same species, generally differing by less than 10% (75). Thus, if a new gene or genomic sequence were to be acquired through HGT, the new DNA would reflect the G+C content of the host genome at that time, however, as time progresses and natural mutations occur at a rate unique to the new host organism, the new DNA will acquire a G+C pattern similar to that of the native DNA, consequently masking a HGT as time progresses (52).
2) Using complex algorithms to develop “genetic fingerprints” based on di-nucleotide frequencies. The distribution of dinucleotides is biased in microorganisms, likely due to the spatial compatibility of two neighbouring t-RNAs (encoded by such dinucleotides) on the ribosomal surface. Such a biased dinucleotide use is encouraged by the resulting improved translation efficiencies, serving as a driving force for evolutionary selection. The preferred dinucleotide frequencies consequently allow for the distinction of foreign DNA from native DNA (11,41,42).
3) Determining differences in preferred codon usage based on availability of different t-RNA species. Studies have indicated that although a number of codons may code for any given amino acid (except Met and Trp), the frequency of their use is determined by the availability of the organism specific isoaccepting t-RNA population (37,51,54).

Examples of such phylogenetically suspected HGT events include the proposed transfer of the P-element or P-factor from a relative of *Drosophila nebulosa* to *Drosophila melanogaster* (30) and the spread of the LINE (long interspersed repetitive element) Juan-A among three non-sibling *Aedes* mosquitoe species. Two trans-kingdom transfers include that of the phosphoglucose isomerase gene between an eukaryote and bacterium, dating back to between 470 and 650 million years ago (43) and an eukaroytic gene for a glycolytic enzyme, fructose bis-phosphate aldolase, to marine cyanobacteria (79). Table 2.2 summarizes several more examples of phylogenetically derived HGT transfer events involving eukaryotes (88).

Table 2.2 Phylogenetic evidence of HGT events involving eukaryotes

Protein	Donor	Recipient	Reference
Thioredoxin m	prokaryotic symbiont	contemporary photosynthetic eukaryotic cells	(32)
Glyceraldehyde-3-phosphate dehydrogenase	Eukaryotic host	*E.coli*	(25,60)
Cytosolic Gap gene	γ-proteobacteria	Trypanosomes	(20)
Dihydroorotate dehydrogenase	*Lactobacillales*	*Saccharomyces cerevisiae*	(31)
Aryl- and alkyl-sulfatase gene	Bacterial	*S. cerevisiae* and *S.bayanus*	(31)

2.5 PITFALLS TO PHYLOGENETIC CONCLUSIONS

When analyzing phylogenetic data to draw conclusions regarding HGT events, certain pitfalls have been identified and computational algorithms have been designed to help with the identification of artefacts. These pitfalls include phenomena such as:

- gene loss, which through natural mutation, may wrongly be interpreted as a HGT event in the organism retaining its ancestral copy. Gene loss occurs through insertion or deletions of various sizes within the open reading frame of functional genes which, through selection over time, may result in the disappearance of the non-functional gene (19);
- gene conversion, where mutations are sustained during an attempt to correct mismatches potentially incurred during cross-over. Such conversions may be wrongly interpreted as HGT events (19);
- convergent evolution, which is driven by selection exerted by the immediate surroundings and could wrongly be interpreted as the acquisition of selectively advantageous genes. An example is seen in the case of the antifreeze glycoproteins (AFGPs) identified in Antarctic notothenioid fish and Arctic cod, both having undergone convergence with respect to these AFGP for survival in their freezing environments (13,24);
- paralogous genes, which are defined as genes that originated from a common ancestral parent that have diverged after a gene duplication event and possibly a subsequent speciation event. The misidentification of such paralogous genes often obscures the accurate identification of HGT events (24);
- “hotspots”, genomic areas prone to extreme mutation rates, have been observed and could potentially also result in the mistaken identification of HGT events (48);
- in addition, conserved sequences are generally not subjected to the same rate of mutation observed in the remainder of the genome. This could mistakenly identify such sequences as being foreign and often housekeeping genes need to be manually removed from the potential HGT candidates identified through such computational analyses (94).

Also, other factors which may result in the misinterpretation of sequence data are based on the use of the software packages available for such computational analyses; incorrect alignment of sequences may result in skewed interpretation. The use of algorithms such as BLAST which generate similarity scores depend entirely on the quality and reliability of sequences available within these databases as well as the evolutionary relationships represented in such databases (94).

2.6 OBSTACLES TO HGT

In a world of self propagating plasmids and transposable elements, one could pose the question as to how or why the impact of HGT appears to have been limited throughout the course of evolution. Indeed, the data described above could suggest that HGT occurs frequently, but that we may be limited in our ability to detect and to analyse such events. Alternatively, the explanation may be that there are significant obstacles that restrict gene transfer events.
In this regard, there are several limiting factors that have been described for natural bacterial gene transfers:

- Bacteria have the ability to detect foreign DNA and often counter its integration by subjecting such alien nucleotide sequences to R.E digestion (27,39).

- The amount of sequence homology between incoming foreign DNA and host genome impacts on the success of its integration (17).
- Another factor that plays a major role in successful natural transformations is host toxicity; any particular gene may code for a protein giving an advantage to one organism, but that very same protein may prove to be lethal, toxic or otherwise detrimental in another organism (48,83).
- The physical proximity, both of the organisms involved in a possible direct DNA transfer, as well as the "free floating DNA" relative to the new host, may also present as a physical barrier to the occurrence of HGT (16).
- Finally, for any newly acquired gene to be maintained within a genome, it needs to provide a selective advantage to its new host and will be lost if not maintained under selective pressure, further lessening its chance of persistence by allowing natural selection to act as a final check-point. This is presumably the most stringent of all selective strategies, owing to the fact that even if a foreign gene is acquired and integrated, its functionality often depends on the support of secondary proteins which, if not present, would result in the gain of a non-functional protein, potentially rendering itself susceptible to complete gene loss through the accumulation of mutations (19).

When the likelihood of successful gene transfers in eukaryotes is considered, several additional factors come into play. On a cellular level, major differences between the mechanisms and machinery involved in eukaryotic and prokaryotic gene expression include:

- prokaryotic promoters differ from eukaryotic promoters in general structure (including elements such as the TATA box, the CAAT box and the GC box), and eukaryotic gene regulatory sequences vary between different eukaryotic species (75);
- eukaryotic polymerases require additional transcription factors for the recognition of promoters (75);
- eukaryotic gene expression is also regulated by additional DNA sequences such as enhancers and upstream activator sequences that may be situated at a significant distance from the gene sequence that is being regulated (75);
- eukaryotic transcription produces heterogeneous nuclear RNA precursors which require post-transcriptional modification in order to yield mRNA (75);
- eukaryotic genes contain introns and exons, which can diverge between eukaryotic genomes, requiring RNA splicing to remove the intronic sequences from the precursor RNA molecules. This splicing is aided by the presence of small nuclear RNA molecules (75).

These limiting factors can be overcome within the laboratory environment through the application of advanced molecular tools. However, each of these factors may prove to be an obstacle when interchanging eukaryotic and prokaryotic DNA through natural transfer.

2.7 EXTENT OF HGT

When foreign DNA is accepted by a new host and incorporated as part of its genome, the newly acquired DNA is subject to the same rate of change through natural mutation as the rest of the genome, gradually resulting in a more "personalized" codon usage profile that is reflected throughout the indigenous recipient genome. This process is referred to as amelioration (52).

To establish the rate of HGT within any given organism, it needs to be determined which alien sequences within the genome have successfully persisted. This is done by estimating the amount of amelioration within the foreign sequence since its time of arrival (50). Lawrence and Ochman used this approach to determine that ~ 1 600kb of foreign DNA had been introduced into the *E. coli* genome since diverging from the *Salmonella* lineage (52,53).

Several other approaches have attempted to estimate the rate of natural HGT. Using software packages that draw conclusions from data sets such as "clusters of orthologous groups of proteins" (COGs) in a collection of gene trees representing a set of taxa, averages of 11 HGT events among the 44 taxa of the COG species tree under investigation have been reported (56).

Other studies have emphasized the diversity among species in terms of the amount of genome sequence obtained through HGT; with close to no HGT sequences in *Rickettsia prowazekii*, *Borrelia burgdoferi* and *Mycoplasm genitalium*, to almost 17% in *Synechocystis* PCC6803 and 18% in *E*. coli (22). The general consensus remains that HGT has played a significant role in the evolution of the microbial world (27,70,90).

2.8 HGT AND GENETICALLY MODIFIED ORGANISMS (GMOs)

As mentioned previously, genetic transformation can be considered as laboratory-induced examples of HGT. Yet, very little information regarding the occurrence of similar events in natural or industrial settings exist. In this regard, a reoccurring argument is based on the perceived risks that may be associated with the application of GMOs in agriculture or industry. The assumption is that the modified and transferred DNA may present a greater risk of HGT. This is of particular relevance since the traits encoded by the foreign DNA in most GMOs would have been designed to give the transformed strain a trait of agronomical or other relevance (33). This DNA may therefore impart a competitive advantage to any recipient specie, which may lead to serious negative consequences to natural ecosystems. Furthermore, many GMOs carry antibiotic resistance genes that were used as markers in the selection process. Such genes are of obvious concern to consumers, although no evidence for any negative impacts subsequent to the presence of such genes in GM crops has thus far been reported.

The use of GMOs has therefore raised concerns regarding the potential hazards involved in such transfers, in particular the transfer of DNA sequences that may present risks (e.g. antibiotic resistance genes). These concerns have resulted in the requirement for extensive trials before commercialization can be considered. However, our lack of knowledge regarding HGT in particular in eukaryotes makes risk assessment in this regard rather difficult, if not impossible.

The molecular techniques applied to generate most GMOs involve using naturally occurring mobile genetic elements. These elements are modified to include the GM genes of interest (and often include the use of antibiotic resistance genes as markers) as well as in some cases interspecies origins of replication, specifically designed to cross species barriers.

Regarding the potential for gene transfer to occur, research has shown that free DNA can be detected in most inhabited environments, aquatic and terrestrial, as a consequence of natural DNA release (see Table 2.3) (58). In addition, studies have confirmed the longevity of such released DNA to be directly influenced by the nature of its environment (57). This suggests that GM DNA (including antibiotic resistance genes) could potentially spread beyond the initially modified organism to other naturally occurring organisms, following the natural release of such GM DNA. This could have a severe impact on the immediate ecosystem, with the far reaching

effects extending to human health issues. A major concern is the potential of pathogens gaining increased resistance to the currently available spectrum of antibiotics used in medicine through such HGT events. There are claims however, that the acquisition of such antibiotic resistance genes would rather occur from naturally occurring bacteria than from GMOs (16). Regardless, from a public point of view there is growing concern that by generating GMOs, the potential occurrence of a HGT event may be enhanced and that spreading of such GM DNA beyond the original boundaries cannot be controlled (33,34).

Table 2.3 High molecular weight DNA present in aquatic systems

Habitat	Molecular size (kb)	DNA concentration (µg/l)	Half-life (h)
Fresh water	ND	0.5-25.6	4-5.5
Estuarine	0.15-35.2	10-19	3.4-5.5
Offshore/ocean	0.24-14.3	0.2-1.9	4.5-83
Freshwater sediment	1.0-23.0	1.0	-
Marine sediment	-	-	140-235

ND = not determined; - = unknown

With respect to the wine industry, introducing GM yeast into the fermentation process could have significant downstream effects. GM DNA could conceivably be introduced into the external environment after having passed through the winery effluent, thus creating a gateway for uncontrolled spread of genes among naturally occurring microbial communities beyond the proximity of the winery. In order to address these uncertainties, it is essential to launch comprehensive risk assessment analyses before any GMO is to be introduced into the commercial environment. However, a mitigating factor in this regard would be the natural presence of the modified gene within the larger ecosystem under consideration. Indeed, if the integrated DNA originates from organisms that are present in the same ecosystem, the release of this DNA would not add to the naturally present DNA sequences. The concept of the hologenome of an ecosystem has been developed to take such considerations into account (98).

2.9 RISK ASSESSMENT STRATEGIES

Risk assessment studies should be designed in such a manner that all the various aspects possibly affected by the release of a GMO into the natural environment will be explored. This includes aspects such as controlled field trials and determining the impact of the use of GMOs on the direct microbial communities such as those performed by Milling *et al.* (64), Timms *et al.* (91), Brusetti *et al*. (12) and Valero *et al.* (93). In the study performed by Milling *et al.,* a comparison based on 16S- and 18S-rDNA DGGE fingerprints indicated no difference in the composition of bacterial and fungal diversity in the rhizosphere and soil of a transgenic potato line when compared to its non-transgenic parental strain. During the study perfomed by Timms *et al.*, a genetically modified *Pseudomonas fluorescens* serving as a biological control agent providing protection to a number of crop plant species from damping-off caused by *Pythium ultimum*, showed to have improved biocontrol activity as compared to the wild type SBW25, effectively suppressing *Pythium* spp. present at up to 100 times normal field infestations. Any observed changes following inoculation with the wild type or GM *P. fluorescens* in microbial diversity (bacteria and fungi) were found to be negligible based on selective plate count and

SSU rRNA based PCR-DGGE analyses. Brusetti *et al*. investigated the effect of transgenic Bt 176 maize on the rhizosphere bacterial community, cultured in a greenhouse. When compared to its non-transgenic counterpart, grown in identical conditions, bacterial counts for several bacterial types showed no significant differences. During a three years study performed by Valero *et al.*, the survival of industrial yeast strains were evaluated in 6 different vineyards, where it was found that these populations were subject to natural annual fluctuations. In 2005, Schuller *et al.* performed a study in which the behaviour of several genetically modified *S. cerevisiae* VIN13 strains were monitored within the microbial communities of a confined cellar as well as artificial greenhouse vineyards. Data yielded from their study indicated no significant difference in occurrence between the parental strain and the GM strains, neither parental commercial nor GM strain had any effect on the natural vineyard-associated flora and, subsequent to spontaneous micro-vinification, no significant difference was observed between the strains in terms of fermentation performance (81).

Examples of such field trials directly related to the wine industry include such studies as the GM grapevine field trial which was launched in Australia in 2003 (14) as well as the proposed field trial for a transgenic grapevine at Wellgevallen, Stellenbosch, South Africa, as part of the Grapevine Biotechnology program at the Institute for Wine Biotechnology, University of Stellenbosch (1). Several other GM crops are also currently undergoing field trials in South Africa as can be seen in Table 2.4 (95). A detailed list of global field trials can be found on the Information Systems for Biotechnology website (4).

It follows then that risk assessment strategies should be designed in such a way to incorporate the previously mentioned phenomena of the ecosystems' hologenome when investigating downstream effects of GMO use within any given environment.

Table 2.4 GM crop field trials in South Africa

Institution	**GM crop field trial**
Monsanto	Cotton, Maize
Delta & Pinelands	Cotton
Syngenta	Cotton
Dow Agro	Maize
ARC SASRI	Soybean, Potato Sugarcane

When dealing with GM crops, further downstream issues that need to be addressed include:

- the safety for human consumption of GM crops
- the use of antibiotic resistance genes, capable of altering the current pathogenic status of microorganisms
- whether the newly produced proteins are safe for human consumption and
- whether allergens may have been introduced into the GMO (26).

The risks involved with the use of GMOs and the subsequent impacts of improper risk assessments was the core issue in a controversial case of introgression (gene flow from one species to the gene pool of another by the backcrossing of an interspecific hybrid with one of the parental strains) to native ancestral varieties of maize crops, reported in remote mountainous areas of Sierra Norte de Oaxaca in South Mexico in November 2001, allegedly following introduction of US transgenic crop species as food aid (77). This was reported after a

moratorium had been placed on the cultivation of GM crops in Mexico since 1998 in an attempt to maintain the diversity of wild maize. However, less than a year after the original introgression claims, the authors and publishers started receiving severe criticism from the scientific community, questioning the validity of the data obtained from an apparently flawed assay, launching a global-scale scientific debate (44). The authors have since claimed to have obtained additional data substantiating their initial claims of introgression. Whether proven or not, the fact remains that risk assessment strategies are absolutely crucial when launching GMOs into any given habitat, as demonstrated in this example.

Whatever the long-term outcome of this debate, it is clear that, but for the phylogenetic evidence, there is very limited knowledge regarding HGT in eukaryotes. As a consequence, there is a significant need to acquire such knowledge, since understanding of these processes within eukaryotic systems will have a significant impact on the risk assessment applied to many future applications of modern biotechnological tools.

2.10 ADVANTAGEOUS COMMERCIAL APPLICATIONS OF HGT

With all the available direct evidence for HGT with respect to prokaryotes, it is surprising that, but for the phylogenetic evidence, there is very limited knowledge regarding HGT in eukaryotes. A significant need to acquire knowledge regarding the occurrence of HGT between and involving eukaryotes is evident.

As a unicellular organism, yeast population dynamics and ecology is closer to many prokaryotes than to higher eukaryotes (75). For this reason, using yeast as a model organism to investigate HGT appears to have both advantages and disadvantages. On the one hand, such events should be easier to record and to analyse in this organism, providing a platform for the study of, in particular, the molecular mechanisms that may be specific to eukaryotes. On the other hand, it is also obvious that the results may be less or not at all representative of HGT in higher eukaryotes. In any case, considering the tremendous biotechnological importance of yeast, the potential for HGT within this group of organisms has to be studied on its own merits.

Understanding these processes within an eukaryotic environment is essential for the development of many commercial and medical applications. Possible applications of controlled HGT could for example provide the foundations for further technological advances in medicine. To highlight a few examples, extensive research is currently underway worldwide to develop more efficient gene therapy treatments. These techniques rely on HGT to either replace or knock-out mutated or non-functional genes responsible for genetic disorders (3) such as:

- Sickle cell anaemia (73)
- Alzheimer's disease (92)
- Lesch-Nyhan syndrome (29)
- Parkinson's disease (21)
- Several cancer types (67)

Shifting focus to some of the environmental issues faced today, the potential advantages of utilizing HGT as a means of spontaneously transforming naturally occurring microbiological communities with pollutant-degrading genes for the purpose of bioremediation or bioaugmentation are vast (74,96). For these purposes, significant effort is being made to improve our understanding of the dynamics of biofilms, which are particularly well suited for this

purpose due to their large biomass and ability to immobilize recalcitrant compounds (7). Many organisms have the ability to adhere to inert surfaces and develop a coating termed the EPS (extracellular polymeric substance), consisting of organic substances such as polysaccharides, proteins, nucleic acids, phospholipids, uronic acid and humic substances (97). The origin of these substances is believed to be a result of secretion, shedding of cell surface material, cell lysis and contributions from the environment, with the major component in this biofilm matrix being water at an estimated 97% (97). The exact composition of a biofilm, however, is unique to each environment, influenced by the microbial community as well as physical and chemical external factors (86), and serves as a protected environment, guarding the underlying microbes from unfavourable environmental factors, such as antimicrobials and chemical biocides (28,78) thus making such structures particularly well suited for the purposes of bioremediation (82). One could consider that if the microbes constituting the biofilm structure are so well protected by this EPS, any naked DNA present within this structure could possibly be just as well preserved, potentially providing intact DNA to a new host. By harvesting data collected from HGT studies within such biofilm structures, large scale cost-effective bioremediation could become a very real possibility within the foreseeable future.

2.11 COMMERCIAL GM YEAST

Currently many GM yeast strains that present some specific and significant advantages when compared to existing commercial strains have been developed, and some of these strains have already gained approval from the necessary authorities and are available on a commercial scale. These include:

1. A GM wine yeast, ML01, that is capable of degrading malic acid (36).
2. A GM wine yeast designed (by First Venture Technologies) to reduce ethyl carbamate levels, a carcinogen naturally found in certain fermented foods and beverages as a result of incomplete urea metabolism. This GM yeast has been altered to increase controlled levels of urea amidolyase expression, the native enzyme responsible for urea metabolism (2).
3. A GM yeast containing the xylose isomerase gene, capable of converting non-fermentable xylose to xylulose, allowing for the production of biofuel from waste materials such as woodchips, straw and cornhusks, through fermentation (49).

With an ever growing consumer driven demand for improved wine, researchers have centred their attention to developing GM *S. cerevisiae* with further improved fermentative abilities and enhanced sensory qualities as well as strains resulting in a product with overall improved health benefits.

A GM *S. cerevisiae* strain, known as ML01, currently in use for wine production in Moldavia, USA and Canada is one example of a GM yeast strain that has successfully met all the requirements associated with the commercialization of a GM product. This strain contains the *Schizosaccharomyces pombe* malate permease gene (*mae1*) as well as the *Oenococcus oeni* malolactic gene (*mleA*) under control of the *S. cerevisiae PGK1* promoter and terminator. These alterations allow for the prevention of harmful biogenic amines produced by lactic acid bacteria in during malolactic fermentation in wine (36).

The availability of modern genetic tools, combined with the current collective knowledge on *S. cerevisiae* genetics and physiology have allowed researchers to generate an array of GM yeast with such improvements. Available in laboratories from across the world, the commercial use of these strains are pending approval based on risk assessment results.

2.12 CONCLUSION

Acknowledging both the advantageous and disadvantageous aspects concerned in the use of GMOs as discussed in this chapter, it is clear that stringent risk assessment strategies are a vital prerequisite to the launch of any GMO on a commercial scale. This study aims at laying the foundation for risk assessment strategies with regards to HGT related to GM yeast, with regards to the wine making process in particular. We aimed to investigate the occurrence of, and mechanisms involved in HGT between a GM *S. cerevisiae* and wild type *S. cerevisiae* present during alcoholic fermentation by focussing our attention on 3 key questions concerning DNA release, DNA stability and DNA uptake and expression by previously untransformed *S. cerevisiae* hosts. By generating circumstances favouring HGT events in combination with simplified screening techniques, we will be addressing crucial aspects regarding the safety of using GM yeast for wine production. Additionally, a basis for establishing yeast as a model eukaryotic system for investigating HGT will be developed.

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Chapter 3

Materials and Methods

Materials and Methods

3.1 DETECTION OF RELEASED MULTI-COPY PLASMID DNA FROM *S. cerevisiae* DURING ALCOHOLIC FERMENTATION

3.1.1 Strains and plasmids

The industrial *S. cerevisiae* strain, Vin13, was the selected yeast strain for all fermentations performed during this study. This decision was based on its popularity in the wine industry and the relative amenability for transformation in the laboratory (11). In addition, this strain has been extensively studied and reasonably well characterized (http://www.uniprot.org/taxonomy/317876) and is frequently applied within this academic environment, thus providing a well optimized tool as an industrial yeast representative. The *E.coli* strain, DH5α (Gibco BRL/life Technologies, Rockville, MD, USA) was used as bacterial host strain for all DNA manipulations.

The multi-copy plasmid pYES2-KanMX/LacZ is an episomal shuttle vector generated from various components of the following plasmids: pHP41 (Figure 3.1a) (9), pYES2 (Figure 3.1b) (Invitrogen Corporation, USA) and pJCEs-TEFpKanMX (this laboratory). Primers were designed for PCR amplification of the LacZ cassette (expressing β-galactosidase) using pHp41 as template (all PCR amplifications were performed in a Biometra T3 Thermocylcer). A HindIII R.E recognition site was incorporated into the forward primer and a BglII R.E recognition site was incorporated into the reverse primer (see primer sequence, PCR cycle and reaction mixture in Table 3.1). The LacZ PCR product was digested with BglII/HindIII R.E combination and pYES2 with HindIII/BamHI R.E combination. Gel purified fragments (Wizard Cleanup Kit - Promega Corporation, USA) were then ligated at 4 °C overnight using ligase (according to the manufacturers recommendation) to incorporate the LacZ cassette under control of the *GAL1* promoter of pYES2, yielding the intermediate plasmid, pYES2-LacZ (Figure 3.1c). Similarly, primers were designed for amplification of the KanMX (conferring geneticin resistance) cassette from pJCEs-TEFp*KanMX (Figure 3.1d) as template (see primer sequence and PCR parameters in Table 3.1) (All primers manufactured by IDT). Following gel purification, this PCR product was ligated into pGEM®-T Easy (Figure 3.1e) (Promega Corporation, USA) at 4 °C overnight and again excised by digesting with SphI/SpeI R.E combination. pYES2-LacZ was digested with XbaI/SphI R.E combination. These two fragments were gel purified and ligated at 4 °C overnight to yield the final construct, pYES2-KanMX/LacZ (Figure 3.1F). Ligated products were transformed into DH5α by adding the ligated DNA to 100µl competent DH5α and mixing by vortex. The suspension was maintained on ice for 1 minute followed by a heat shock incubation for 45sec at 42 ℃. The heat shocked su spension was immediately incubated on ice for 2minutes followed by a room temperature incubation of 2minutes. A volume of 900µl LB broth was added to the suspension and mixed by inversion, followed by an incubation period of 30minutes at 37℃. Transformed cultures were subse quently plated on selective media (12).

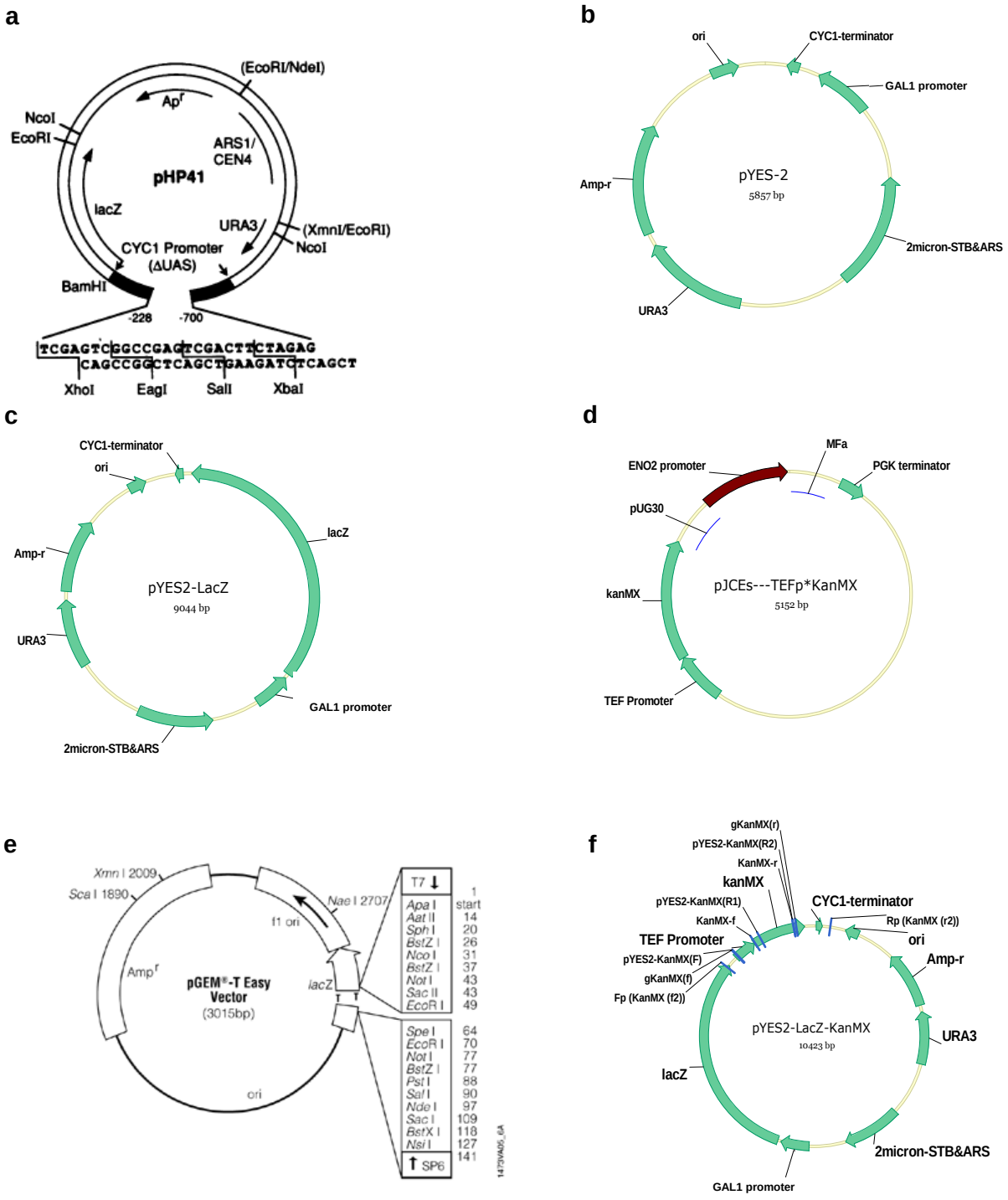

Figure 3.1 Plasmids used for construction of pYES2-KanMX/LacZ **(a)** pHP41 (9), **(b)** pYES2, **(c)** pYES2-LacZ, **(d)** pJCEs-TEFp*KanMX, **(e)** pGEM®-T Easy vector (Promega Corporation), USA) and **(f)** pYES2-KanMX/LacZ.

Table 3.1 PCR parameters for cloning

Primer sequence	Template	PCR Cycle	PCR Mix (50µl total volume)
LacZ(f):5'GCTCAAGCTTATGACCGGATCCGGAGCTTG3' LacZ(r):5'GATCAGATCTGGCAGTACATAATGGATTTCCTT3'	pHP41	94 °C, 2min 94 °C, 20sec 55°C, 30sec 72 °C, 3min ×10 cycles 94 °C, 20sec 62 °C, 30sec 72 °C, 3min ×20 cycles 72 °C, 5min 4 °C, ∞ Heatinglid,105°C	3-5µg template DNA 0.2mM primer(f) 0.2mM primer(r) 0.1mM dNTP's (each) 1× ExTaq buffer 1U ExTaq
KanMX(f2):5' CTGCCCGTATTTCGCGTAAGGAAAT 3' KanMX(r2):5' CTTCGAGCGTCCCAAAACCTTCTCA 3'	pJCEs-TEFpKanMX	94 °C, 2min 94 °C, 20sec 56 °C, 30sec 72 °C, 3min ×10 cycles 94 °C, 20sec 64 °C, 30sc 72 °C, 3min ×20 cycles 72 °C, 5min 4 °C, ∞ Heating lid @ 105 °C	3-5µg template DNA 0.2mM primer(f) 0.2mM primer(r) 0.1mM dNTP's (each) 1× ExTaq buffer 1U ExTaq

3.1.2 Culture conditions, DNA extractions, transformations and overlay assays

Transformed *E. coli* DH5α was cultured in Luria-Burtani (LB) media (10g/l peptone, 5g/l yeast extract, 5g/l NaCl, pH 7.2) supplemented with 100µg/ml ampicillin at 37 °C whilst continuously shaking overnight. The cultures were collected the following day by transferring 2ml successively into 2ml microfuge tubes followed by centrifugation at 12000rpm for 30sec in a Sorvall MC 12V benchtop centrifuge. The media was removed by aspiration and the remaining pellet was resuspended in 100µl of solution I (50mM glucose, 25mM Tris-Cl pH8.0 and 10mM EDTA pH8.0) by vigorous vortexing. Once completely resuspended, 200µl of solution II (0.2N NaOH and 1% SDS) was added and mixed by inversion. This was followed by addition of 150µl ice-cold solution III (60 ml of 5M potassium acetate, 11.5ml of glacial acetic acid and 28.5ml H_2O) and mixed by inversion, followed by a 10min incubation at -80℃. The suspension was then centrifuged at 12000rpm in a benchtop centrifuge for 10minutes after which the supernatant was aspirated and added to a new 2ml microfuge tube containing 1ml 100% EtOH and mixed by inversion. The suspension was once again incubated at -80℃ for 10minutes, followed by benchtop centrifugation at 12000rpm for 10min. The supernatant was aspirated and the pellet resuspended in 100µl dH_2O containing RNaseA at a final concentration of 0.2µg/µl (12).

Once having correctly identified the extracted plasmid DNA through R.E digestion screening, it was transformed into *S. cerevisiae* by resuspending a large Vin13 colony (5mm diameter) in 1ml dH_2O. The cells were washed by pelleting in a benchtop centrifuge at 12000rpm for 30seconds followed by aspiration of the supernatant. The pellet was then resuspended in 1ml 100mM LiAc and incubated at 30℃ for 5minutes. The cells were once again pelleted in a benchtop centrifuge at 12000rpm for 30seconds followed by aspiration of the supernatant. The following was then added in this particular sequence: 240µl 50% PEG, 36µl1M LiAc, 5µl 10mg/ml denatured cold herring sperm DNA, 10µl plasmid DNA and 45µl dH_2O. This mixture was thoroughly mixed by vortexing for at least 1minute followed by incubation at 42℃ for 30minutes. Following incubation, the cells were pelleted in a benchtop centrifuge at 12000rpm for 30sec, the supernatant was removed and the pellet resupended in 200µl dH_2O and subsequently cultured on YPD (10g/l yeast extract, 20g/l peptone, 20g/l glucose) supplemented with 100µg/ml G418 (solubilised in dH_2O) at 30 °C (5). When culturing on solid media, 20 g/l agar was added to the various media types. Vin13 cultures were incubated at 30 °C for 2-5 days.

In addition, an X-Gal overlay assay was performed by preparing 0.5M Potassium Phosphate buffer, pH7.0 (61ml 1M K_2HPO_4, 39ml KH_2PO_4 and 100ml dH_2O) which was used to prepare a 0.5% low melting temperature agarose by heating in a microwave. Once cooled, 600µl 99% N,N-dimethylformamide, 100µl 10% SDS and 100µl 100mg/ml 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside resupsended in 99% N,N-dimethylformamide was added, mixed and poured (10) over transformed Vin13 cultured on YPGal agar (where the 20g/l glucose was replaced by 20g/l 0.45µm filter sterilized galactose). Colonies displaying ß-galactosidase activity were identified by a blue colour change after incubation at 30 °C for 24 hours. The resulting strain was named Vin13pYES2-KanMX/LacZ.

3.1.3 Fermentation trials

Benchtop fermentations (Figure 3.2) were performed at room temperature in MS300 (synthetic must containing 125g/l glucose and 125g/l fructose) prepared according to Bely, 1990 (2), YPD (with 10% glucose) and 0.45µm filter sterilized grape must as follows: 4x100ml media in 500ml Erlenmeyer flasks were inoculated with Vin13pYES2-KanMX/LacZ to an initial OD_{600nm} = 0.1 (Shimadzu UV-1601 UV visible spectrophotometer) and sealed fermentation caps that had been soaked in 70% ethanol overnight. Fermentations were exposed to 3 different stress conditions:

For increased exposure to ethanol, the compound was directly added to the medium to the final concentration of 7% ethanol at the initiation of fermentation. To increase cell density, fermentation media types were inoculated to an initial OD_{600nm} = 0.6-0.8. The relevant cultures were heat shocked by incubation at 42 °C for 30minutes. Standard benchtop fermentations were included as controls. An additional set of 4x100ml fermentations were inoculated with untransformed Vin13 as a control set and exposed to the same stress conditions.

Figure 3.2 Benchtop fermentations representing two YPD with 10% glucose (1 & 2) and two filter sterilized grape must fermentations (3 & 4).

Growth curves were established for each of the media types by inoculating 100ml of the various media with ~3 000 000 (Vin13) cells/ml (corresponding to OD_{600nm}=0.1) and measuring the OD_{600nm} on a daily basis. These data were used to determine key points at which stress treatments would be made and to determine sampling points.
These fermentations were allowed to proceed for 10-14 days, whilst continuously monitoring CO_2 production through weight loss. The fermentations exposed to heat shock were incubated at 42 °C for 30min on days 1, 5, 6, 7 and 11.

3.1.4 Sampling and PCR screening

The fermentations were sampled on days 1, 2, 4, 5, 6, 8, 11 and 13 by first resuspending the sedimented yeast cells, followed by extraction of approximately 1 ml culture broth which was subsequently filter sterilized through 0.45 µm acetate syringe filters. These filter sterilized samples were spotted on YPGal agar supplemented with 100µg/ml G418 solubilised in dH_2O and incubated at 30 °C for 2-5 days in order to identify the presence of any unfiltered Vin13 cells in the supernatant which could lead to false positive results during the subsequent PCR screen.

The filter sterilized extracted samples were subjected to PCR screening in order to detect the presence of free floating DNA released by fermenting Vin13pYES2-KanMX/LacZ (and untransformed Vin13 fermentations as control). A set of nested primers were designed to detect the presence of the LacZ cassette of pYES2-KanMX/LacZ in increments of ~ 500bp (Table 3.2). In order to determine the PCR sensitivity of this screen, a template dilution range (1µg – 100fg) was included in each sample set as an indicator.

Table 2.2 PCR parameters for screening of fermentation supernatants for multi copy plasmid DNA (pYES2-KanMX/LacZ)

Primer sequence	**Product size**	**PCR Cycle**	**PCR Mix (100µl total volume)**
Forward primer:		94 °C, 2min	2µl fermentation supernatant
LacZ(f):5' GCTGTTGCCCGTCTCACTGGTGAA 3'		94 °C, 20sec }	0.2mM primer(f)
Reverse primers:		43 °C, 30sec } ×30 cycles	0.2mM primer(r)
LacZ(r1):5' CGCGTAAAAATGCGCTCAGGTCAA 3'	~500bp	72 °C, 3min }	0.1mM dNTP's (each)
LacZ(r2):5' CGCACGGCGTTAAAGTTGTTCTGC 3'	~1000bp	72 °C, 5min	1.75 – 3.25mM $MgCl_2$
LacZ(r3):5' GACCACGGGTTGCCGTTTTCATCA 3'	~1500bp	4 °C, ∞	0.01mg/ml BSA
LacZ(r4): 5' CGGGGAGCGTCACACTGAGGTTTT 3'	~2000bp	Heating lid @ 105 °C	1× Supertherm buffer
			1U Supertherm Taq

3.2 DETECTION OF RELEASED INTEGRATED PLASMID DNA FROM *S. cerevisiae* DURING ALCOHOLIC FERMENTATION

3.2.1 Strains and plasmids

The industrial *S. cerevisiae* strain Vin13pDLG31 (6) (Figure 3.3) was the selected yeast strain for all fermentations performed during this study.

Vin13pDLG31 possesses the sulfometuron methyl resistance gene (*SMR1-410*) as well as the ability to grow on starch as sole carbon source due to the presence of the *LKA1* gene (α-amylase) from *Lipomyces kononenkoae* (6).

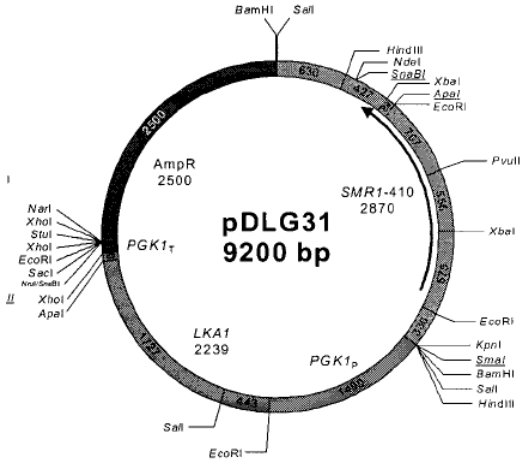

Figure 3.3 The integration plasmid, pDLG31 (6), used to transform the industrial strain, Vin13, yielding Vin13pDLG31.

3.2.2 Media and culture conditions

Vin13pDLG31 was cultured in YPD media supplemented with 100µg/ml sulfometuron methyl (SMM; solubilised in 99% N,N-dimethylformamide at 95 ℃ for 10min) at 30 ºC for 4 – 6 days. The production of α-amylase was screened on SC Phadebas media (6.7g/l yeast nitrogen base, 5 tablets/l Phadebas starch and 20g/l agar) supplemented with 100µg/ml SMM at 30 ºC for 2-5 days.

3.2.3 Fermentation trials

Benchtop fermentations were prepared and stressed as discussed in 3.1.3. Growth curves and fermentations proceeded as previously described in 3.1.3.

3.2.4 Sampling and PCR screening

The fermentations were continuously sampled as described in 3.1.4. These filter sterilized samples were spotted on SC Phadebas media supplemented with 100µg/ml SMM and incubated at 30 ºC for 2-5 days in order to identify the presence of any unfiltered yeast cells in the supernatant which would lead to false positive results during the subsequent PCR screen (as described in 3.1.4). Nested primers were designed to detect the presence of the LKA cassette of pDLG31 at increments of approximately 500bp and are listed in Table 3.3.

Table 3.3 PCR parameters for screening of fermentation supernatants for integrated plasmid DNA (pDLG31)

Primer sequence	**Product size**	**PCR Cycle**	**PCR Mix (100µl total volume)**
Forward primer:		94 °C, 2min	2µl fermentation supernatant
PGK(f):5' CCCAGACACGCTCGACTTCC 3'		94 °C, 20sec }	0,2mM primer (each)
Reverse primers:		43 °C, 30sec } ×30 cycles	0,2mM dNTP's (each)
LKA1(r1):5' CTCAGGGGAGGACAAGACCG 3'	~500bp	72 °C, 3min }	1.75 – 3.25mM $MgCl_2$
LKA1(r2):5' GCCGGTGTCATCCGGAATAT 3'	~1000bp	72 °C, 5min	0.01mg/ml BSA
LKA1(r3):5' GACCCACCAACACGCTGAAA 3'	~1500bp	4 °C, ∞	1× Supertherm buffer
LKA1(r4): 5' TCGGTAAGCCACCGGACATT 3'	~2000bp	Heatinglid @ 105 °C	1U Supertherm Taq

3.3 DETECTION OF THE UPTAKE AND FUNCTIONAL EXPRESSION OF MULTI-COPY PLASMID DNA BY *S. cerevisiae* DURING ALCOHOLIC FERMENTATION

3.3.1 Strains and plasmids

The industrial *S. cerevisiae* strain Vin13 was the selected yeast strain for all fermentations performed during this study (http://www.uniprot.org/taxonomy/317876).

pYES2-KanMX/LacZ (discussed in 3.1.1) was selected as the episomal multi-copy plasmid used in this experiment.

3.3.2 Media and culture conditions

In order to determine whether a 1 in 1 000 000 uptake event could be detected, transformed Vin13pYES2-KanMX/LacZ was co-cultured with untransformed Vin13 on selective YPGal agar supplemented with 100µg/ml G418. A dilution series of transformed to untransformed Vin13 were plated at ratios of approximately 1:1 000 000 and 10:1 000 000. After incubation at 30 ^{o}C for 2-6 days, an X-gal overlay was performed, according to the Herskowitz Lab protocol (10), in order to identify ß-galactosidase activity.

3.3.3 Fermentation trials

A series of benchtop fermentations were performed at room temperature as previously described in 3.1.3 with the exception that untransformed Vin13 was used as inoculum. The appropriate fermentation volumes were spiked with approximately 5µg purified pYES2-KanMX/LacZ plasmid DNA on days 0, 2, 3, 6, 8, 10 and 13. An additional set of 4x100ml fermentations were inoculated with untransformed Vin13 as a control set and exposed to the same stress conditions, however, without DNA spiking.

In the 2nd and 3rd fermentation sets, fermentation volumes were adjusted to 20ml and spiking occurred at a final concentration of 5µg/ml of fermenting media. YPD (10% glucose) was omitted from the repeat sets.

Growth curves and fermentations proceeded as previously described in 3.1.3.

3.3.4 Selective screening for uptake and expression of spiked DNA

The fermentations were continuously sampled by first resuspending the sedimented yeast cells, followed by 2 extractions of approximately 1ml each per fermentation. One millilitre, representing approximately 1% of each of the fermentations was pelleted at 12000 rpm in a benchtop centrifuge (Eppendorf 5415D, Germany) after which 850µl of the supernatant was extracted. The pellet was resuspended in the residual 150µl and plated on selective YPGal agar supplemented with 100 µg/ml G418, followed by an X-gal overlay (10). This was done in order to identify the presence of any Vin13 cells that may have accepted and expressed the spiked DNA.

All samples were maintained at room temperature and again sampled approximately 3 weeks after inoculation in order to determine the effect of a longer exposure time to high concentrations of ethanol.

3.3.5 PCR screening (Primary)

Representative colony forming units (cfus) obtained from initial selective plate screenings were subjected to colony PCR screening with Δ1 and Δ2 primers (8) in order to verify that cfus were *S. cerevisiae* strain Vin13. See Table 3.4 for primer sequences and PCR conditions.

3.3.6 CHEF analysis

Karyotyping through CHEF analysis (13) was performed for additional verification of *S. cerevisiae* Vin13, eliminating possible false positive results due to contamination. CHEF plugs were prepared by inoculating the cultures to be analysed into 5ml YPD broth and incubating at 30 ℃ overnight. The following day, the culture was pelleted in a benchtop centrifuge at 12000rpm for 30sec after which the supernatant was removed. This was followed by addition of 1ml 50mM EDTA pH8.0. The suspension was gently vortexed and centrifuged using a benchtop centrifuge at 12000rpm for 30sec. After removing the supernatant, 100µl 50mM EDTA pH 8.0 and 50µl Zymolyase solution (1ml SCE solution (1 M sorbitol, 0.1M sodium citrate and 60mM EDTA), 9mg Zymolyase 20T, 50µl β-mercaptoethanol freshly prepared) were added. Low melting temperature agarose (1% LM agarose in 125mM EDTA, pH 7) was immediately added and mixed through pipetting. The mixture was pipetted into plugs and allowed to set in a freezer. Once set, the plugs were removed and placed in the barrel of a 5ml syringe to which 2.5ml ETB solution (9ml 0.5M EDTA, pH 8, 1ml 1M Tris-HCL, pH 8 and 0.5ml β-mercaptoethanol, freshly prepared) was added. The plunger was then inserted, the barrel inverted and the nozzle sealed with parafilm. The plugs were then incubated at 37 ℃ with rotation overnight. The ETB solution was removed the following day and plugs washed twice with 5ml 50mM EDTA followed by the addition of 2.5ml proteinase solution (9ml 0.5M EDTA, 1ml 10% N-Lauroylsarcosine, 10mg proteinase K, 1mg RNase, freshly prepared). The nozzle was once again sealed with parafilm and plugs were incubated at 37 ℃ while rotating overnight. The following day the plugs were rinsed with 5ml 50mM EDTA after which 4ml 1xTE 10mM Tris-HCL, 1mM EDTA, pH 8) was added and allowed to incubate at 37 ℃ for 1 hour. The plugs were then ready to be used in a 1.2% agarose MP gel, run on a BIO-RAD CHEF MAPPER for 24 hours (24ma, angle = 60°, 6.0V/cm).

Table 3.4 PCR parameters for identification of *S. cerevisiae* strain Vin13

Primer sequence	Template	PCR Cycle	PCR Mix (50µl total volume)
Δ1: 5'CAAAATTCACCTATWTCTCA3' Δ2: 5'GTGGATTTTTATTCCAACA3'	*S. cerevisiae* genomic DNA	94 °C, 2min or 5min for cfu 94 °C, 1min 45 °C, 30sec } ×30 cycles 72 °C, 90sec 72 °C, 5min 4 C, ∞ Heating lid,105 °C	1 cfu or 200ng DNA 0,2mM primer(each) 0,1mM dNTP's (each) 2mM $MgCl_2$ 1× Supertherm buffer 1U Supertherm Taq
KanMX(f): 5'GACTCACGTTTCGAGGCCGC3' KanMX(r): 5'CTGCGATTCCGACTCGTCCA3'	pYES2-KanMX/LacZ (product size ~650bp)	94 °C, 2min or 5min for cfu 94 °C, 1min 57 °C, 30sec } ×30 cycles 72 °C, 1min 72 °C, 5min 4 °C, ∞ Heating lid,105 °C	1cfu or 200ng DNA 0,2mM primer (each) 0,2mM dNTP's (each) 2mM $MgCl_2$ 1× Supertherm buffer 1U Supertherm Taq

3.3.7 Genomic DNA extraction and PCR screening (Secondary)

In order to identify the presence of pYES2-KanMX/LacZ plasmid sequences, nested primers for the lacZ cassette, described in Table 3.2, were used for colony PCR screening. Additionally, primers KanMX(f) and KanMX(r) were designed for the detection of the KanMX cassette, amplifying the open reading frame in a subsequent colony PCR screen. See Table 3.4 for primer sequences and PCR conditions.

PCR screening with Δ1 and Δ2 as well as KanMX(f) and KanMX(r) were also performed on genomic DNA, extracted by inoculating an overnight culture in YPD at 30 ℃. The cultures were pelleted the following day transferring successively into 2ml microfuge tubes and centrifugation in a benchtop centrifuge at 12000rpm for 30sec. After the supernatant had been discarded, 200µl phenol:chloroform:iso-amylalcohol (25:24:1), 200µl glass beads and 200µl smash-and-grab buffer (1% SDS, 2% Triton X-100, 100mM NaCl, 10mM Tris pH8.0 and 1mM EDTA pH8.0) were added followed by vortexing vigorously for 3min. This was followed by adding 200µl TE and benchtop centrifugation at 12000rpm for 5min. The aqueous phase was then removed and transferred to a new microfuge tube containing 1ml 100% EtOH, which was mixed by inversion and again subjected to benchtop centrifugation at 12000rpm for 2min. The supernatant was then discarded and the pellet resuspended in 400µl TE to which 10µl 4M NH_4OAc and 1ml 100% EtOH was added and mixed by inversion. After benchtop centrifugation at 12000rpm for 2min, the supernatant was discarded and the pellet allowed to dry before resuspending in 100µl dH_2O (7).

3.3.8 Southern Blot hybridization

Southern Blot analysis was initiated by extracting total genomic DNA according to Hoffman and Winston (7) of which 160µg was digested with 5µl ScaI R.E and 1× R.E buffer in a total volume of 100µl. Of this digestion, 40µl was loaded on a 0.7% agarose gel which was electrophoresed overnight for optimal fragment separation. The gel was then submerged in depurination solution (0.25M HCl) for approximately 10min, until the loading buffer front turned yellow). The gel was then rinsed with dH_2O followed by submerging in denaturation solution (0.5N NaOH and 1.5M NaCl) for two repeats of 15min each at room temperature whilst continuously shaking. The gel was again rinsed in dH_2O followed by submerging in neutralization solution (0.5M Tris/HCl pH7.5 and 3M NaCl) for two repeats of 15min each at room temperature whilst continuously shaking. The DNA contained on the gel was overnight blot transferred by capillary action to a Hybond™-N^+ membrane (Amersham, United Kingdom). The DNA was UV-crosslinked to the wet membrane for 3min (DNA side down) followed by a brief rinse with dH_2O. The membrane was then allowed to air-dry before submerging in pre-hybridization solution (5× SSC (20× SSC: 3M NaCl and 0.3M sodium citrate at pH7.0), 50% formamide, 0.1% Na-lauroylsarcosine, 0.02% SDS and 2% blocking reagent) at 42 ℃ overnight. The following day, the pre-hybridization solution was replaced with hybridisation solution (DIG-labelled probe diluted in new pre-hybridization solution; the probe consisted of a ~650bp PCR DIG-labelled probe generated with KanMX(f) and KanMX(r) primers (as recommended by the manufacturers (3); see Table 3.4 for PCR parameters) which was incubated at 42 °C overnight whilst continuously shaking. The membrane was then washed twice for 15min in 2× wash solution (2× SSC and 0.1% SDS) at room temperature whilst continuously shaking. This was followed by two washes of 15minutes in 0.5× wash solution (0.5× SSC and 0.1% SDS) at 68 ℃ whilst continuously shaking. The membrane was equilibrated in DIG washing buffer (maleic acid buffer (0.1M maleic acid and 0.15M NaCl at pH7.5) containing 0.3% Tween 20) for 1minute at room

temperature with shaking. The membrane was blocked by gently agitating in 1× blocking solution (10% w/v skimmed milk in maleic acid buffer; autoclaved) for 2 hours at room temperature. The anti-digoxygenin-AP solution was centrifuged for 1minute at 12000rpm immediately before diluting it 1:10 000 in 1× blocking solution. The blocking solution was discarded and replaced with the antibody solution for 30min at room temperature whilst continuously shaking. The antibody solution was discarded and the membrane gently washed twice in DIG washing buffer for 20min and room temperature with shaking. The DIG washing buffer was discarded and the membrane equilibrated in detection buffer 100mM NaCl and 100mM Tris/HCl at pH 9.5) for 2min at room temperature with shaking. Approximately 30 drops of CSPD-star was added to the detection buffer and allowed to continue shaking at room temperature for a further 5min. The membrane was then removed from the solution, placed between two sheets of transparency film and a liquid seal created around the membrane by wiping the top sheet with tissue paper, removing excessive liquid. The membrane (still between the transparencies) was then placed in an autoradiograph cassette with a standard autoradiograph and exposed for varying periods (from 15min to 24 hours) to obtain optimal exposure. The film was developed by submerging the exposed autoradiograph in fixing solution for 2-5min, rinsing in dH_2O followed by submerging in developer solution until the desired amount of developing has occurred (12).

3.3.9 Determining the site of integration

Determining the site of integration was initially attempted through inverse PCR (differing from conventional PCR in that the primers are designed such that they anneal to the terminals of the know sequence, amplifying in an outwardly fashion, thus amplifying the sequences flanking the known sequence) (4). However, difficulties encountered during the application of this approach led to the use of an alternative method; a subgenomic library was constructed by gel purifying the band indicated by the Southern Blot to contain the inserted KanMX fragment. This purified DNA was then ligated into a pre-existing pGEM®-T Easy construct generated in this lab (pGEM®-T Easy-SMR) by replacing the initial inserted fragment (SMR). Final constructs were transformed into DH5α which was plated on LB agar media containing ampicillin at 100µg/ml (12). Transformants were restreaked on identical media and subjected to colony hybridization by cutting a piece of nylon membrane and 4 pieces of blotting paper to the size of the petri dish to be probed. The 4 pieces of blotting paper were then individually soaked in petri dishes in each of solutions A – D (solution A: 10% SDS; solution B: 0.5N NaOH and 1.5M NaCl; solution C: 0.5M Tris-HCl and 1.5M NaCl at pH7.4 and solution D (2× SSC). The nylon membrane was then pressed against the plate to be probed, generating a replica, which was then placed cells side up over the 4 blotting papers in alphabetical sequence for: solution A = 3min, solution B = 5min, solution C = 5min and solution D = 5min. The membrane was then placed over a dry piece of blotting paper, cells side up, and allowed to air-dry for 30minutes. The membrane was then UV-crosslinked, probed and exposed as discussed in 3.3.8, using the same probe that was used for the Southern Blot hybridization (generated using primers KanMX(f) and KanMX(r)) (12).

Transformants identified to contain the KanMX insert were selected for DNA sequencing (Stellenbosch University Central Analytical Facility. http://academic.sun.ac.za/saf/dnas/services.html), using primers gKanMX(f) {5'GCTGCGCACGTCAAGACTGT3'} and gKanMX(r) {5'CCTATGGAACTGCCTCGGTG3}'.

Additionally, a second chromosome walking based approach was included in order to identify the site of integration for each of the isolates. In this approach, the integrated genomic *S. cerevisiae (*1µg) was digested with 5U SacI, PstI and SphI R.Es individually, in a final volume of 50 µl for 2 hours at 37°C, resulting in fragments with 3' overhangs. Following digestion, the R.Es were inactivated by incubation at 65°C for 10min. A set of single stranded primers (OHP) with 3' overhangs complementary to the 3' overhangs generated by SacI, PstI and SphI digestion were ligated to the digested genomic DNA fragments (10pmol primer and 10µl digested DNA) in the presence of 1×T4 DNA ligase buffer and 3U of T4 ligase in a final volume of 20µl at 4°C overnight. This resulted in a genomic DNA library consisting of fragments with 5' overhanging primer sites. Adapter primers (AP), complementary to these overhangs were then used in combination with gene specific primers (gKanMX(f) and gKanMX(r)) for PCR amplification, allowing specific amplification of only fragments containing the inserted cassette (KanMX). See Table 3.5 for primer sequences and PCR parameter details (1).

Amplified products were gel purified using the illustra™ GFX™ PCR DNA and gel Band Purification kit (GE Healthcare) as recommended by the manufacturer and subcloned into the pGEM®-T Easy vector at a ratio of 1 pGEM®-T Easy vector:3 purified PCR product using 1U T4 ligase and 1× T4 ligase buffer in a total reaction volume of 10µl at 4 °C overnight. Ligations were subsequently transformed into competent DH5α and cultured as discussed in 3.1.1. Plasmid DNA was then extracted as discussed 3.1.2 and insertion was verified through PCR screening with the AP and respective GSP as listed in Table 3.5. Positively identified plasmids were submitted for sequencing to the University of Cape Town sequencing facility (http://www.nucleics.com/DNA_sequencing_support/sequencing-service/university-cape-town-sequencing.html).

Table 3.5 OHP primer, AP and GSP sequence and PCR parameters for chromosome walking

Primer sequence	PCR Cycle	PCR Mix (100µl total volume)
OHP SacI: 5'GAATTCGAGCTCGCCCGGGATCCTCTAGAAGCT3' OHP PstI: 5'GAATTCGAGCTCGCCCGGGATCCTCTAGATGCA3' OHP SphI: 5'GAATTCGAGCTCGCCCGGGATCCTCTAGACAGT3' AP: 5'GAATTCGAGCTCGCCCGGGAT3'	94 °C, 15min 94 °C, 30sec 52 °C, 30sec } ×35 cycles 72 °C, 4min 72 °C, 5min 4 C, ∞ Heating lid,105 °C	1µl DNA library 10pmol AP 10pmol GSP 10mM dNTP's (each) 1× DreamTaq buffer 1U Supertherm Taq

3.4 DETERMINING THE STABILITY OF SPIKED MULTI-COPY PLASMID DNA DURING ALCOHOLIC FERMENTATION

The second 1ml supernatant sample aliquots were used as template in PCR screens to determine the stability of the spiked DNA. Using the nested LacZ primers listed in Table 3.2, the DNA integrity of the spiked multi-copy pYES2-KanMX/LacZ plasmid DNA was establish in ~500bp increments. PCR parameters for this screen are also listed in Table 3.2.

3.5 DETECTION OF THE UPTAKE AND FUNCTIONAL EXPRESSION OF SPIKED LINEAR DNA BY *S. cerevisiae* DURING ALCOHOLIC FERMENTATION

3.5.1 Generating linear DNA

Primers KanMX(f2) and KanMX(r2) (Table 3.1) were used to amplify the KanMX cassette from pYES2-KanMX/LacZ (discussed in 3.1.1) as template DNA in order to generate the linear DNA which could be used to spike the fermentations.

3.5.2 Media and culture conditions

In order to determine whether a 1 in 1 000 000 uptake event could be detected, the linear KanMX PCR product was ligated into an episomal cassette (YEp351-SMR/KanMX) and transformed into *S. cerevisiae* Vin13 to test for functionality (http://www.uniprot.org/taxonomy/317876). Once functionality had been established, this transformed Vin13YEp351-SMR/KanMX was co-cultured with untransformed Vin13 as described in 3.3.2 on selective YPD supplemented with 100µg/ml G418.

3.5.3 Fermentation trials

The fermentation trials were performed as described in 3.3.3, with the exception that all fermentations were performed in 100ml aliquots and KanMX PCR product was used to spike the fermentations.

3.5.4 Selective screening for uptake and expression of spiked linear DNA

Sampling and selective plating was performed as described in 3.3.4 with the exception that the pelleted cells were plated on YPD supplemented with 100µg/ml G418.

All samples were maintained at room temperature and again sampled approximately 3 weeks after inoculation in order to determine the effect of a longer exposure time to high concentrations of ethanol.

3.5.5 PCR screening for spiked DNA stability

The second supernatant sample aliquots were used as template in PCR screens to determine the stability of the spiked DNA. Using the pYES2-KanMX primers listed in Table 3.6, the DNA integrity of the spiked linear DNA (KanMX cassette) was established in ~500bp increments. PCR parameters for this screen can also be found in Table 3.6.

Table 3.6 PCR parameters for amplification and screening of stability of linear KanMX cassette

Primer sequence	**Product size**	**PCR Cycle**	**PCR Mix (50µl total volume)**
Forward primer:		94 °C, 2min	1cfu or 200ng DNA
pYES2-KanMX(f):		94 °C, 20sec }	0,2mM primer (each)
5’ CGCAGCTCAGGGGCA TGATG 3’		58 °C, 30sec } ×30 cycles	0,2mM dNTP’s (each)
Reverse primers:		72 °C, 90sec }	2mM $MgCl_2$
pYES2-KanMX(r1):		72 °C, 5min	0.01mg/ml BSA
5’ CGCATCGGGCTTCCCATACA 3’	~500bp	4 °C, ∞	1× Supertherm buffer
pYES2-KanMX(r2):		Heating lid,105 °C	1U Supertherm Taq
5’ CGGTCTGCGATTCCG ACTCG 3’	~1000bp		

3.6 DETECTION OF WHOLE PLASMID TRANSFER BETWEEN TWO *S. CEREVISIAE* STRAINS VIN13 DURING ALCOHOLIC FERMENTATION

3.6.1 Strains and plasmids

The SMR cassette was PCR amplified using primers SMR-Fp and SMR-Rp from plasmid pDLG42 (this laboratory; Figure 3.4a) as template. See PCR parameters in Table 3.7. The gel purified (Wizard Cleanup Kit) PCR product was then subcloned into pGEM®-T Easy (according to the manufacturers' recommendations), yielding pGEM®-T Easy-SMR (Figure 3.4b). The SMR cassette was then excised from pGEM®-T Easy-SMR by digestion with SphI/PstI R.E combination. YEp351 was also digested with the same R.E combination and the two fragments were ligated at 4 °C overnight, yielding YEp351-SMR (Figure 3.4c). The LacZ cassette was then excised from pYES2-LacZ (discussed in 3.1.1) by digestion with SphI/EcoRV R.E combination and ligated into YEp351-SMR (digested with the same R.E. combination) at 4°C overnight. The resulting plasmid YEp351-SMR/LacZ (Figure 3.4d) was transformed into competent DH5α according to Sambrook *et al*. (12).

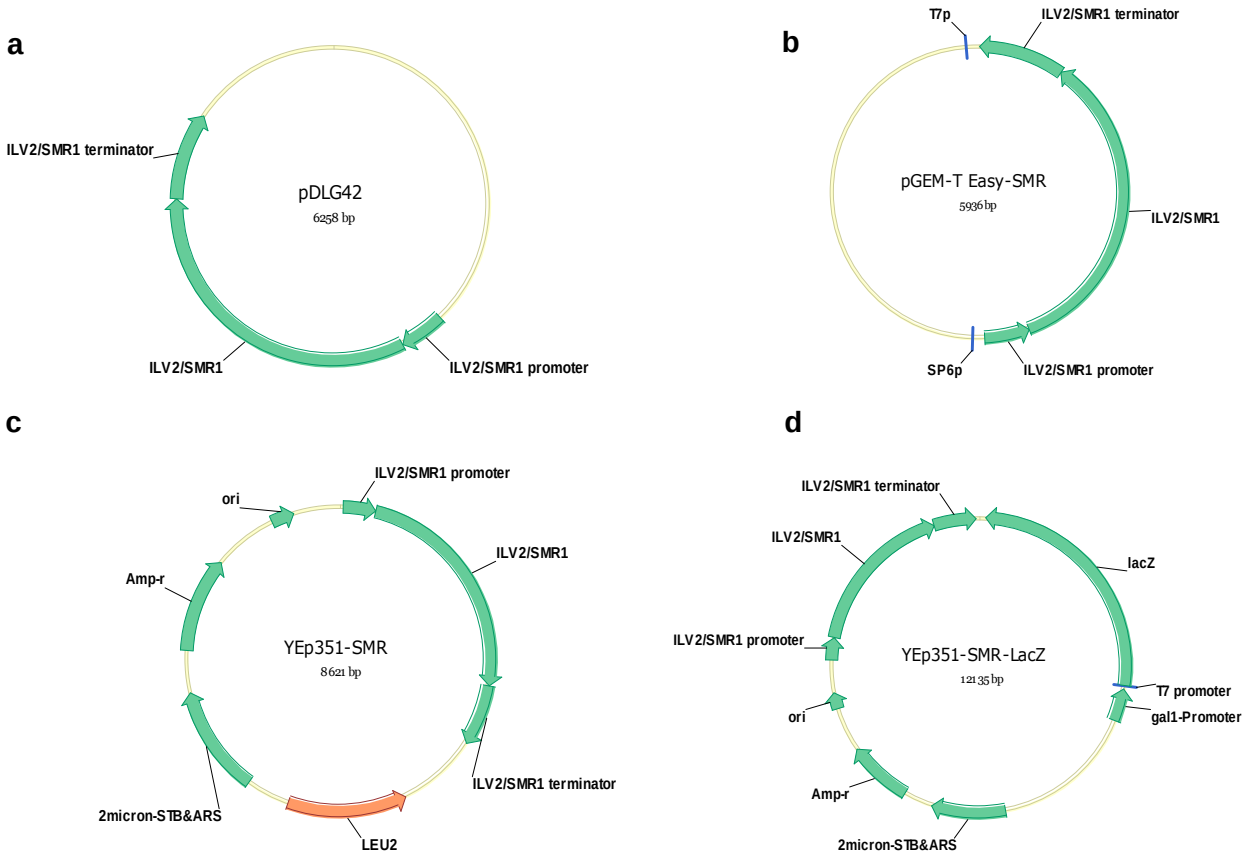

Figure 3.4 Plasmids used for construction of YEp315-SMR/LacZ **(a)** pDLG42 (this laboratory) **(b)** pGEM®-T Easy-SMR **(c)** YEp351-SMR and **(d)** YEp351-SMR/LacZ.

Table 3.7 PCR parameters for SMR cloning and YEp361-SMR/LacZ screening

Primer sequence	**Template**	**PCR Cycle**	**PCR Mix (50µl total volume)**
SMR-Fp: 5'CAGTTGCTGATCTCGGCGCG 3' SMR-Rp: 5'GGTGATGCCGGCCACGATGC 3'	pDLG42 (~2360 bp)	94 °C, 2min 94 °C, 1min 65 °C, 30sec } ×30 cycles 72 °C, 1min 72 °C, 5min 4 °C, ∞ Heating lid,105°C	1 cfu or 200ng DNA 0,2mM primer (each) 0,2mM dNTP's (each) 2mM $MgCl_2$ 1×ExTaq buffer 1U ExTaq Pol
ILV2-F: 5' TAATAATTCAGATCTACGTCACACCGTAATTTG 3' ILV2-R: 5' TACCGATTTGGTAGAATGTCATATATAAGAAGCA 3'	SMR cassette (~2360 bp)	94 °C, 2min 94 °C, 1min 58 °C, 30sec } ×30 cycles 72 °C, 90sec 72 °C, 5min 4 °C, ∞ Heating lid,105 °C	1 cfu or 200ng DNA 0,2mM primer (each) 0,2mM dNTP's (each) 2mM $MgCl_2$ 1× Supertherm buffer 1U Supertherm Taq

3.6.2 Media, culture conditions and overlay assays

Transformed *E. coli* DH5α was cultured in LB media supplemented with 100µg/ml ampicillin at 37 °C whilst continuously shaking. Once the correct construct had been identified through R.E digestion, it was transformed into Vin13 as described by Gietz (5), with the exception that cells were resuspended in a final volume of 100µl distilled water and cultured on YPD media supplemented with 100µg/ml SMM at 30 °C. When culturing on solid media, 20g/l agar was added to the various media types. Vin13 cultures were incubated at 30 °C for 2-5 days.

In addition, an X-gal overlay assay was performed according to the Herskowitz Lab protocol (11) over transformed Vin13 cultured on YPD agar. Colonies displaying ß-galactosidase activity were identified by a blue colour change after incubation at 30 °C for 24 hours. The resulting strain was named Vin13YEp351-SMR/LacZ.

The strain previously described in 3.1.2, Vin13pYES2-KanMX/LacZ, was the second strain to be used for this experiment.

3.6.3 Fermentation trials

The fermentation trials were performed as described in 3.1.3, with the exception that all fermentations were performed in 20ml aliquots. Both strains Vin13YEp351-SMR/KanMX and Vin13pYES2-KanMX/LacZ were simultaneously co-inoculated as described in 3.1.3.

3.6.4 Selective screening for transfer of multi-copy shuttle vectors

Sampling and selective plating was performed as described in 3.1.4 with the exception that only 1ml sample was extracted which was pelleted and plated on YPD agar 100µg/ml G418 as well as 100µg/ml SMM, followed by an X-gal overlay assay (10).

All samples were maintained at room temperature and again sampled approximately 3 weeks after inoculation in order to determine the effect of a longer exposure time to high concentrations of ethanol.

3.6.5 PCR screening

The presence of the KanMX cassette was screened for using primers KanMX(f) and KanMX(r) as described in Table 3.4 and the presence of the SMR cassette using primers ILV2-F and ILV2-R. See Table 3.7 for details. The LacZ cassette was also screened for by using primers LacZ(f) and LacZ(r) described in Table 3.2.

3.6.6 Plasmid extraction from putative whole plasmid transferred Vin13 isolates and subsequent culturing in DH5α

DNA was extracted from the putatively HGT Vin13 cultures according to Winston and Hoffman (7) with the exception that the cultures were first treated with 50µl zymyolyase solution (1M sorbitol, 0.1M sodium citrate, 60mM EDTA, adjusted to pH 7.0, 9mg zymolyase 20T and 50µl β-mercopatoethanol) at 37 ℃ for 1 hour. After havin g precipitated the nucleic acids, the pellet was resuspended in 500µl dH_2O and RNase A treated at a final concentration of 0.1mg/ml for 10min at room temperature. This was followed by an additional ethanol precipitation as before, however, the pellet was resuspended in 50µl dH_2O, which was transformed into competent DH5α according to Sambrook *et al*. (12). Transformed DH5α were cultured on LB media supplemented with 100µg/ml ampicilin.

3.6.7 Plasmid verification through PCR screening and R.E digestion

Transformed DH5α colonies were screened for plasmid presence using primer sets KanMX(f) and KanMX(r) (see Table 3.4 for PCR parameters) and ILV2-F and ILV2-R (see Table 3.7 for PCR parameters).

Isolates indicating the presence of either plasmid were cultured in LB broth (100µg/ml ampicillin) overnight, followed by plasmid extraction according to Sambrook *et.al*. (12). Purified plasmid DNA was subjected to verification through R.E digestion with a HpaI/BamHI R.E combination.

3.6.8 CHEF analysis

Karyotyping through CHEF analysis was performed as discussed in 3.3.6 in order to verify that each isolate was indeed *S. cerevisiae* Vin13.

3.7 ASSESSING THE OCCURRENCE OF HGT WITHIN A BIOFILM

3.7.1 Optimizing molecular probes

Molecular probes were optimized individually and in combination in order to determine which two-dye-combination would yield results. This was done by spotting overnight Vin13 cultures (cultured in 10%YPD and 1% YPD) on glass slides and staining with various molecular probes, followed by confocal scanning laser microscopy (CLSM) imaging using a Zeiss LSM 510 (Carl Zeiss International). See Table 3.8 for details on staining optimization.

3.7.2 Flowcell design

Perspex flowcells were designed and manufactured to contain a 25mm x 4mm x 6mm chamber with a volume of approximately 800µl. The chamber was silicone sealed with two glass microscopic slides on either side, allowing for attachment of a Vin13 biofilm as well as direct visualization through the glass. See Figure 3.5.

3.7.3 Biofilm establishment

The flowcell setup was sterilized by passing 10% bleach through the system at a flowrate of 200µl/min overnight. The bleach was subsequently replaced with distilled water and flushed at the same flowrate overnight. The water supply was then replaced with a YPD (10% and 1%) media supply and allowed to pass completely through the system.

Once YPD media was emitted from the waste outlet, the peristaltic pumps were switched off and the chamber was inoculated with Vin13. By using a syringe and needle, ~3 000 000 cells were injected into the chamber, taking care to avoid inserting bubbles and to seal the incision point in the silicone tubing with a silicone sealant. The peristaltic pumps were switched on, maintaining a flowrate of 200µl/min for four consecutive days, allowing for the development of a mature Vin13 pure culture biofilm on the floor of the flowcell chamber.

3.7.4 Biofilm staining

Based on optimization on microscopic glass slides (see 3.7.1), it was decided to use a Draq5™/Concanavalin A (Sigma-Aldrich Corporation) (Biostatus Limited) combination.

Once biofilm maturity had been reached, the peristaltic pumps were switched off and, using a needle and syringe to inject the probes as described in 3.7.3, staining proceeded according to Table 3.8.

Table 3.8 Molecular probes optimized for staining *S. cerevisiae* Vin13 for CLSM imaging

Molecular Probe	Concentration	Volume Vin13(o/n culture):Probe	Duration	Comment
Nile Red[2]	1mg/ml	1:7	10min	Light sensitive, stain in dark room
Acridine Orange[1]	250µg/ml	9:1	10min	
Concanavalin A, Flourescein conjugate[3]	1mg/ml	1:3	20min	
Ethidium Bromide[1]	5mg/ml	1:1	10min	
Syto9[1]	0.05mM	9:1	10min	Component of Invitrogen Baclight Bacterial Viability Kit
Propidium Iodide[1]	20mM	1:5	1hour (@ 37 °C)	Component of Invitrogen Baclight Bacterial Viability Kit
Draq5™ [1]	5µM	5×10^4cells:15µl	3-30min	

1 - Nucleic Acid probe; 2- Lipid probe; 3 - Cell-surface carbohydrate probe

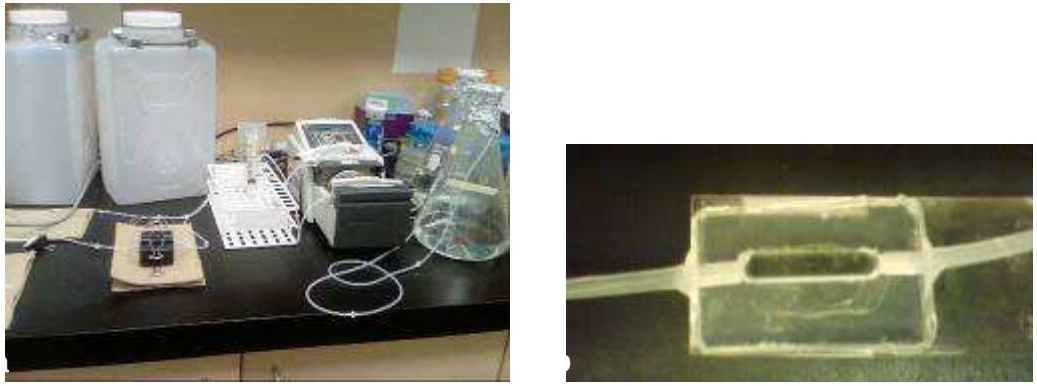

Figure 3.5 Flowcell design for developing biofilms (a) Flowcell setup with fresh media continuously being pumped through the flowcell by means of a peristaltic pump, to the outflow of waste on the left. **(b)** Perspex flowcell containing a 25mm x 4mm x 6mm chamber, sealed with glass microscopic slides on either side.

3.7.5 Biofilm visualization through CLSM imaging

Stained biofilms were visualized on a Zeiss LSM 510 CLSM (Carl Zeiss Inc. – Germany) equipped with Argon (458nm, 488nm, 514nm), HeNe1 (543nm), HeNe2 (633nm) and Titanium\Sapphire (780nm) lasers. The Draq5™ probe was excited with an Argon laser at 488nm and emission was detected using a 715LP filter. Similarly, the Concanavalin A-flourescein conjugate was excited with an Argon laser at 488nm and emission was detected using a 505LP filter.

Mature biofilms were established as discussed in 3.7.3 (using 10% YPD and 1% YPD) with the exception that after day 2 of biofilm development, the YPD media supply was spiked with pYES2-KanMX/LacZ at a concentration of 5µg/ml. This plasmid containing YPD was passed over the growing biofilm at a flowrate of 200µl/min for an additional two days after which the biofilm was harvested from the glass slide, resuspended in 200µl and plated on YPD agar supplemented with 100µg/ml G418.

3.8 LITERATURE CITED

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Chapter 4

Research Results

Horizontal Gene Transfer in wine fermentation environments

Results

4.1 INTRODUCTION

Horizontal gene transfer can theoretically occur through a number of ways. The process may involve physical contact between two cells or simply rely on the release of DNA into the environment followed by its uptake by another (30,48). Another well documented method of HGT involves viral mediated transfers (30). In the case of yeast, the latter is unlikely to be of relevance, since no viruses are known to infect this organism. However, it should be noted that the yeast genome contains a significant number of transposable elements that show certain molecular features usually associated with viral replication and transmission (3). Whether such elements could be functioning as carrier molecules that support horizontal transfers has not been investigated thus far.

In the present study, the possibility of the occurrence of HGT between cells of the yeast *S. cerevisiae* was investigated. Since the study places itself within the context of the assessment of potential risks associated with genetically modified organisms, the investigation focused in particular on establishing the likelihood of the transfer of a trait that has been genetically engineered. The study also focused on assessing such risks in an environment close to those found in industrial fermentations, and more specifically, in those found during wine making.

Individual steps that were monitored include the possible release of free DNA by industrial strains of *S. cerevisiae* into the environment, the stability of such free DNA in the environment, the possibility that such free DNA could be taken up by other viable cells under such conditions, and finally whether events of direct transfer of DNA between cells co-existing in the industrial environment, including in the form of biofilms, could be identified.

The release of DNA could be favoured by several factors, including stressful environmental conditions that may result in compromised cell walls and/or organelle membranes, resulting in DNA leakage into the environment (9,24). Another phenomenon that could contribute to DNA release is autolysis. While the total cellular content is released under these conditions, it is usually assumed that macromolecules, in particular DNA, are digested before release, resulting in only small fragments being spilled into the surrounding environment (42). If larger fragments of DNA containing significant biological information, i.e. entire genes or fragments encoding functional domains therefof, were released, viable cells within the environment may possess a natural competency and enable them to accept such released DNA (36).

Since the aim of this study was to investigate the potential for HGT under wine making conditions, experimental conditions were adjusted to imitate such environments. However, since various stress conditions appear to favour DNA transfers in laboratory transformation protocols used for the experimental introduction of free DNA into cells in a process referred to as transformation or transfection, several stress conditions were also experimentally applied.

Three conditions in particular were selected for this purpose, including sudden increases in ethanol concentrations at the beginning of fermentation, the application of periodic heat shocks and increases in cell densities beyond the usually observed values. In theory, by exposing the fermenting cultures to increased levels of ethanol, cellular membrane integrity would be further compromised, generating permeable cells which could be prone to both increased release and uptake of DNA (22). Also, during routine laboratory transformation protocols, cultures are transformed by a process involving heat shock treatment (33). For this reason, heat shock was included as a stress condition. Industrial wine fermentations may sometimes experience significant temperature variations, for example in cases of malfunctioning cooling systems, and

such variations have the potential to induce serious heat stress. In addition, increasing the numbers of cells should lead to enhanced physical contact, while also leading to rapid nutrient depletion, enhancing competitive behaviour for survival (28).
Furthermore, this study investigated several factors that may contribute to HGT. The stability of free-floating DNA within the fermenting media was determined within various fermenting media throughout the course of alcoholic fermentation. In addition, an initial model system to study the possible occurrence of HGT in a biofilm environment was established. Based on the close physical proximity of cells and harsh competition for survival within biofilm structures, it is believed to provide another potentially favourable environment for the occurrence of HGT events (34).

4.2 DETECTION OF DNA RELEASE FROM *S. cerevisiae* DURING ALCOHOLIC FERMENTATION

A first step of horizontal transfer of DNA may involve the release by damaged or dead cells of free DNA into the environment (42). For such a release to lead to an effective transfer of genetic information, certain requirements would have to be met. Firstly, for a transfer to be of measurable impact from a pheno- and genotypic perspective, the released DNA should consist of fragments above a certain size. Indeed, DNA fragments shorter than those that can code for a functional protein or domain thereof, even if taken up and integrated into the genome of a recipient cell will not lead to a transfer of a specific character or trait. While it can be argued that such transfers of short fragments may have some mutagenic potential, they would certainly not meet the standard definition of a horizontal gene transfer since no specific transfer of biological information would be associated with the event. While the “functional” size of a DNA fragment varies according to the encoded character, this uncertainty can be overcome when monitoring the transfer of genes from a genetically modified background, where the minimum size can be clearly defined. In a risk assessment process like this, this size would be defined by the minimal length of DNA fragment that would contain the relevant coding regions of the gene(s) encoding the modified trait.

A second consideration that impacts on the probability of free DNA being taken up is related to the stability of such DNA in the specific environment. Indeed, it can be argued that the likelihood of an HGT event will increase with the time that the free DNA will persist in the environment without being too severely degraded. The agent most suspected of impacting this scenario is the presence of DNases (42). Additionally, if indeed released DNA is maintained stably in sizes of genetic significance, the success of an HGT involving this DNA relies on further physical attributes of the DNA in question. It is yet unknown whether compounds in the environment would impact the ability of release such DNA to be taken up by yeast.

Another factor to consider is the natural “competence” of the cultures present during such DNA release events. Although no data is available regarding the existence of naturally “competent” yeasts, this study will unveil whether this can be overcome by means yet unknown.

If in fact DNA is found to undergo HGT into a new host, its perseverance is subsequently influenced largely by selective pressure. If the DNA under investigation is of such a nature that its presence provides a selective advantage to those organisms which have accepted and can express it, selection pressure will allow these organisms to out-perform its untransformed counterparts, permitting persistence of the newly transferred DNA in the new host organism.

To monitor whether free DNA was released into the environment by yeast cells under conditions mimicking wine fermentation and to assess the average length of such DNA, a series of nested PCR experiments was designed. To increase the likelihood of such a release, the

cultures were subjected to certain stress factors, increasing the likelihood of dead or damaged cells in the environment, which could lead to more DNA being released (13,20,22,35). While unlikely, it is noteworthy that such stresses occasionally occur during industrial fermentations (27), and that the experimental setup is therefore not far removed from true conditions in such settings.

4.2.1 Detection of released multi-copy plasmid DNA from *S. cerevisiae* during alcoholic fermentation

The first type of DNA to be investigated in terms of release from *S. cerevisiae* Vin13 during alcoholic fermentation was the multi-copy episomal plasmid, pYES2-KanMX/LacZ. Plasmids are small circular nucleic acid structures, containing several genetic elements allowing for autoreplication, increased genetic stability and selective markers. The combinations of such elements enable plasmids to exist as independent molecular structures within the nucleus. Several aspects of plasmid biology make them ideal tools for the investigation of HGT. Certain plasmids types, such as the multi-copy plasmid (as used in this case), exist in large numbers, from 40 to more copies within host cells. In addition, such plasmids often contain genetic elements enabling them to persist within unrelated organisms, an important aspect in standard laboratory transformation processes where plasmids are routinely shuttled between prokaryotes and eukaryotes (30).

For these same reasons, plasmids may serve as ideal tools for the monitoring of HGT. Their circular nature may also favour their stability as free DNA and enhance their potential to be involved in this process. One has however to consider that the probability of plasmid structure being maintained within the harsh fermenting environment is dependent on DNase activity (42).

This section is thus aimed at investigating the probability of the release of plasmid DNA from Vin13 cultures that had been transformed with a multi-copy plasmid and subsequently subjected to stress conditions believed to be to favour such events.

4.2.1.1 Strain identification and selective plate screening

S. cerevisiae strain Vin13 was transformed with the multi-copy plasmid pYES2-KanMX/LacZ, generating strain Vin13pYES2-KanMX/LacZ which was plated on selective YPGal agar supplemented with G418 (100µg/ml). Plasmid presence and functionality was verified by performing an X-gal overlay assay over the cultured strain (Figure 4.1).

Figure 4.1 Vin13pYES2-KanMX/LacZ on YPGal agar supplemented with 100µg/ml G418 with an X-gal overlay assay.

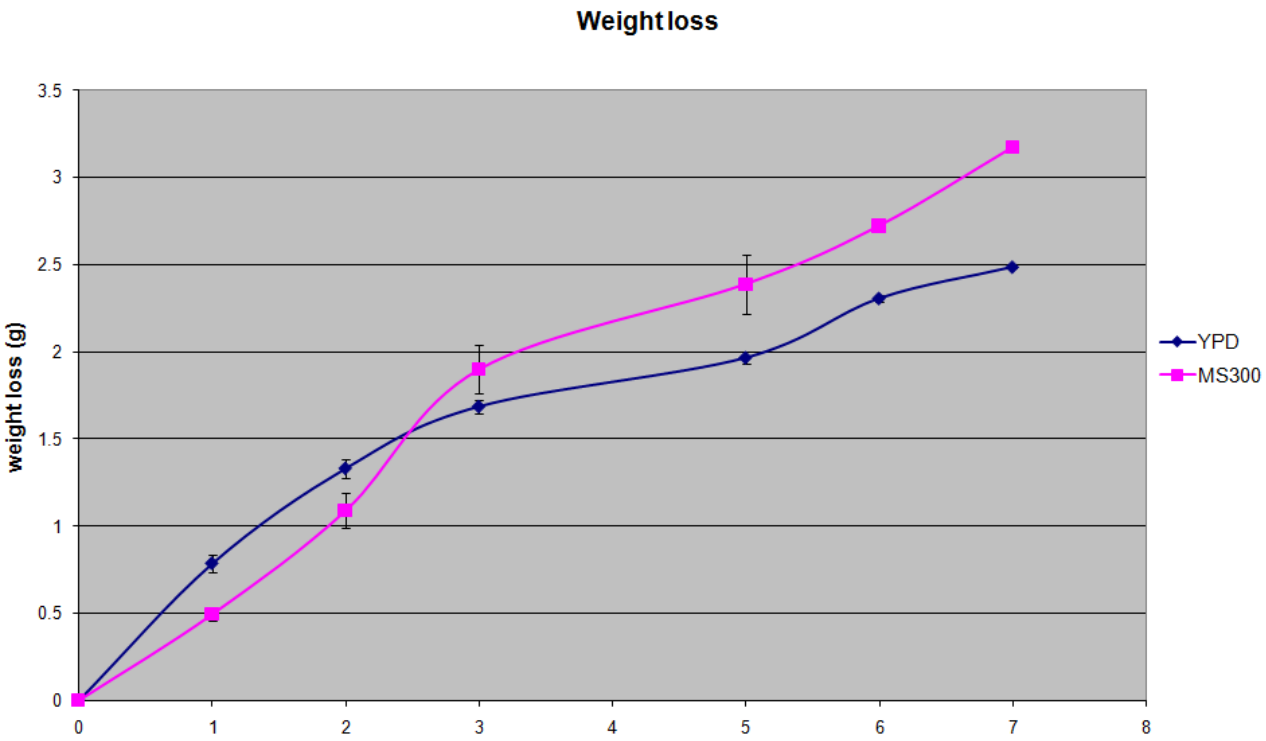
The ability of this strain to grow on this media indicates the newly acquired resistance of Vin13 to G418 through the presence of the KanMX cassette from pYES2-KanMX/LacZ and the observed colour change from white to blue indicates the functional expression of β-

galactosidase from the LacZ cassette of pYES2-KanMX/LacZ. When untransformed Vin13 was plated on the same selective media, no growth was observed, indicating true selective conditions imposed by the presence of G418 antibiotic rendering untransformed susceptible Vin13 not viable.

4.2.1.2 Fermentation trials

In order to determine whether cells within a particular growth phase were more or less susceptible to the selected stress conditions in terms of DNA release, or otherwise stated, whether cultures were more or less prone to release nuclear content during certain growth phases, stresses were imposed upon the fermenting cultures on selected days. As discussed earlier, the stress conditions decided upon consisted of increased ethanol concentrations and periodic heat shocks. The heat shocks, however, required manual administration. In order to determine the key points at which fermentations would be heat shocked, the growth curves for Vin13 were established in MS300 and YPD (10% glucose). These media types were selected because they are of controlled composition and therefore allow reproducible measurements (Figure 4.2). While MS300 mimics the composition of grape must, at least with regard to its major components, real grape must was included at a later stage as a fermentation medium. The chemical composition of natural grape must is highly variable and complex, and it may contain compounds that could contribute to or generally impact on the occurrence of gene transfers.

(a)



(b)

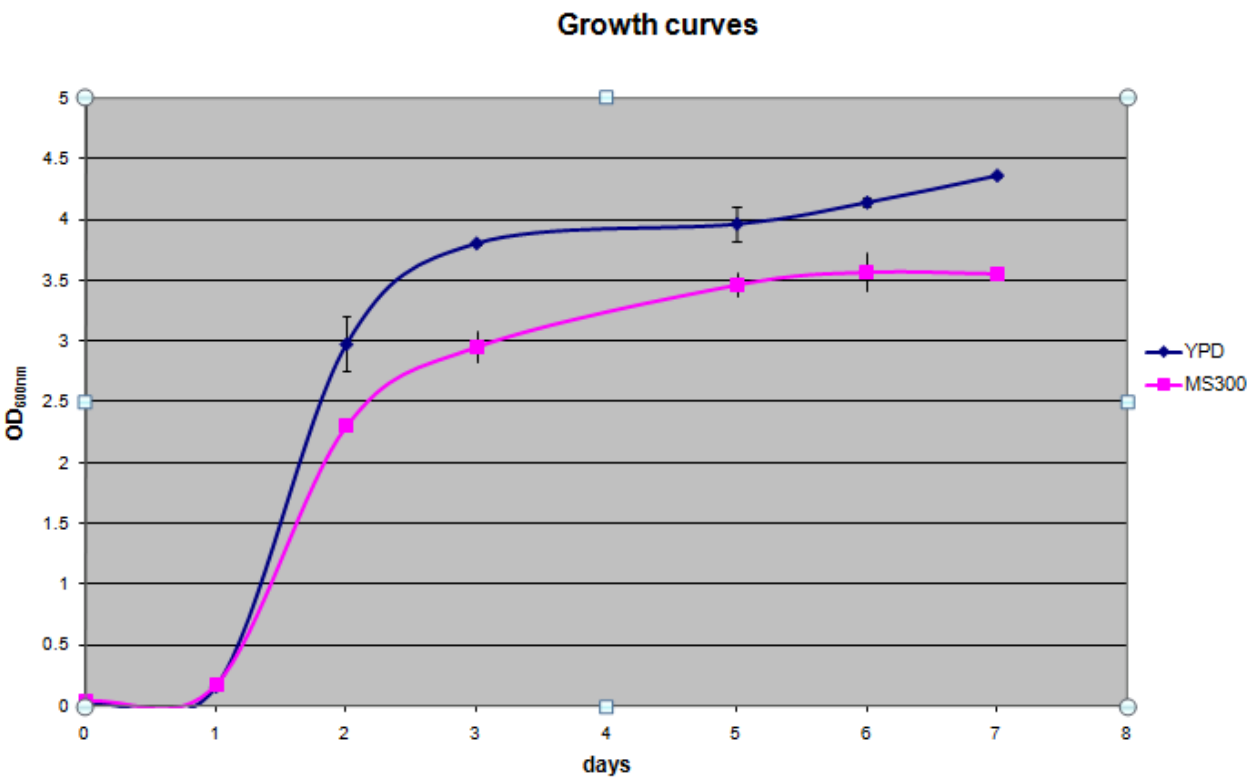


Figure 4.2 Fermentation data reflecting the **(a)** weight loss and **(b)** growth curves observed in both YPD and MS300. The data reflect the progress of fermentation by *S. cerevisiae* across a period of 9 days, allowing for the identification of each growth phase experienced and duration thereof.

The data indicate that the strains show an overall lag phase of 24 hours followed by a sharp increase in cell numbers which starts to plateau by day 3. As the weight-loss indicates, CO_2-release continues at this stage. This behaviour is a typical feature of high-sugar fermentations, where entry into stationary phase is induced by mounting ethanol concentration, and not nutrient depletion. In these conditions, the cellular growth slows and ceases entirely after some time, yet the cells remain metabolically active and continue to ferment the remaining sugar. By day 7, measurement of sugar concentrations indicated that all sugar had been exhausted at this stage (data not shown). This final stage therefore corresponds more to the classical stationary phase characterised by reduced metabolic activity observed under laboratory conditions.

From these data, it was decided to sample and heat shock the cultures at several stages representative of all growth phases. The selected time points were after 24 hours, at days 2, 4, 5 and 6, 8, 11 and 13.

All fermentations were conducted at room temperature. Stresses due to increasing ethanol and high cell densities occur as a normal consequence of the fermentation process. However, heat shock, while sometimes occurring during industrial fermentations (27), had to be applied manually. The shock was administered at the predetermined time points by submerging the whole fermenting culture into a waterbath at 42 ℃ for 30min.

The fermentations were allowed to proceed for 13 days, during which time CO_2 production was monitored through determining the total weight loss of fermenting culture volumes, thus reflecting fermentation progress (data not shown).

4.2.1.3 PCR optimization

Based on the robustness, speed, sensitivity, accuracy and versatility of PCR technology (16), it was an obvious choice for the method of screening for released DNA. Before initiating these PCR screenings on the filtered supernatants, the functionality of the specially designed LacZ nested primers were verified (Figure 4.3), yielding products increasing with ~500bp with the use of each successive reverse primer.

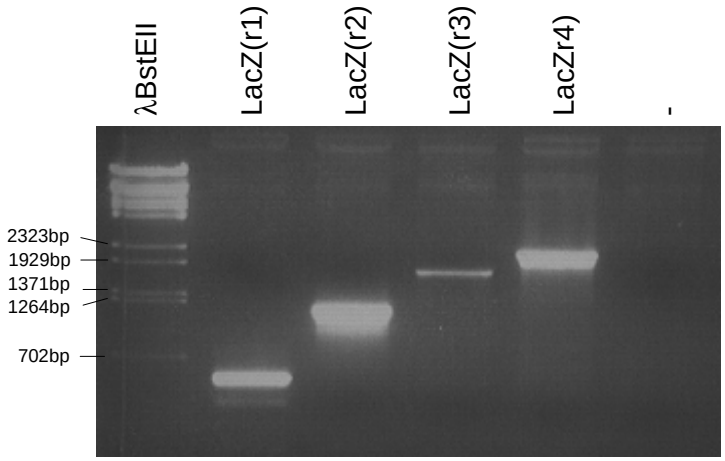
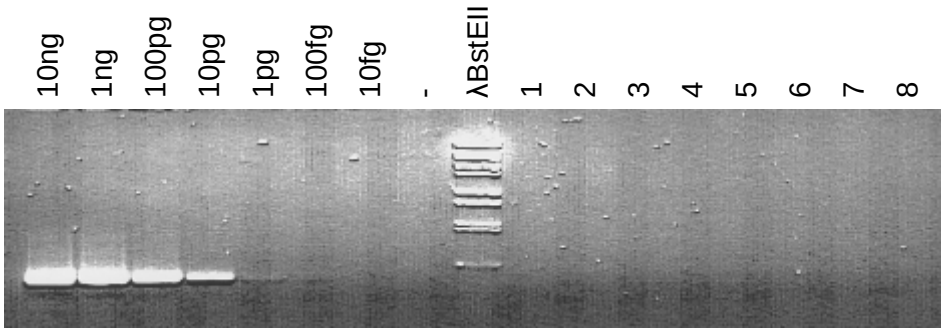


Figure 4.3 PCR with nested LacZ primers yielding products incrementing by ~500bp. i.e. using LacZ(f) in combination with: LacZ(r1) primer yields a product of ~500bp, LacZ(r2) primer yields a product ~1000bp, LacZ(r3) primer yields a product ~1500bp and LacZ(r4) yields a product of ~2000bp. The absence of a band in the – lane Refer to Table 3.2 for primers and PCR conditions.

The results indicated successful detection of the LacZ cassette from pYES2-KanMX/LacZ as template. However, applying these primer sets for the detection of the LacZ cassette in fermentation supernatants proved more complex. In order to accurately interpret the results, a reference was required in terms of the sensitivity of each PCR screen. By including a dilution template range in each screening of the samples, the minimum detection levels within the various fermenting backgrounds could be established. The parameters assessed throughout these optimizations included annealing temperatures, dNTP, $MgCl_2$, primer and supernatant concentrations (as template) as well as the final PCR volume. PCR enhancing agents such as bovine serum albumin were also employed. It was observed that samples taken in the earlier stages of fermentation required less optimization to achieve the desired sensitivity threshold than samples taken during the later stages of fermentation, possibly explained by the inhibiting effect of accumulated phenolic compounds as fermentation progressed (31,41). After having determined the optimal conditions for each primer set for each of the various time-points sampled, the general sensitivity of the PCR screens were optimized to detect ~100fg template DNA per 100µl PCR volume (Figure 4.4).

(a)



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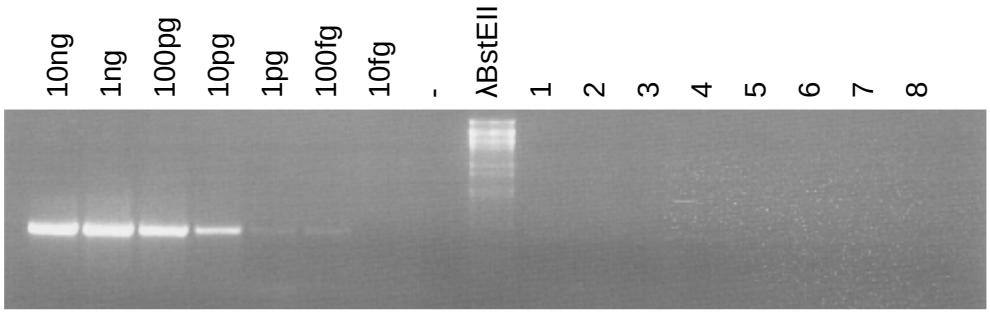


(c)

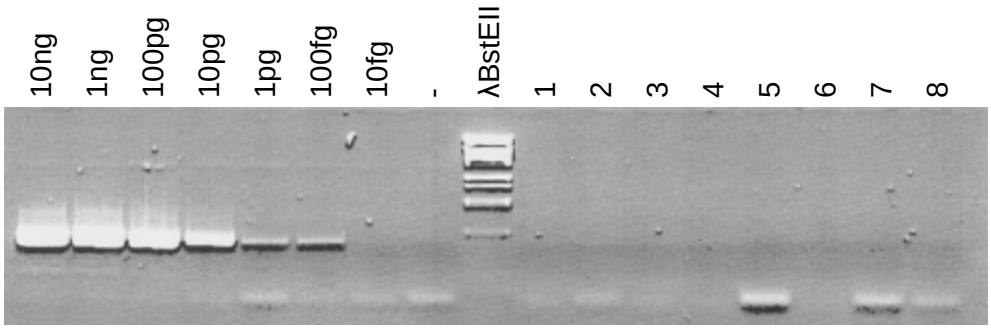


Figure 4.4 Representative PCR screens of supernatants from fermenting **(a)** MS300 taken on day 2, **(b)** YPD (10% glucose) taken on day 4 and **(c)** filter sterilized grape must taken on day 9, using primers LacZ(f) and LacZ(r1). Lanes 1 - 8 represent the screened filtered supernatants from each stress condition, with 1 & 5 taken from the standard fermentation set, 2 & 6 from the increased ethanol set, 3 & 7 from the increase OD_{600nm} set and 4 & 8 from the heat shock set (1 – 4 = Vin13pYES2-KanMX/LacZ fermentations and 5 – 8 = Vin13 controls); 10ng - 10fg indicates the template (purified pYES2-KanMX/LacZ plasmid DNA) dilution range screened in 2% fermenting media background taken from the standard Vin13 fermentation set on the same day as each of samples 1 - 8, yielding a product of ~500bp, as expected. – represents the negative control containing only ddH_2O as template.

As demonstrated in Figure 4.4, the decrease in intensity of the PCR product corresponds to the decrease in template concentration, reflecting the sensitivity of each individual screen. The negative control indicated PCR reactions were free of contamination.

By using online algorithms for calculating molecular conversions (http://www.unitconversion.org/weight/daltons-to-femtograms-conversion.htm and http://www.rcgld.org/otherwebs/labfaqs/main/MW.php) it was determined that 100fg pYES2-KanMX/LacZ corresponds to approximately 17.55×10^4 copies of pYES2-KanMX/LacZ,

suggesting that all PCR screens were sensitive enough to detect the presence of approximately 17.55×10^4 copies of pYES2-KanMX/LacZ. Referring to Figure 4.2, if the OD_{600nm} of cultures fermenting in YPD (10% glucose) is estimated to be approximately 3.9 on day 4, this value corresponds to the presence of approximately 7.5×10^6 cells/ml of fermenting media, given that an OD_{600nm} of 0.1 is equal to approximately 3×10^6 cells/ml. Bearing in mind that each cell contains approximately 60 copies of the multi-copy pYES2-KanMX/LacZ plasmid (12), Figure 4.4 shows that our PCR screen was sufficiently sensitive to detect the presence of DNA in the fermenting supernatant if only 0.25% of the fermenting culture had lysed to release this plasmid DNA into the fermenting supernatant. It was also established that a maximum of 2µl supernatant could be used as template in a minimum of 100µl PCR reaction volume. Using more template/fermentation supernatant or a smaller total PCR volume resulted in insufficient dilution of the inhibiting fermented background media, yielding unsatisfactory PCR sensitivities or often no PCR product (data not shown).

4.2.1.4 Sampling and PCR screening

In order to determine whether DNA was released to the detectable levels described above during the course of alcoholic fermentation, the stressed fermenting broths were continuously sampled. Assuming that the supernatants contained released DNA and could thus serve as a template for a PCR screen to detect the presence of the LacZ cassette from pYES2-KanMX/LacZ using specially designed primers, the sampled supernatants were immediately 0.45 µm filter sterilized and used in subsequent PCR screens. It was also assumed that if plasmid DNA had indeed been released, all regions of the plasmid would be equally represented in the supernatant. In order to account for the possible degradation of released DNA (through mechanical damage or digestion through R.E activity (42)), a set of nested primers were designed for the detection of the LacZ cassette, capable of detecting the presence thereof in a series of products increasing with ~500bp with the use of each successive reverse primer.

During such PCR screens, the presence of any (viable or non-viable) transformed Vin13 cells within the sampled supernatant could effectively serve as DNA template and lead to false positive results, thus, in order to verify the absence of any Vin13pYES2-KanMX/LacZ, the filter sterilized supernatants were spotted on selective YPGal agar supplemented with G418 at a concentration of 100µg/ml (Figure 4.5).

Figure 4.5 Filter sterilized fermenting YPD (with 10% glucose) supernatants of the fermentations under discussion (1 = Vin13pYES2-KanMX/LacZ standard, 2 = Vin13pYES2-KanMX/LacZ increased ethanol, 3 = Vin13pYES2-KanMX/LacZ increased $OD_{600nm,}$ 4 = Vin13pYES2-KanMX/LacZ heat shocked, 5 = Vin13

standard, 6 = Vin13 increased ethanol, 7 = Vin13 increased $OD_{600nm,}$ 8 = Vin13 heat. Vin13pYES2-KanMX/LacZ was included as a positive control (+) shocked).

Selective plate screening displayed no growth from any of the spotted filtered supernatants, indicating that all viable cells were successfully removed before commencing with subsequent PCR screens. It is also assumed that if all intact viable cells were successfully removed through this technique, all intact non-viable cells were also successfully removed. Identical results were observed for the filter sterilized fermenting MS300 and filtered grape must supernatants (data not shown).

Each of the various fermenting medium types under each of the various stress conditions (including the untransformed Vin13 fermentations as controls) were continuously sampled, filter sterilized and subjected to PCR screening for released pYES2-KanMX/LacZ DNA, using the nested LacZ primers (Table 3.2). The results displayed in Figure 4.4 are a representative example of the results that were obtained, since all screens showed identical results and no released DNA could be detected within the fermenting supernatants under these conditions. It is possible that larger DNA fragments could have been present within the fermenting culture media, but may have been trapped along with other cellular constituents within the filter matrix, thus removing such possible DNA templates from the subsequent PCR screening procedures.

4.2.2 Detection of released integrated DNA from *S. cerevisiae* during alcoholic fermentation

In order to evaluate whether the DNA type plays a role in the frequency of released DNA or the accessibility thereof within the various fermenting medium types under investigation, genomic DNA was also considered in terms of release from *S. cerevisiae* Vin13 during alcoholic fermentation. Although genomic DNA would *a priori* not appear to present the same potential of release than plasmid DNA, this study nevertheless addressed this scenario by performing fermentations with Vin13pDLG31. This is a genetically modified Vin13 strain containing an integrated copy of the LKA1 and SMR cassettes (15). Assuming that DNA release into the environment brought about by cellular damage or autolysis provides accessibility to all regions of the genome (22,37), the fate of the transgenic DNA can be traced through PCR screening with specially designed primers and in doing so, assess the risk involved with regards to HGT when using integrated DNA.

4.2.2.1 Strain identification and selective plate screening

Before initiating the various fermentations, the identity of the Vin13pDLG31 strain was confirmed by karyotyping (data not shown) and the functionality of the integrated cassette was assessed through selective plating on YPD agar supplemented with 100µg/ml SMM (Figure 4.6a). In addition, the production of α-amylase (encoded by the LKA1 cassette) allows for starch degradation, enabling the transformed Vin13 to utilize starch as sole carbon source. This was confirmed on SC Phadebas agar supplemented with 100µg/ml SMM at 30 ^{o}C for 2-5 days (Figure 4.6b), noted by the formation of halos around transformed cfus as starch is degraded for its use as a carbon source.

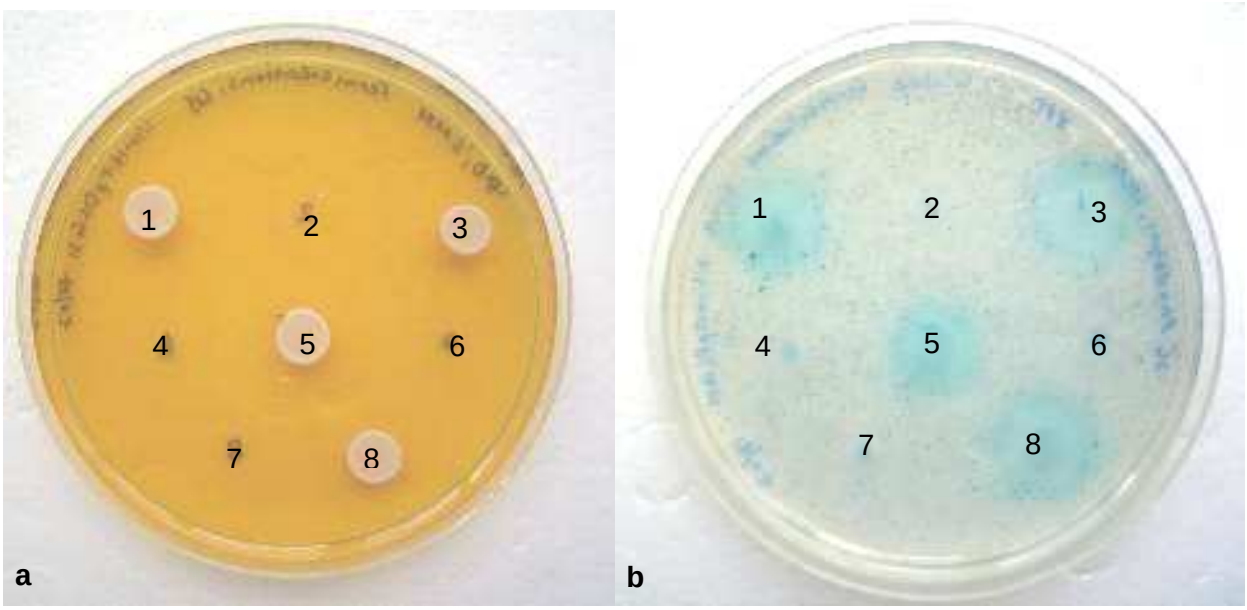


Figure 4.6 Vin13pDLG31 functionality verified by spotting fermenting cultures on **(a)** YPD agar supplemented with 100µg/ml SMM (1, 3, 5 and 8) and **(b)** SC Phadabas agar supplemented with 100µg/ml SMM (1, 3, 5 and 8). As control, Vin13 was spotted (2, 4, 6 and 7).

The ability of the spotted Vin13pDLG31 cultures to grow on YPD agar supplemented with 100µg/ml SMM (Figure 4.6a) indicates the functional expression of the SMR cassette, conferring SMM resistance. The clear hallow visible around Vin13pDLG31 cultures spotted on SC Phadebas agar supplemented with 100µg/ml SMM (Figure 4.6b) is a result of starch degradation and utilization, proving functionality of the LKA1 cassette. When untransformed Vin13 was spotted on either of these medium types, no growth was observed, indicating true selective conditions.

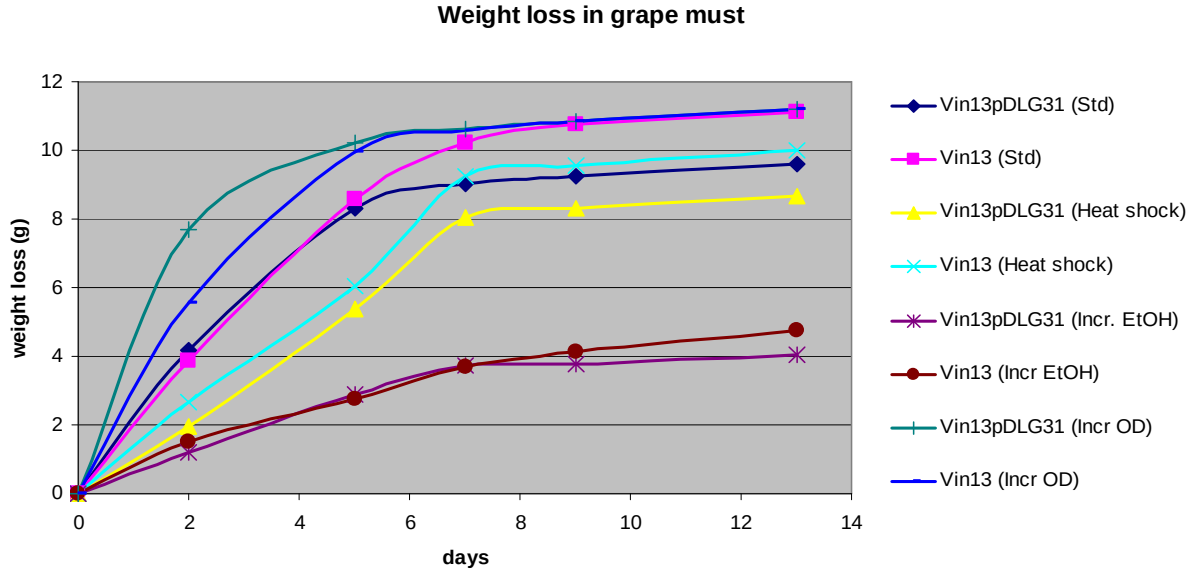
4.2.2.2 Fermentation trials

As in 4.2.1.2, all stressed and control fermentations were allowed to proceeded for 10-14 days, during which weight loss was continuously monitored (Figure 4.7). YPD (10% glucose) was omitted as a fermentation medium type from this study.

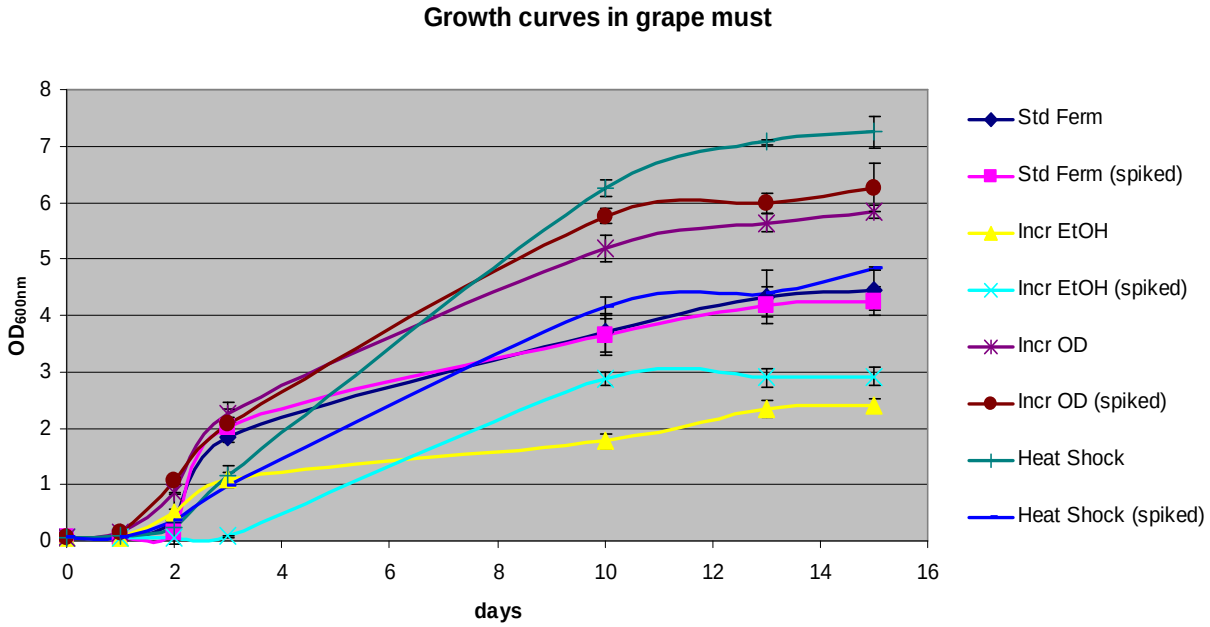
(a)


Weight loss in MS300
weight loss (g)
days
0
1
2
3
4
5
6
7
8
Vin13pDLG31 (Std)
Vin13 (Std)
Vin13pDLG31 (Heat shock)
Vin13 (Heat shock)
Vin13pDLG31 (Incr. EtOH)
Vin13 (Incr EtOH)
Vin13pDLG31 (Incr OD)
Vin13 (Incr OD)

(b)


Weight loss in grape must
weight loss (g)
days
0
2
4
6
8
10
12
14
Vin13pDLG31 (Std)
Vin13 (Std)
Vin13pDLG31 (Heat shock)
Vin13 (Heat shock)
Vin13pDLG31 (Incr. EtOH)
Vin13 (Incr EtOH)
Vin13pDLG31 (Incr OD)
Vin13 (Incr OD)

(c)



(d)

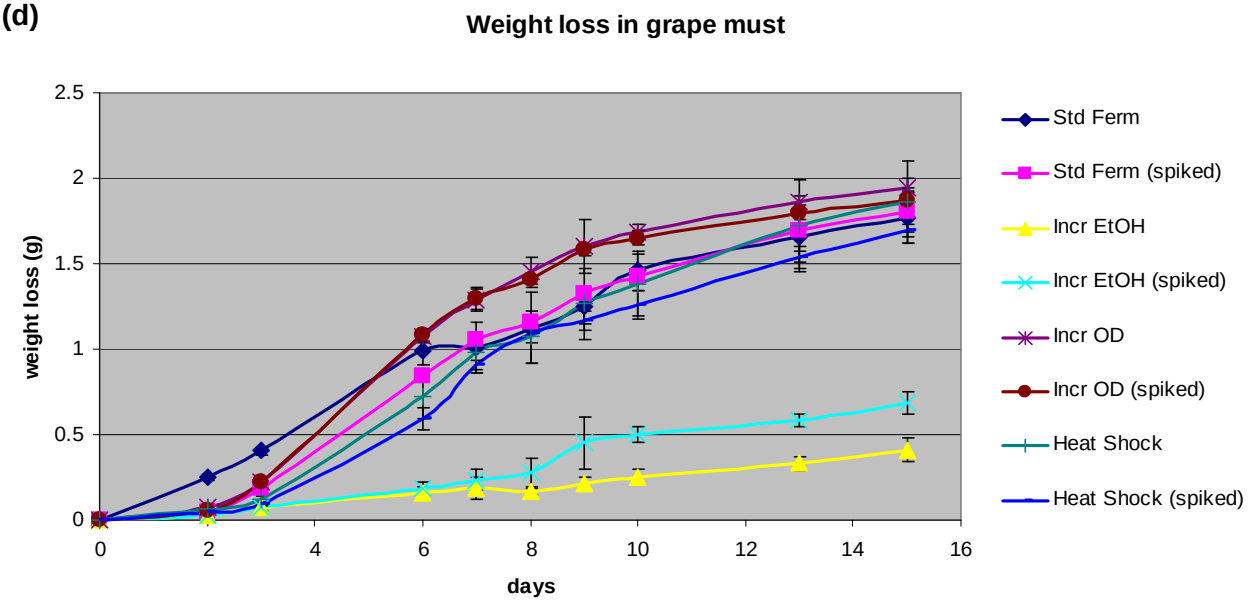


Figure 4.7 Fermentation data of Vin13pDLG31 fermenting media; weight loss observed in **(a)** MS300 and **(b)** 0.45µm filter sterilized grape must and growth curves determined in **(c)** MS300 and **(d)** 0.45µm filter sterilized grape must. Single measurements were taken for each fermentation set.

Data shown in Figure 4.7 demonstrates a general trend of the culture weight loss to plateau by day 2 or 3 for the MS300 fermentation sets, however, the filtered grape must sets show the

increase in weight loss to continue for several days after weight loss had ceased in MS300. This is possibly a consequence of higher sugar concentrations in the filtered grape must. We also observe a lower total weight loss for the increased ethanol fermentation sets, demonstrating the inhibiting effect of the ethanol on yeast cultures. This is reflected in the corresponding growth curves in both media types (c & d), where the cultures with initially increased ethanol concentrations experience difficulty growing exponentially. This scenario is similar to sluggish fermentation, a common occurrence in wine making conditions (32).

4.2.2.3 PCR optimizing

As with the LacZ primers in 4.2.1.3, PCR screens were designed to detect the presence of the LKA1 cassette from Vin13pDLG31 through a series of nested primers, with each successive reverse primer yielding a product incrementing by ~500bp. Thus, before initiating PCR screening on these filtered supernatants, PCR conditions were optimized for the nested LKA1 primers (Table 3.3) (Figure 4.8).

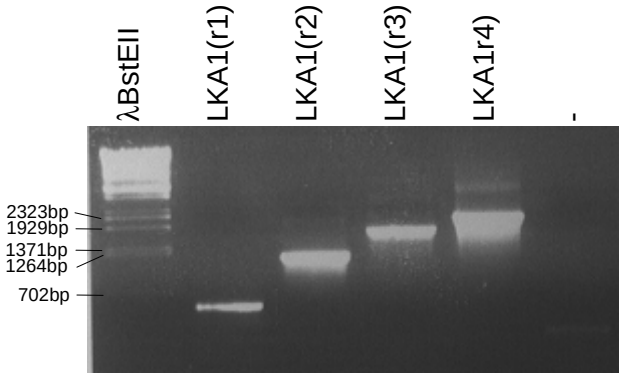


Figure 4.8 PCR with nested LKA1 primers yielding successive products incrementing by ~500bp, i.e. using the LKA1(f) primer in combination with: LKA1(r1) yields a product of ~500bp, LKA(r2) primers yields a product of ~1000bp, LKA(r3) primer yields a ~1500bp product and LKA1(r4) primer yields a ~2000bp product from purified Vin13pDLG31 genomic DNA as template, as expected. Refer to Table 3.3 for details on primers and PCR conditions. – represents the negative control where only ddH_2O was used as template.

The results displayed in Figure 4.8 indicate successful PCR in terms of detecting the LKA1 cassette from genomic DNA isolated from Vin13pDLG31 as template, yielding products incrementing by ~500bp with each successive reverse primer used. The absence of a PCR product in the negative lane demonstrates that the PCR screen was contamination free.

As in 4.2.1.3, the minimum detection levels within each of the various fermenting media types required extensive optimizing and were successfully optimized to detect ~1ng purified Vin13pDLGG31 genomic DNA per 100µl PCR volume (Figure 4.9).

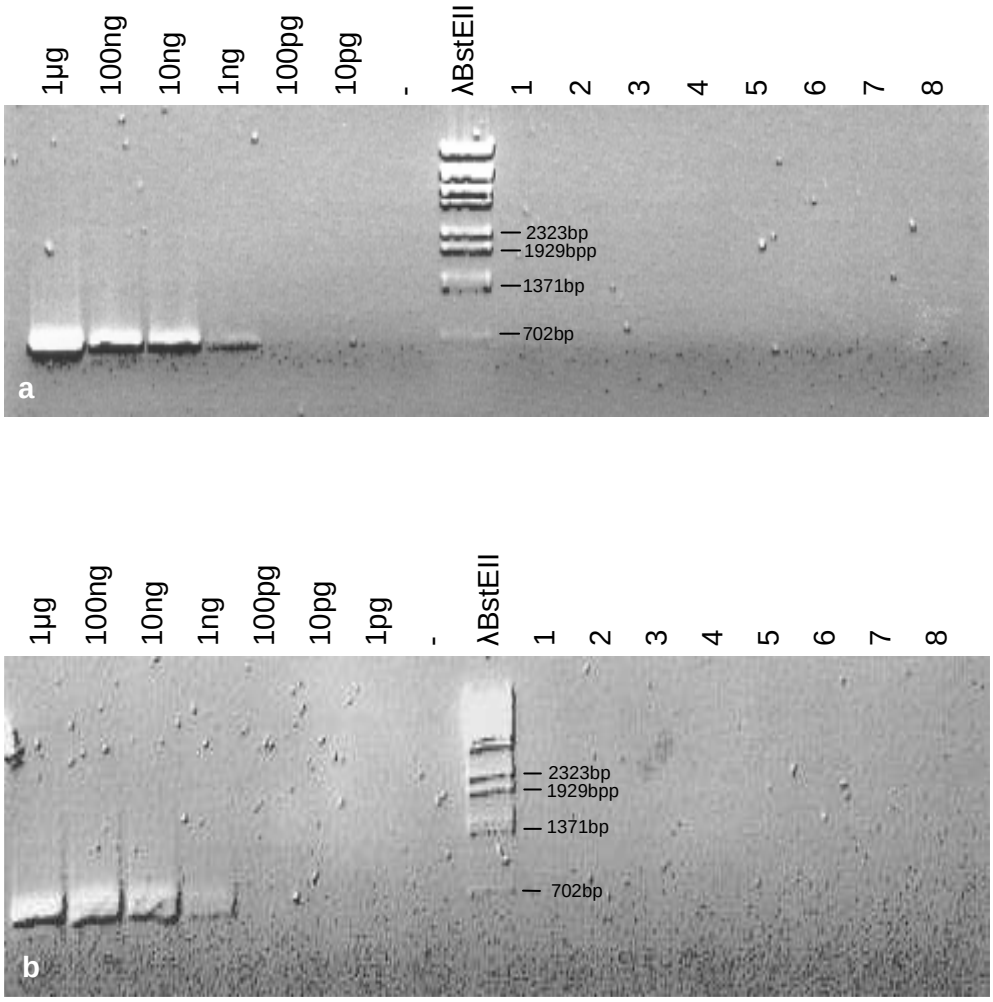


Figure 4.9 Representative PCR screens for the LKA1 cassette with primers LKA1(f) and LKA1(r1) in filtered supernatants from fermenting **(a)** MS300 taken on day 8 and **(b)** filter sterilized grape must taken on day 3. 1 – 8 represent the screened filtered fermentation supernatants from each stress condition; 1 & 5 from the standard fermentation set, 2 & 6 from the increased ethanol set, 3 & 7 from the increase OD_{600nm} set and 4 & 8 from the heat shock set (1 – 4 = Vin13pDLG31 fermentations and 5 – 8 = Vin13 controls); 1µg - 1pg represents the template dilution range screened in a 2% fermenting media background taken from the standard Vin13 fermentation set on the same day as samples 1 -8. – represents the negative control containing only ddH_2O as template.

As demonstrated in Figure 4.9, the intensity of the ~500bp PCR product obtained by using primers LKA1(f) and LKA1(r1), decreases as the amount of template DNA decreases, indicating the sensitivity of each individual PCR screen. The absence of a PCR product in the negative control indicates no contamination of the PCR reactions. Using online algorithms for calculating molecular conversions (http://www.unitconversion.org/weight/daltons-to-nanograms-conversion.html?dalton%3Bfocus%3D1%3Bbase%3D1=4290000000&nanogram and http://www.rcgld.org/otherwebs/labfaqs/main/MW.php) it was determined that the sensitivity obtained in this screening process corresponds to approximately 14.03×10^4 copies of the Vin13 genome and thus the integrated LKA1 cassette. As discussed in 4.2.1.3, if one were to apply the same rationale whereby the estimated OD_{600nm} of cultures fermenting in MS300 is estimated to be 3.6 by day 8, this translates to approximately 1.08×10^8 cells/ml. When considering our PCR detection limits of 14.03×10^4 copies of the Vin13 genome, Figure 4.9 shows this to be sufficiently sensitive to detect DNA in the supernatant if only 0.12% of the fermenting culture had lysed to release the nuclear content into the fermenting supernatant.

4.2.2.4 Sampling and PCR screening

Supernatants were continuously sampled from each of the stressed fermentations in different media, filter sterilized and used as templates in PCR screens using these optimized LKA1 nested primers. In order to eliminate the possibility of obtaining false positive results during such PCR screens as a result of residual cells, the filtered supernatants were spotted on selective YPD agar supplemented with 100µg/ml SMM. (Figure 4.11).

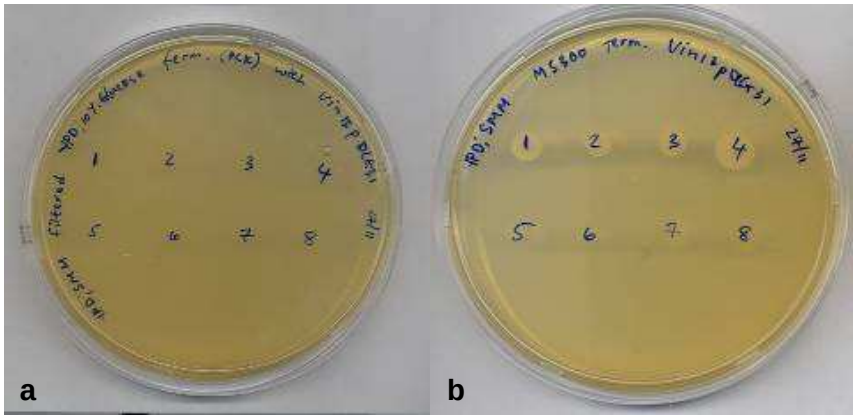


Figure 4.10 (a) Filtered and **(b)** unfiltered Vin13pDLG31 fermenting grape must supernatants (1-4) and Vin13 controls (5-8) spotted on YPD agar supplemented with 100µg/ml SMM. Samples 1 & 5 were taken from the standard fermentation set, 2 & 6 from the increased ethanol set, 3 & 7 from the heat shocked set and 4 & 8 from the increased OD_{600nm} set.

Results indicated that sampled supernatants were effectively filtered of all viable Vin13 cells, as evident by the lack of growth for samples 1 – 4 (Figure 4.11a) as compared to the unfiltered counterparts 1 – 4 in (Figure 4.10b). Similarly, if this technique was capable of successfully removing all viable cells, it is assumed that all non-viable cells were also removed, resulting in a cfu free supernatant. As before, these data suggest that if the filtering technique was stringent enough to eliminate intact viable cells, all intact non-viable cells would also have been removed.

The filtered supernatants were then used as templates in a series of PCR screens for released pDLG31 integrated DNA using the nested LKA1 primers (Table 3.3). Although the data given in Figure 4.10 are only representative and not all data are shown, all screens in both MS300 and filtered grape must fermentations showed the same results, i.e. no DNA could be detected within these sensitivity ranges. As discussed in 4.2.1.4, this does not preclude the scenario of larger fragments being present at lower concentrations or smaller fragments being present at higher concentrations, however, the fact remains that no DNA could be detected within the sensitivity range established within this study.

4.3 DETECTION OF UPTAKE AND FUNCTIONAL EXPRESSION OF SPIKED DNA BY *S. cerevisiae* DURING ALCOHOLIC FERMENTATION

A second step in horizontal transfer of DNA is the uptake and functional expression of foreign donor DNA by a new host. During routine laboratory transformation of *S. cerevisiae*, investigators rely on the addition of carrier molecules to cells in order to facilitate transport of DNA molecules into cell nuclei (33). However, in a wine fermentation environment, these traditional carrier molecules would not be present. Nevertheless, with the complex nature of a fermenting broth, the possibility of yet unknown molecules fulfilling this role cannot be disregarded. In addition, increasing ethanol concentration during alcoholic fermentation

destabilizes cellular membranes and increase the permeability of the cells, particularly for small compounds (22,42), potentially presenting an uptake opportunity. This study therefore aimed at examining the possible frequency of such events. The rationale was to spike Vin13 cultures within various fermenting media types (33) with DNA at different time points, whilst continuously exposing the fermentations to the stress conditions discussed in 4.1.

4.3.1 Detection of the uptake and functional expression of multi-copy plasmid DNA by *S. cerevisiae* during alcoholic fermentation

To investigate whether *S. cerevisiae* cells are able to take up and functionally express DNA during alcoholic fermentation, such fermentations were spiked with the multi-copy episomal plasmid, pYES2-KanMX/LacZ. As discussed in section 4.2.1, plasmids are ideal tools for monitoring such events due to the nature of their molecular design (30), allowing for the development of simplified screening techniques. Fermentations with the wine yeast Vin13 were spiked with purified pYES2-KanMX/LacZ plasmid DNA at several intervals. The plasmid spiking concentration (5µg/µl) was similar to those used in routine laboratory protocols for yeast transformations (33). This level is significantly higher than the level of released DNA in wine fermentation, as shown by the previous sections of this study, but was chosen to assess the theoretical capability of *S. cerevisiae* cells to accept free DNA under these conditions.

Furthermore, as described in section 4.2.1.2, fermentations would be exposed to a set of stress conditions which my facilitate uptake of DNA. The uptake of DNA approach was therefore designed to provide a theoretically ideal environment for the occurrence of uptake events (22,42). Untransformed and unspiked Vin13 fermentations were included as controls.

4.3.1.1 Plasmid functionality and selective conditions

Strain and plasmid functionality had previously been established in 4.2.1.1. In order to demonstrate that selective conditions were sufficiently stringent to detect what was expected to be low natural transformation frequencies, screening techniques were assessed for their ability to detect a single transformed cell within 1 000 000 cfus. Vin13pYES2-KanMX/LacZ transformants were co-cultured with untransformed Vin13 (5 days) at various ratios and these mixed cultures were plated on YPGal agar supplemented with 100µg/ml G418 (Figure 4.11).

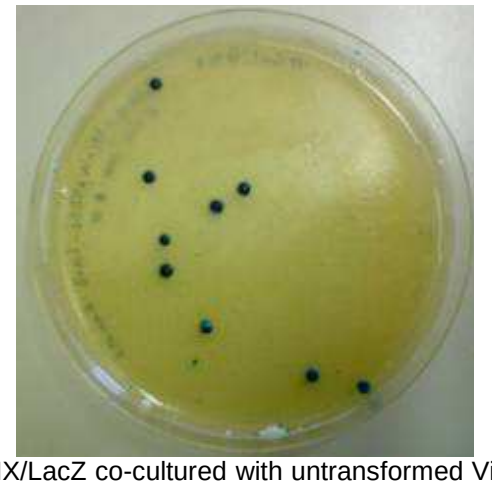


Figure 4.11 Vin13pYES-KanMX/LacZ co-cultured with untransformed Vin13 at a ratio of 10:1 000 000 plated on YPGal agar supplemented with 100µg/ml G418 followed by an X-gal overlay assay.

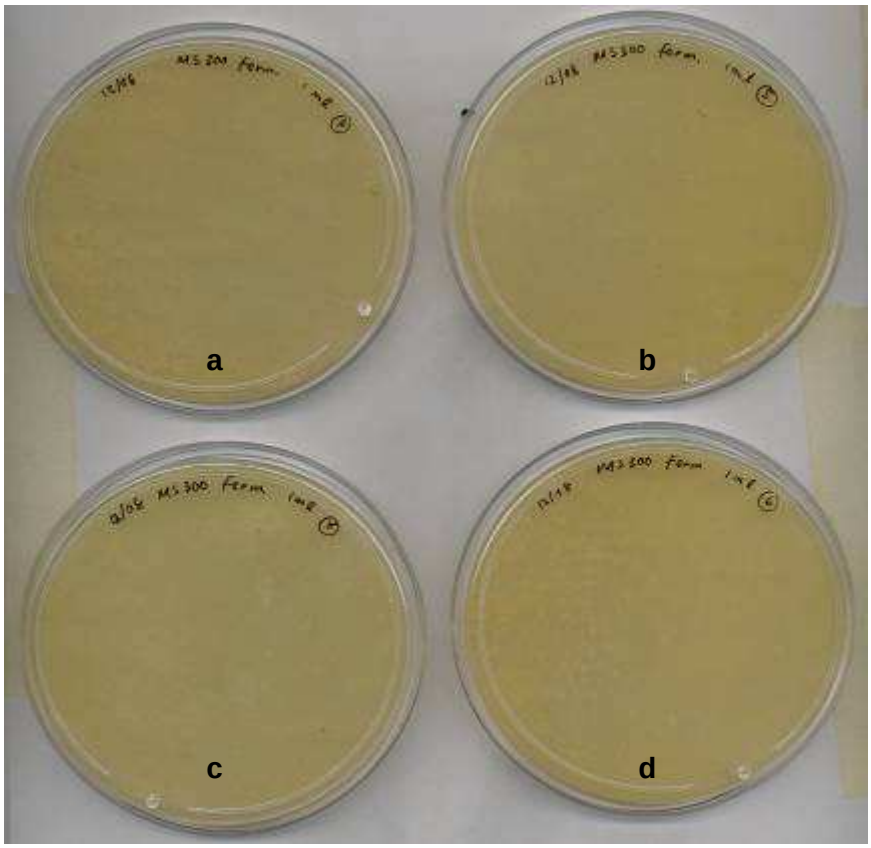
These results indicated that selective conditions were sufficiently sensitive to detect ~10 transformed cells (displaying G418 resistance) among ~1 000 000 untransformed/susceptible co-cultured Vin13 cells. The blue colour change observed by the successive X-gal overlay assay confirmed functionality of the LacZ cassette, expressing β-galactosidase (Figure 4.11) and successful co-expression of the two selective genes that are combined on the plasmid.

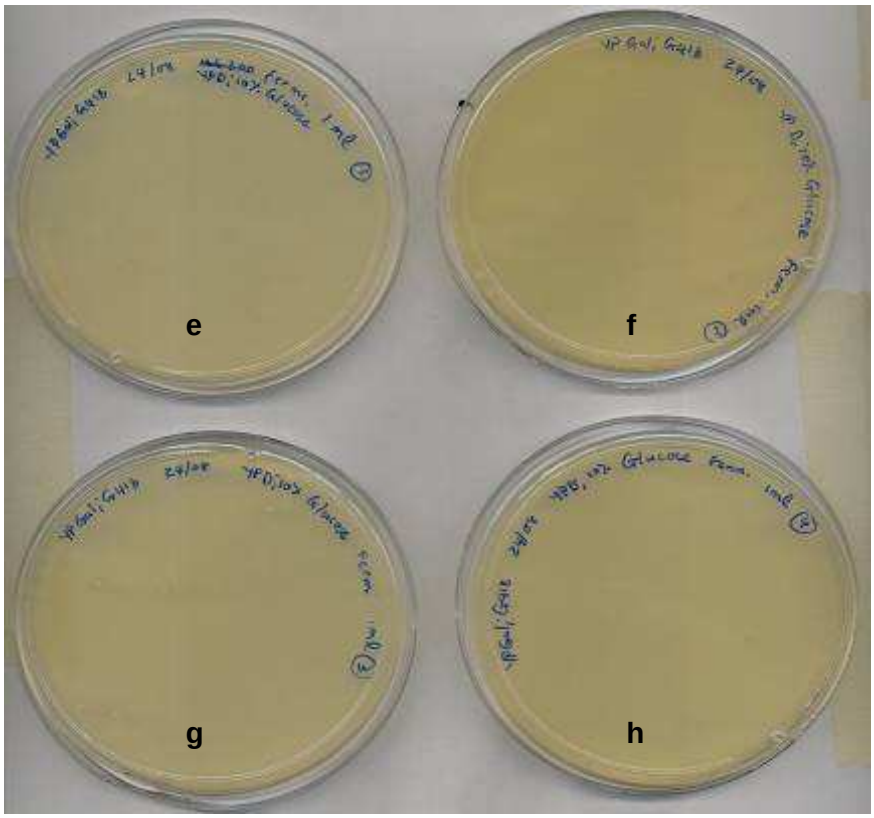
4.3.1.2 Fermentation trials

All stressed and control fermentations proceeded for 10 - 14 days, during which time culture growth curves and fermentation weight loss through CO_2 production was monitored. Initial fermentations were performed in 100ml fermentation medium volumes (MS300, YPD (10% glucose) and 0,45µm filter sterilized grape must) spiked with a total of ~5µg pYES2-KanMX/LacZ per spiking event, leading to a concentration incrementing with 50ng/ml with each successive spiking event. This concentration was considered to represent a level of DNA that can be reached if a significant number of cells were to undergo autolysis. However, in order to include worst case scenario fermentation, an additional concentration was selected at ~5µg/ml per spiking event, a scenario which would not occur in a natural or industrial fermentation. These worst case scenario fermentations were performed in duplicate in order to establish reproducibility if indeed HGT events were observed. YPD (10% glucose) was omitted as a fermentation medium (data not shown).

4.3.1.3 Selective screening for uptake and expression of spiked pYES2-KanMX/LacZ

All fermentation sets were continuously sampled and screened for the presence of Vin13 cells that would express the phenotypic traits associated with plasmid pYES2-KanMX/LacZ DNA. The initial screening process occurred by plating on YPGal agar supplemented with 100µg/ml G418. Representative examples of these screening procedures are shown in Figure 4.12.


a
b
c
d


e
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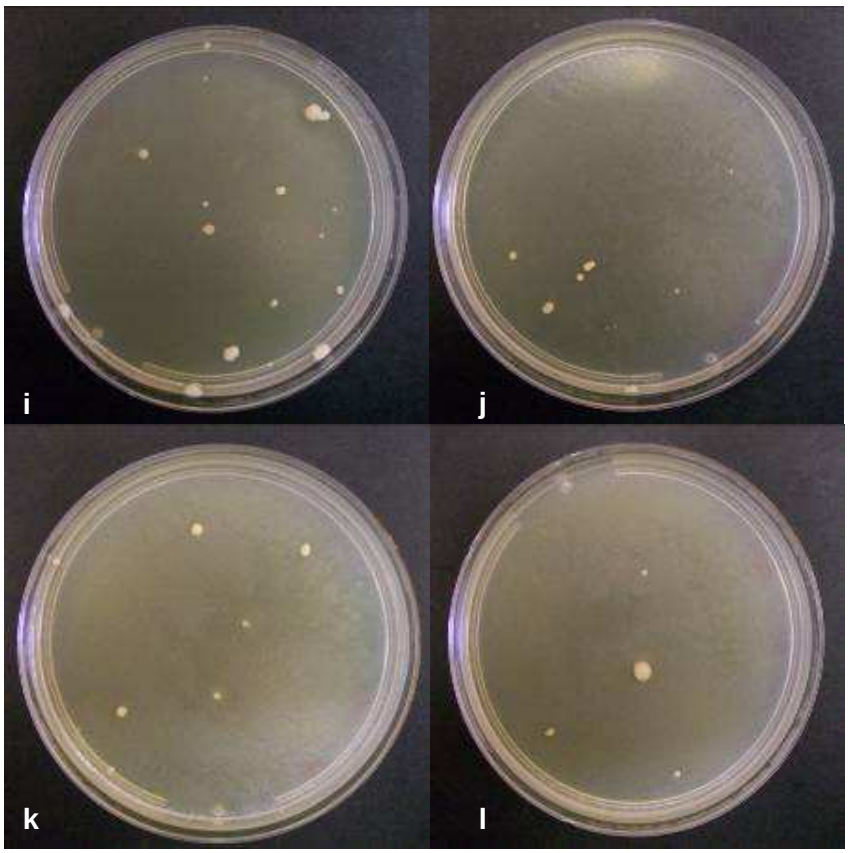


Figure 4.12 Representative Vin13 fermentation (spiked with pYES2-KanMX/LacZ) samples plated on YPGal agar supplemented with 100µg/ml G418**.** In each example, ~1% of the total fermentation cultures were plated. Samples were taken on day 10 from the MS300 fermentations **(a)** heat shocked, **(b)** increased OD_{600nm}, **(c)** increased ethanol and **(d)** standard fermentation sets; on day 5 from the YPD (10% glucose) fermentations **(e)** heat shocked, **(f)** increased OD_{600nm}, **(g)** increased ethanol and **(h)** standard fermentations sets; on day 8 from the filtered grape must fermentations **(i)** increased $OD_{600nm,}$ **(j)** heat shocked, **(k)** increased ethanol and **(l)** standard fermentation sets.

Based on the sensitivity established in Figure 4.11, it was assumed that if cells had accepted and were functionally expressing the plasmid DNA, they would be observed as the only G418 resistant and thus viable cells among all plated untransformed and susceptible Vin13 cells. In several cases, growth of individual colonies was observed on these plates. However, yeast are known to be able to acquire a certain degree of resistance to antibiotic treatments (18). To verify whether such growth corresponds to a true DNA uptake event or from spontaneous acquisition of G418 resistance, the resistant colonies were assessed for β-galactosidase activity. Indeed, the data show that many of the G418 resistant colonies (see for example Fig. 4.14 i, j, k and l) did not display β-galactosidase activity subsequent to performing X-gal overlay assays, suggesting false positive results due to naturally acquired G418 resistance rather than plasmid presence (data not shown).

As control, the unspiked fermentation sets were screened in the same manner however, no putative uptake events were observed (data not shown). Conversely, several positive cfus were observed from the spiked fermentations in 0.45 µm filter sterilized grape must (Figure 4.13).

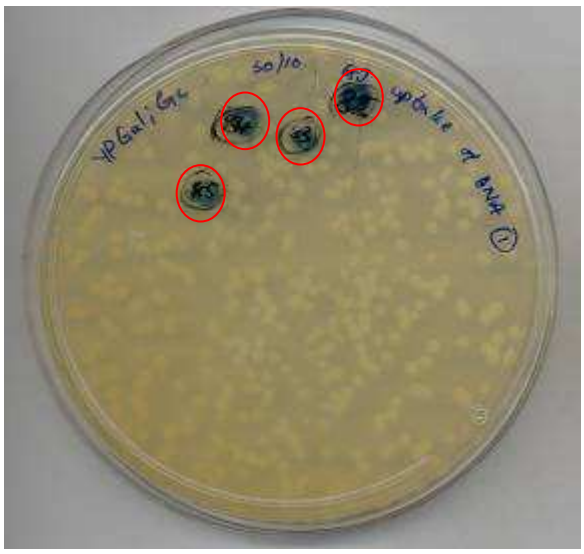


Figure 4.13 Representative putative positive uptake of spiked pYES2-KanMX/LacZ; taken on day 6 from the standard fermentation set of filtered grape must (100ml) inoculated with Vin13 and spiked with ~5µg pYES2-KanMX/LacZ per spiking event; plated on YPD agar supplemented with 100µg/ml G418 followed by an X-gal overlay assay.

The results seen in Figure 4.13 represent the initial colonies obtained that displayed β-galactosidase activity in combination with G418 resistance, thus implying uptake of spiked plasmid DNA. By day 6, a total of 4 spiking events had occurred, i.e. a total of 20µg had been spiked; this represented a final plasmid concentration of 2µg/ml fermenting media. As mentioned earlier, a degree of suspected spontaneously acquired G418 resistance was observed, as seen in Figure 4.13. Nevertheless, for the subsequent genetic screens, representatives were selected from both those cfus displaying, as well as those not displaying β-galactosidase activity.

At this point it was observed that re-streaking of any putative positive cfus onto identical selective media showed no β-galactosidase activity, suggesting a loss of the LacZ cassette or mutation(s) within the LacZ open reading frame (orf) to such an extent that the resulting protein product had lost functionality.

4.3.1.4 PCR screening for pYES2-KanMX/LacZ plasmid sequences

Based on the observation that β-galactosidase activity appeared lost upon re-streaking of these isolates, the presence of the LacZ cassette was assessed in each, using the nested LacZ primers (Table 3.2) in a PCR screen. Negative results suggested the absence of the LacZ cassette (data not shown).

Various explanations could account for the observed loss of activity. The first possibility is that the initial display of β-galactosidase activity was observed during a period of transient expression from what would most likely have been a linearized fragment containing the LacZ cassette. Transient expression of such linear fragments has been observed in laboratories, but no published data exist to our knowledge. It has to be assumed that this corresponds to a linear fragment being maintained in the cell after having been taken up, but without being integrated into the genome. Such fragments may be maintained over several generations, but will ultimately be lost. Another explanation would be that the linear fragments generated through digestion of the spiked plasmid DNA were successfully integrated through recombination into the *S. cerevisiae* genome. Upon subsequent re-culturing on selective media containing G418,

the LacZ gene loss was encouraged by its inability to contribute an advantage to its host under these conditions. It has been shown that the *S. cerevisiae* genome contains both short tandem repeat sequences as well as long inverted repeat sequences, both contributing to genome instability accompanied by DNA deletions of varying lengths (14,23,39).

Regardless of the apparent loss of the LacZ, the G418 resistance appeared to be maintained. By using primers designed to detect the presence of the KanMX cassette (Table 3.4) it was determined that the full KanMX orf was indeed present in the 5 isolates (Figure 4.14).

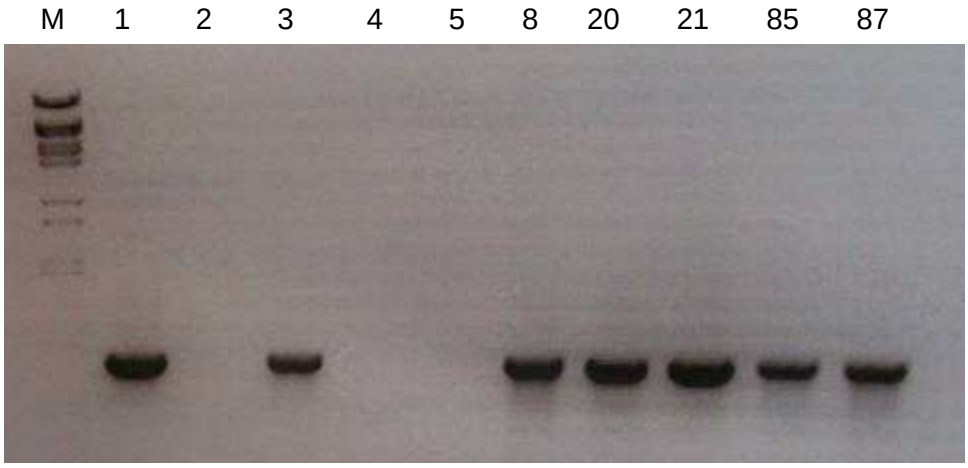


Figure 4.14 PCR screen for the presence of the KanMX orf using primers KanMX(f) and KanMX(r) (refer to Table 2.4) and the following templates: 1 = 10ng purified pYES2-KanMX/LacZ plasmid DNA, 2 = negative control containing only ddH_2O as template, 3 = single Vin13pYES2-KanMX/LacZ cfu, 4 = purified Vin13 genomic DNA, 5 = negative control containing only ddH_2O as template, 8, 20, 21, 85 and 87 = genomic DNA extracted from each of the 5 isolates. M = genomic ladder λBstEII.

In Figure 4.14, the PCR positive controls (lanes 1 & 3) yielded a product of ~ 650bp, as expected, and all negative controls indicating no PCR contamination. Furthermore, results indicated each of the 5 isolates under investigation to contain the KanMX orf, suggesting not only uptake of spiked DNA, but integration of at least the KanMX orf.

All 5 of these putative uptake events occurred in the 100ml fermentation volumes of filter sterilized grape must, spiked with ~5µg pYES2-KanMX/LacZ plasmid DNA per spiking event. Of the 5 isolates, 2 were obtained from spiked heat shocked fermentations (obtained on days 13 and 49), 2 from the spiked increased OD_{600nm} fermentations (obtained on days 6 and 49) and 1 from the spiked standard fermentations (obtained on day 6).

Attempts to reproduce these results in decreased fermentation volumes (20ml) with increased total spiking concentrations at ~5µg/ml fermenting media per spiking event proved unsuccessful (data not show).

4.3.1.5 PCR verification of *S. cerevisiae* Vin13

In order to eliminate the possibility of these results being a consequence of contamination, PCR screening with Δ1 and Δ2 primers (Figure 4.15) was employed for verification that all 5 isolates were indeed Vin13 (25). Results demonstrated in Figure 4.15 confirmed this when compared to *S.cerevisiae* strain W15 (as a negative control).

When compared to strain Vin13, isolates 8, 20, 21, 85 and 87 show identical Δ primer PCR banding patterns, as opposed to the pattern obtained for strain W15 which was incorporated in

`order to demonstrate the ability of this screen to distinguish between two closely related strains (Figure 4.15). This confirmed that these isolates are indeed strain Vin13 *S. cerevisiae.* Strain W15 was included as a negative control, demonstrating the ability to clearly distinguish the banding pattern observed for Vin13 from that observed for other *S. cerevisiae* strains.

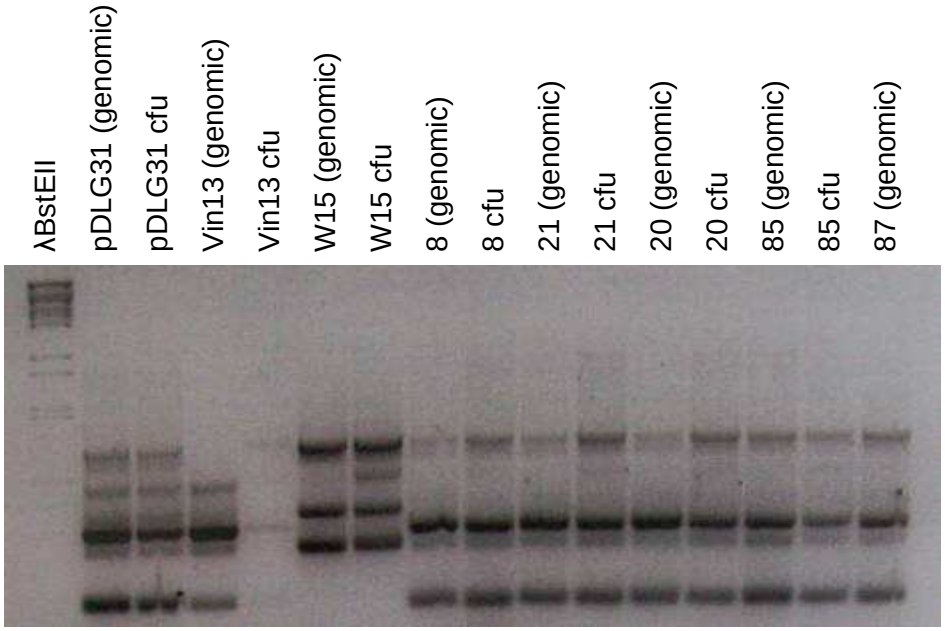


Figure 4.15 PCR screen using Δ1 and Δ2 primers in order to verify that all 5 putative positive cfus were *S. cerevisiae* Vin13 (designated numbers 8, 20, 21, 85, 87). Colony PCR screens were performed on each of the 5 isolates, designated 8 cfu, 20 cfu, 21 cfu, 85 cfu and 87 cfu. As additional verification, purified genomic DNA was included as templates in this PCR screen, designated 8 (genomic), 20 (genomic), 21 (genomic), 85 (genomic) and 87 (genomic). Also included in this PCR screen was Vin13pDLG31 cfu (for colony PCR) and purified pDLG31 genomic DNA, as a positive control and strain W15 as a negative control.

4.3.1.6 CHEF analysis

In addition to PCR identification (using the Δ1 and Δ2 primers (25)) karyotyping was performed through CHEF analysis allowing strain identification through chromosomal banding patterns (Figure 4.16) as confirmation that all 5 putative uptake events occurred in Vin13 (40).

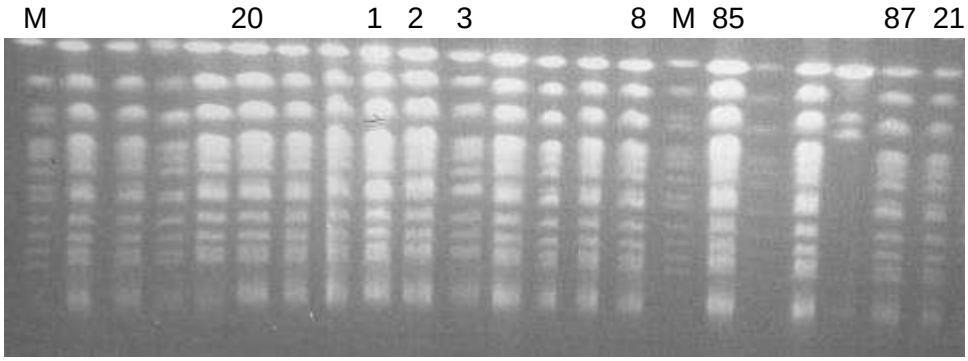


Figure 4.16 Karyotyping through CHEF analysis of all 5 isolates (8, 20, 21, 85 and 87); M = *S. cerevisiae* marker, 1 = Vin13, 2 = Vin13pYES2-KanMX/LacZ, 3 = FY23Δcat. The remaining lanes represent the full set of cfus screened, however only those that proved to have successfully accepted spiked DNA are identified here.

The CHEF analysis indicated that each of the 5 isolates show identical banding patterns to that observed for *S. cerevisiae* strain Vin13, again confirming their genotypic background to be that of Vin13.

4.3.1.7 Southern Blot hybridization

From the observation of the LacZ cassette/β-galactosidase activity loss as well as lack of detection of the LacZ cassette through PCR screening and accompanied by simultaneous maintenance of the KanMX cassette/G418 resistance, it was suspected that pYES2-KanMX/LacZ plasmid was no longer present in its entirety, but that the KanMX cassette had rather integrated into the Vin13 genome. Based on the antibiotic selection pressure applied during the screenings subsequent to the fermentation trials, selective maintenance of the KanMX cassette was a possible outcome. Therefore, in order to prove the presence of the KanMX orf within the Vin13 genome, Southern Blot hybridizations were performed (33). Total genomic DNA extractions (Figure 4.17a) and a DIG-labelled PCR KanMX orf probe were verified, as demonstrated in Figure 4.17b.

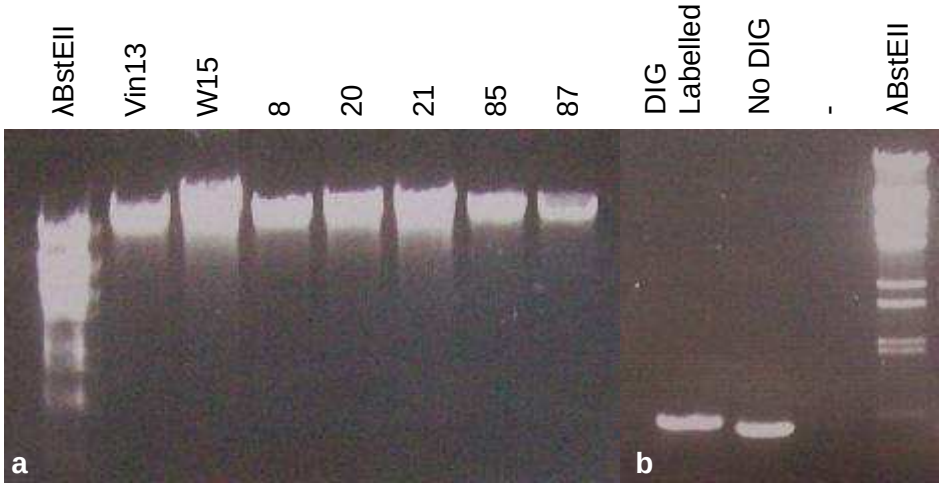


Figure 4.17 (a) Verification of genomic DNA extraction for Southern Blot hybridization of the 5 isolates (8, 20, 21, 85 and 87), including *S. cerevisiae* strains Vin13 and W15 as positive and negative controls respectively. **(b)** DIG-labelled KanMX orf probe.

The data presented in Figure 4.17a indicate sufficient quantity and acceptable quality of extracted and purified genomic DNA to be used in subsequent Southern Blot hybridizations. The non DIG-labeled product (Figure 4.17b) is of expected size (~650bp) and the subsequent shift in this band size in the adjacent DIG labeled version thereof is clearly visible as a consequence of the successful DIG labeling.

Once having verified the extracted genomic DNA and DIG-labelled probe, Southern Blot hybridization identified the presence of the KanMX orf within purified genomic DNA isolated from each of the 5 cfus under discussion (Figure 4.18) (33). These data suggested integration of only a segment of the spiked plasmid DNA, containing at least the KanMX orf, into the genome of Vin13, thus making it resistance to G418.

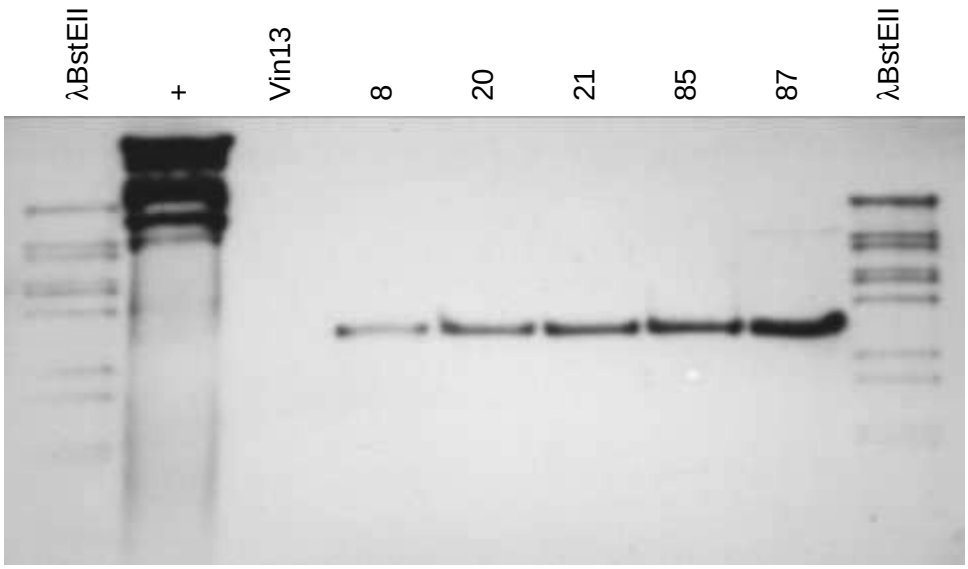


Figure 4.18 Southern Blot hybridization of ScaI digested genomic DNA extracted from the 5 putative positive isolates (8, 20, 21, 85 and 87) probed with a DIG-labeled KanMX orf probe. The + lane contains purified plasmid pYES2-KanMX/LacZ DNA as positive control for hybridization efficiency in detecting the KanMX orf. Total genomic DNA from Vin13 was also extracted, digested with ScaI and included here as a negative control.

The presence of a band in the lanes representing each of the isolates (8, 20, 21, 85 and 87) confirmed the presence of the KanMX orf in each isolate. This confirmation of the presence of the KanMX orf, in combination with the inability to detect the presence of the LacZ cassette/β-galactosidase activity through PCR screening and X-gal overlay assays, may be accounted to selective pressure, whereby, if the LacZ cassette had also been integrated into the Vin13 genome at some point, it may have been lost through successive selection based on G418 resistance. For this reason, the KanMX cassette was likely to be preferentially maintained. These data also suggest that the intact circular plasmid DNA did not transform the cultures as such, but was rather degraded into shorter fragments before integration. Indeed, if circular DNA had entered the cell, it would have been maintained as such. If DNA was degraded before uptake, it should be assumed that the resulting fragments would be of various lengths, containing genetic information of varying significance. Any one of these linear fragments could then serve as candidates for integration. Only those fragments carrying the relevant genetic information would pass the selective screening procedure, identifying those isolates displaying G418 resistance through the presence of the KanMX cassette.

4.3.1.8 Determining the site of integration

Results obtained from the Southern Blot hybridization indicated integration of plasmid DNA sequence containing the KanMX orf, however, in order to verify this, the site of integration and nature of the integrated fragment needed to be identified. Using the Southern blot hybridization data to estimate the band size of R.E digested Vin13 genomic DNA containing the integrated KanMX orf, sub-genomic DNA libraries were constructed to contain these sequences as described in Material and Methods. Positive colonies within these libraries were identified through colony hybridization (Figure 4.19a) using the same DIG labelled KanMX probe as used in the Southern Blot hybridization (33).

DNA was recovered from the DH5α colonies positively identified to contain the Vin13 genomic DNA with integrated KanMX orf through probe hybridization and subsequently sequenced (http://academic.sun.ac.za/saf/dnas/services.html) using primers gKanMX(f) and gKanMX(r) to obtain the sequences of the regions flanking the KanMX orf, thus identifying the site of integration into the Vin13 genome for isolate 21 (Figure 4.20 (a-b)). Isolates 8 and 20 were identified through a chromosome walking based approached whereby genomic DNA was digested to generate fragments with 3' overhangs. Single-stranded oligonucleotides with complementary 3' ends were then ligated to these libraries generating a digested genomic library with single-stranded 5' overhangs, serving as annealing sites for subsequent PCR amplifications using adaptor primers (complementary to the overhanging oligonucleotides) in combination with gene specific primers gKanMX(f) and gKanMX(r) (1) (Figure 4.19(b)). These PCR products were subcloned in the pGEM®-T Easy vector system and subsequently submitted for sequencing (http://www.nucleics.com/DNA_sequencing_support/sequencing-service/university-cape-town-sequencing.html). These sequences can be seen in Figure 4.20 (c-f).

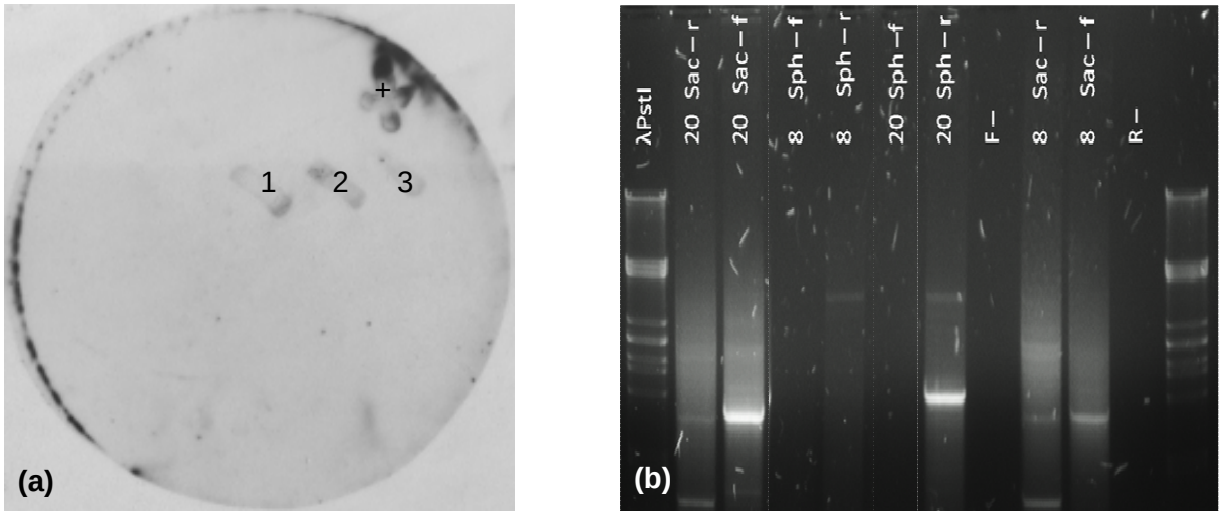
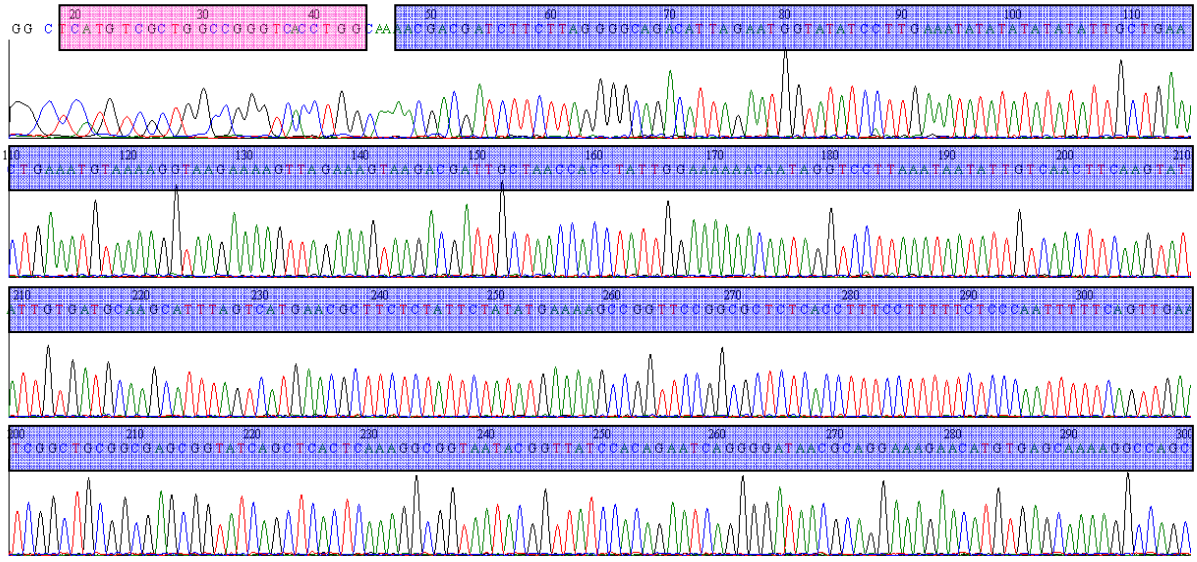
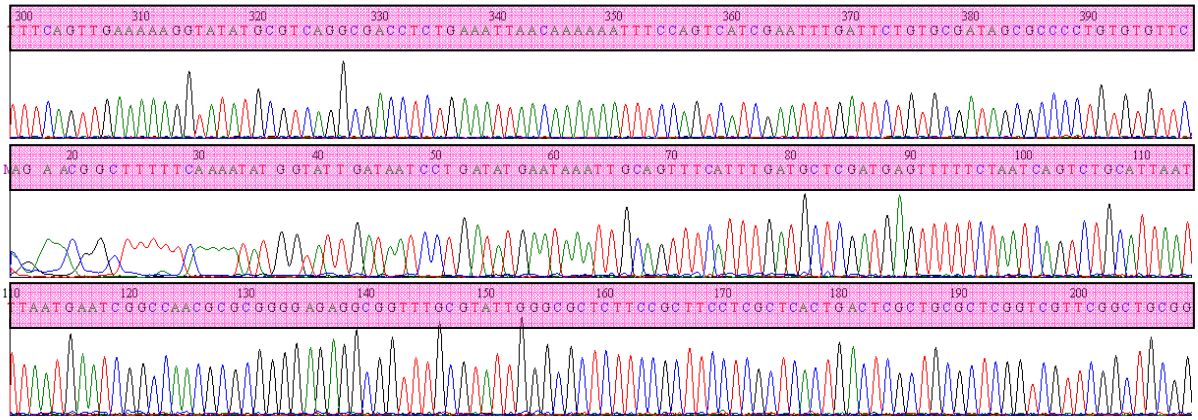


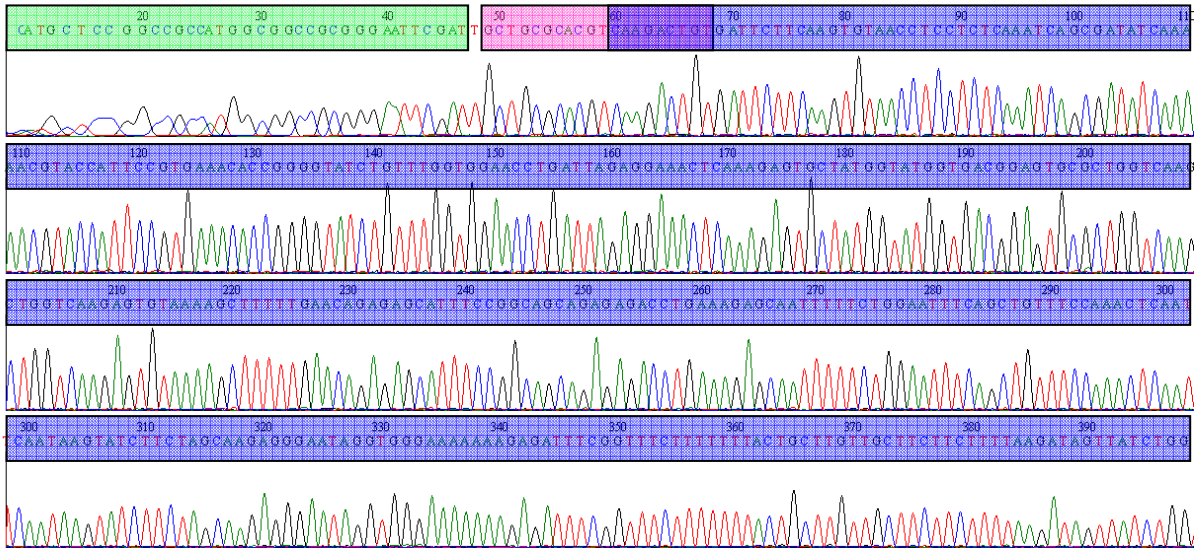
Figure 4.19 (a) Representative colony hybridization. DH5α colonies obtained from generating a subgenomic library containing the ScaI digested Vin13 genomic DNA believed to contain the integrated KanMX orf had been re-streaked and hybridized using the same KanMX orf probe used for the Southern Blot hybridization. This Figure indicates 3 cfus to be positive for the presence of the KanMX orf (designated 1, 2 and 3). As positive control, DH5αpYES2-KanMX/LacZ was streaked (+), indicating functional sensitivity of this screening technique. **(b)** 1% Agarose gel demonstrating PCR products obtained from amplification using the AP and GSP set, where 20 and 8 represent the isolate number, Sac and Sph represent the DNA library used as template and f and r represent the gKanMX(f) and gKanMX(r) primers respectively used as GSP. PstI digested lambda DNA was used as molecular marker in this image.



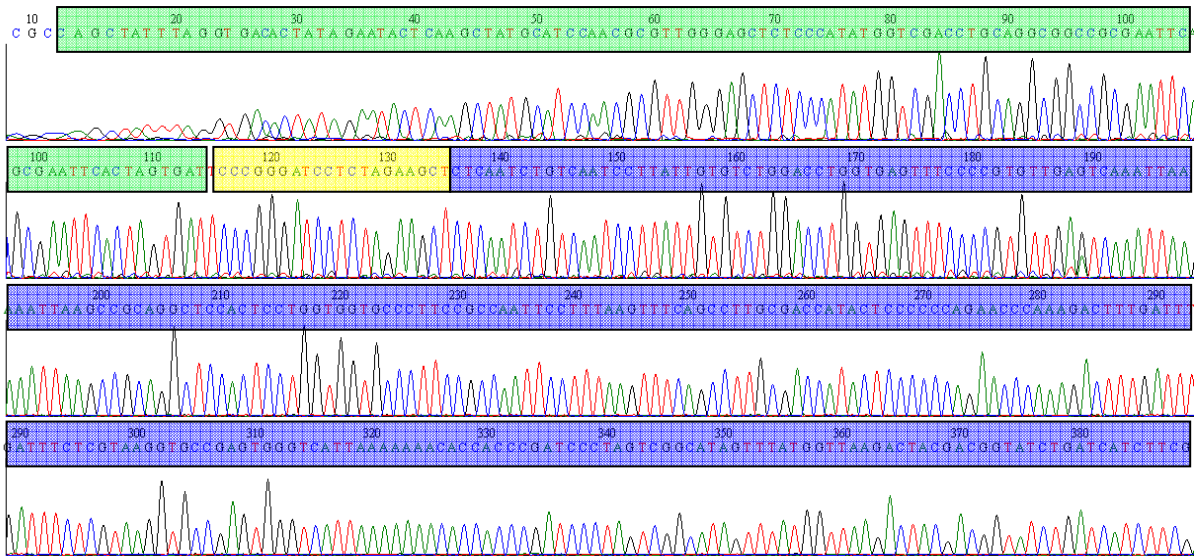
(a)



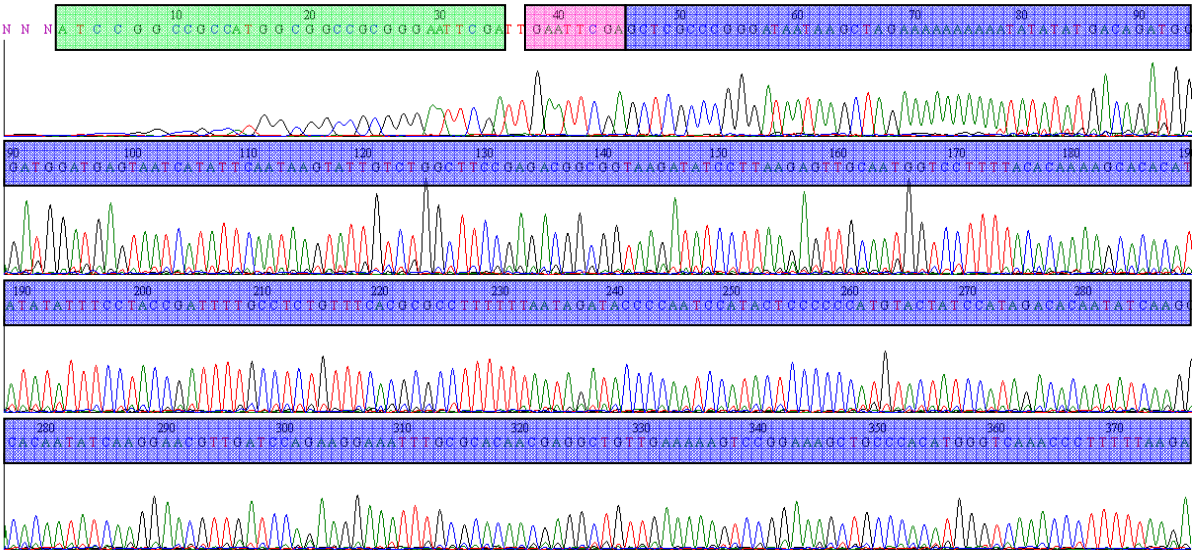
(b)



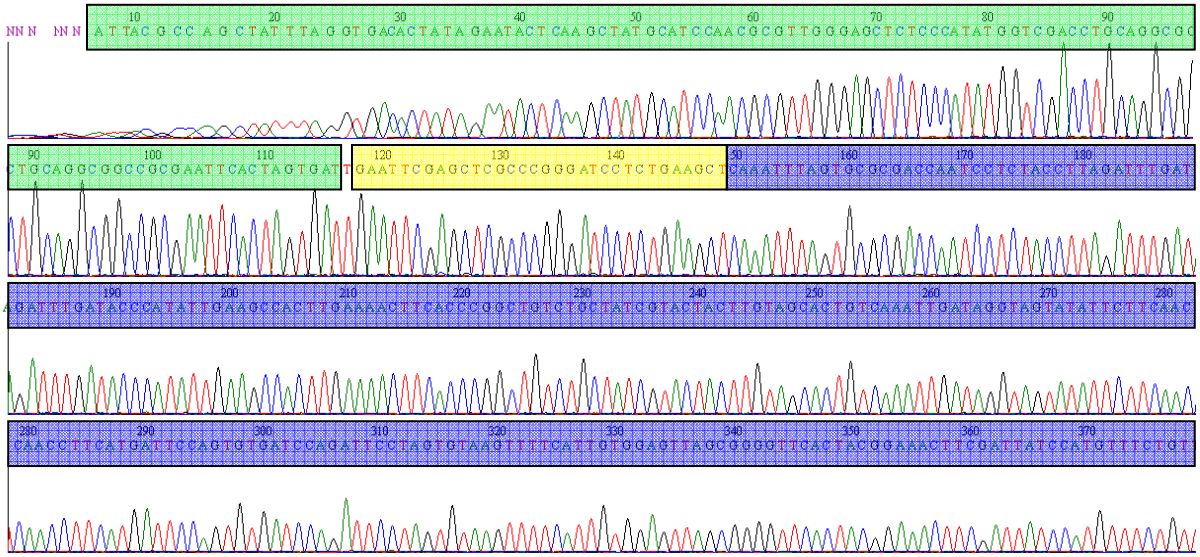
(c)



(d)



(e)



(f)

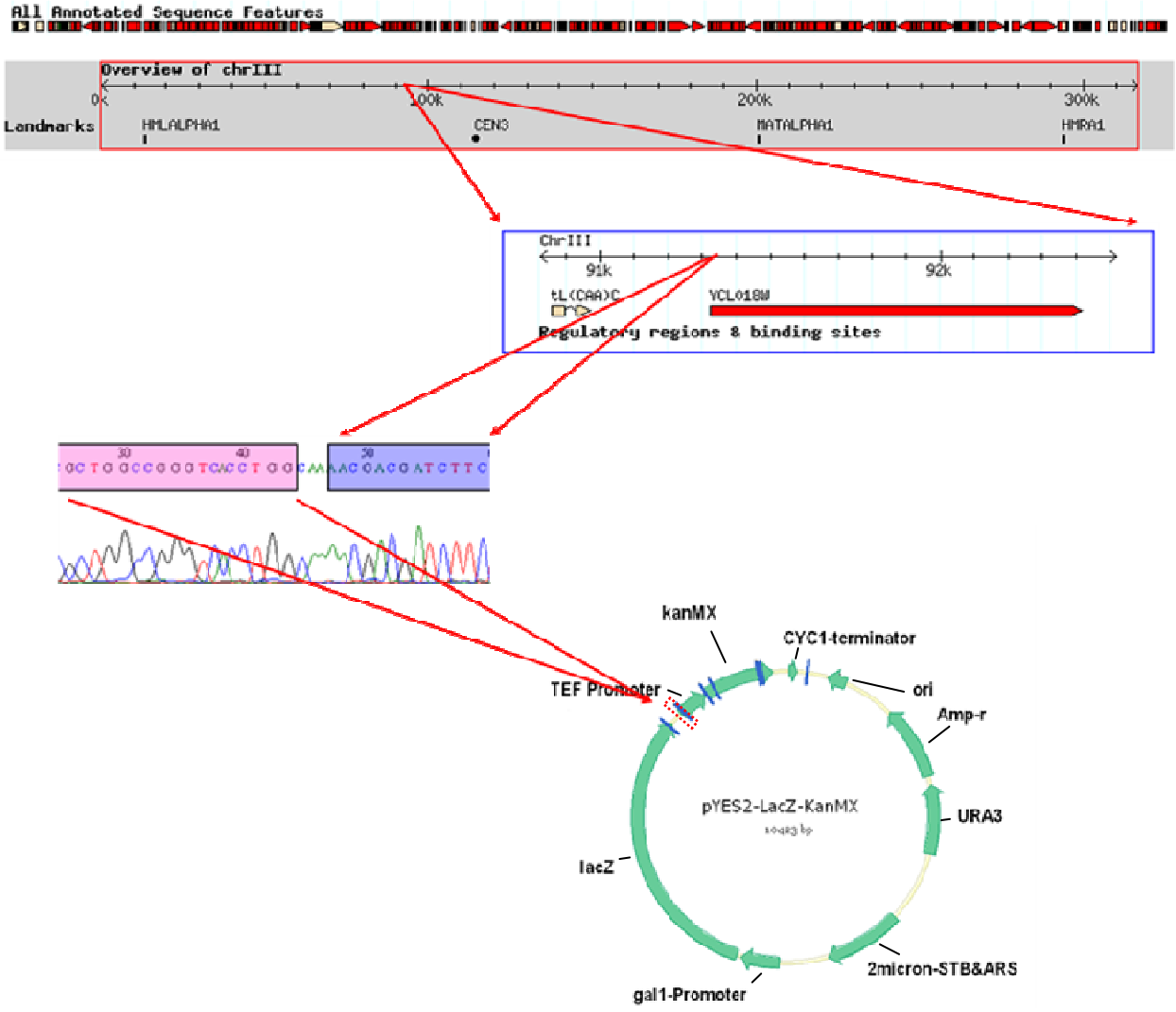
Figure 4.20 Identification of the individual sites of KanMX cassette integration for each of the isolates into the *S.cerevisiae* genome through BLAST analysis in *Saccharomyces* Genome Database. **(a)** Isolate 21 sequence obtained using primer gKanMX(f), where the pink region indicates the reverse complement sequence of position 9105 – 9128 of pYES2/KanMX/LacZ, found 23 bp upstream of the gKanMX(f) recognition site and the blue region indicates integration in the *S.cerevisiae* genome at position 91363 of chromosome III into the *LEU2* (*YCL018W*, position 91324 – 92418). **(b)** Isolate 21 sequence obtained using primer gKanMX(r), where the pink region indicates pYES2-KanMX/LacZ sequence from 20bp downstream from the gKanMX(r) primer recognition site. **(c)** Isolate 20 sequence obtained using primer T7promoter, where the green region indicates position 23 – 59 of the pGEM®-T Easy vector, located 17bp downstream of the T7promoter primer recognition site, the pink region indicates the gKanMX(f) primer recognition sequence found on pYES2-KanMX/LacZ, the blue region indicates position 466864 – 467209 of chromosome XII, partially including the gene encoding 18S ribosomal RNA (*RDN18*, position 466870 to 465071) and the purple region indicates the region of homology between pYES2-KanMX/LacZ and *RDN18*. **(d)** Isolate 20 sequence obtained using primer M13(rev), where the green region indicates position 67 -170 of the pGEM®-T Easy vector, located 6bp upstream of the M13(rev) primer recognition site, the yellow region indicates the OHP SacI and the blue region indicates position 465628 – 465881 of chromosome XII, *RDN18-2* (position 466870 to 465071). **(e)** Isolate 8 sequence obtained using primer T7promoter, where the green region indicates position 28 - 60 of the pGEM®-T Easy vector, located 25bp downstream of the T7promoter primer recognition site, the pink region indicates pYES2-KanMXZ/Lac position 9067 – 9074 and the blue region indicates position 540323 – 540650 of chromosome XII, partially including the gene encoding *UPS1* (*YLR193C*, position 540011 – 540538). **(f)** Isolate 8 sequence obtained using primer M13(rev), where the green region indicates position 68 – 177 of the pGEM®-T Easy vector, 1bp upstream of the M13(rev) primer recognition site, the yellow region indicates the OHP SacI and the blue region indicates position 540091 – 540321 of UPS1 (YLR193C, position 540011 – 540538).

These results provided evidence for the integration of at least the KanMX cassette of the 2µ-based pYES2-KanMX/LacZ at varying positions within the *S. cerevisiae* genome. Furthermore, from these results it appears that plasmid DNA was first degraded in the medium (42), yielding fragments of varying lengths, before being taken up by the yeast. Any of these linear fragments can then be integrated into the *S. cerevisiae* genome, but only those fragments containing relevant genetic information would have been detected through the subsequent screening procedures. This relevant DNA would therefore have to contain at least the KanMX cassette and/or the LacZ cassette. A Saccharomyces Genome Database (SGD) BLAST analysis reveals that there is 1445bp homology between the entire 10423bp pYES2-KanMX/LacZ multi-copy plasmid and the *RAF1* gene located on the 2 micron plasmid, a 131bp homology to the *REP1* gene on the 2 micron plasmid, a 1034bp homology to the *URA3* gene (*YEL021W*) on chromosome V, a 460bp homology to chromosome II, and a 233 bp homology to *UTR1* gene on chromosome X (position 528468 – 526876) (http://www.yeastgenome.org/). The 2 micron homologies come as no surprise due to the design of the multi-copy plasmid utilized in this study, which was 2 micron based. Additionally, when doing a similar SGD BLAST analysis using position 8911 – 112 of the pYES2-KanMX/LacZ multi-copy plasmid, a 1625bp fragment containing the entire KanMX cassette, including an additional 556bp upstream and 260bp downstream, a 99bp homology was found on chromosome X (position 526681 – 526780) (http://www.yeastgenome.org/). However, the sequence data obtained indicates integration into chromosome III and chromosome XII, neither of which were identified in homology BLAST analyses. This implies that integration occurred through a process known as illegitimate recombination whereby recombination can occur without the need for extended homologous sequences (8).

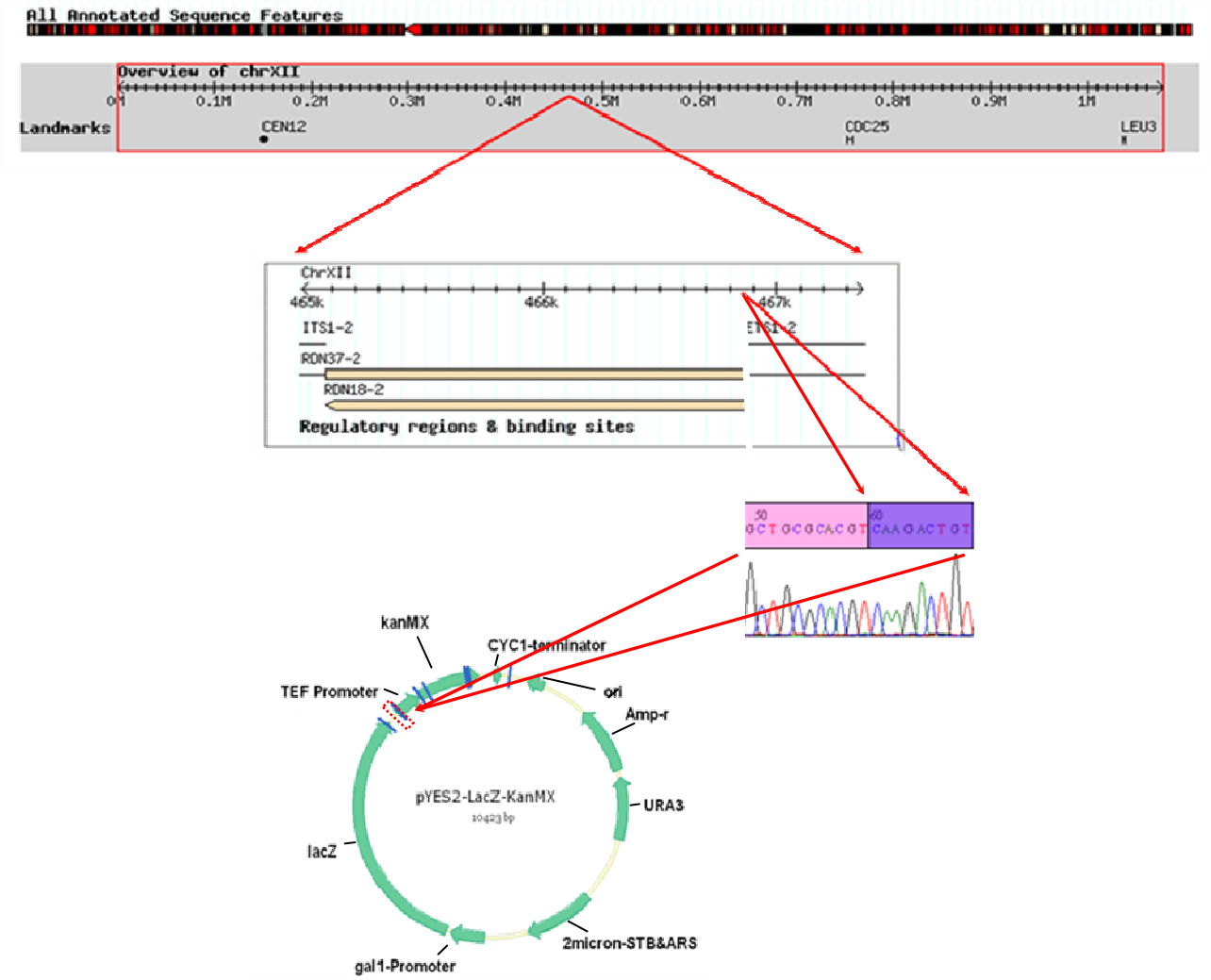
When analysing the part of plasmid DNA that had integrated into the genome, the data show that all three integrated fragments had closely linked plasmid integration sites. Indeed, the integrated plasmid DNA in all cases was within a 100 bp of a sequence representing the 3' part of the *TEF* promoter and the closely linked plasmid backbone sequence. Two explanations can be advanced for this finding: Firstly, this sequence may correspond to a recombination hotspot,

as has been described for some chromosomal elements. More likely, however, is that transforming DNA fragments were all of short size, and only few of the fragments would be of the required size to transfer active geneticin resistance. In this scenario, all such information-rich fragments would be cut closely to the part of the promoter that is required for efficient transcription of the resistance gene. If this hypothesis is true, it would be expected that all the 5' ends of the transforming fragments would also be closely associated with the region surrounding the STOP codon of the gene.

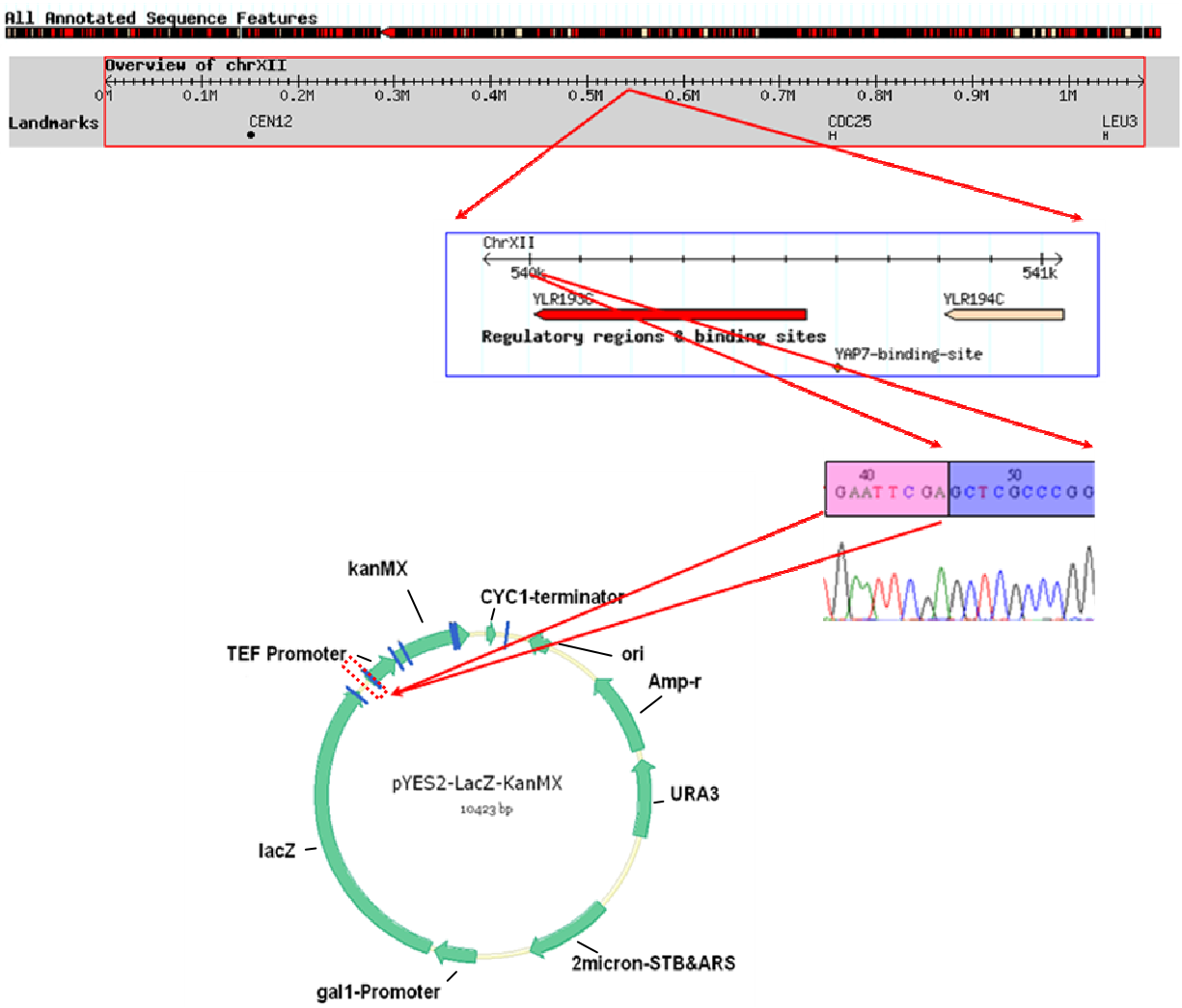
(a)



(b)


All Annotated Sequence Features
Overview of chrXII
0M
0.1M
0.2M
0.3M
0.4M
0.5M
0.6M
0.7M
0.8M
0.9M
1M
Landmarks
CEN12
CDC25
LEU3
ChrXII
465k
466k
467k
ITS1-2
RDN37-2
RDN18-2
Regulatory regions & binding sites
kanMX
CYC1-terminator
TEF Promoter
ori
Amp-r
pYES2-LacZ-KanMX
10423 bp
URA3
lacZ
2micron-STB&ARS
gal1-Promoter

(c)



(d)

8 21 20

ccgcgggaattcgatctgggcccgtttagcttgcctcgtccccgccgggtcacccggccagcgacatggaggcccagaat
accctccttgacagtcttgacgtgcgcagctcaggggcatgatgtgactgtcgcccgtacatttagcccatacatccccatgt
ataatcatttgcatccatacattttgatggccgcacggcgcgaagcaaaaattacggctcctcgctgcagacctgcgagca
gggaaacgctcccctcacagacgcgttgaattgtccccacgccgcgcccctgtagagaaatataaaaggttaggatttgc
cactgaggttcttctttcatatacttccttttaaaaatacagttctcacatcacat KanMX

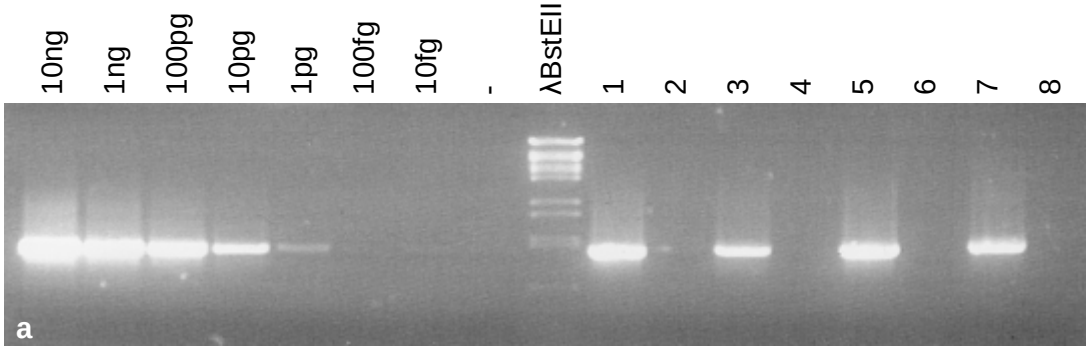
Figure 4.21 A graphical illustration summarizing the chromosomal integration events for isolates **(a)** 21 **(b)** 20 and **(c)** 8, and **(d)** the integration sites indicated on the sequence of the plasmid DNA for all three isolates. The chromosomal position of the KanMX integration site relative to the relevant chromosomes as a whole is represented. The KanMX cassette sequence relative to the pYES2-KanMX/LacZ plasmid is also shown and correlated this to the same chromatogram data of figure 4.20. This illustration was compiled using images from http://www.yeastgenome.org/ and Vector NTI Advanced (TM) 11.0 (Invitrogen Corporation).

Isolates 85 and 87 appeared to have lost the KanMX cassette and subsequent G418 resistance after several generations of proliferation. This was observed both through selective culturing on YPD agar containing G418 at 100µg/ml, which showed no growth, as well as through PCR screening of each isolate with KanMX(f) and KanMX(r) PCR primers (data not shown). It is possible that a similar mechanism of transient expression as discussed for the LacZ gene was involved here.

Furthermore, because this study was initially designed to only detect the occurrence of HGT events and possibly identify the mechanisms involved, the techniques employed to obtain these integration sites were focused solely on identifying the sites of KanMX cassette integration into the *S. cerevisiae* genome. The extent of flanking plasmid sequence accompanying such KanMX cassette integrations on the 3' side of the detected integrations remains to be determined.

4.3.1.9 Determining of the stability of spiked multi-copy plasmid DNA during the process of alcoholic fermentation

Under conditions experienced by microorganisms during alcoholic wine fermentation, autolysis occurs. While we have been unable to demonstrate the presence of large fragments of yeast DNA during wine fermentation, the most likely explanation was the lack of sensitivity of the PCR methods as optimized here. Indeed, normal cell death and autolysis occurs during wine fermentation, and is likely to lead to at least some release of DNA into the environment. Whether this DNA contains fragments of the size required to transmit significant biological information as discussed in the introduction remains to be demonstrated. However, assuming that such releases occur, an important question relates to assessing the stability of such fragments within the fermentation environment. Indeed, it can be assumed that the more stable such DNA is, and the longer it therefore remains present in the environment, the higher the likelihood of a gene transfer event taking place. The fate of such "free-floating" genetically modified DNA is thus a crucial aspect to consider when attempting risk assessment studies. Therefore, an additional aspect to this study was to determine the stability of "free-floating" DNA within the surrounding fermenting media. This was assessed by determining the stability of the spiked pYES2-KanMX/LacZ throughout the course of alcoholic fermentation through a PCR screening technique (Figure 4.22), using the nested primers discussed in Table 3.2.



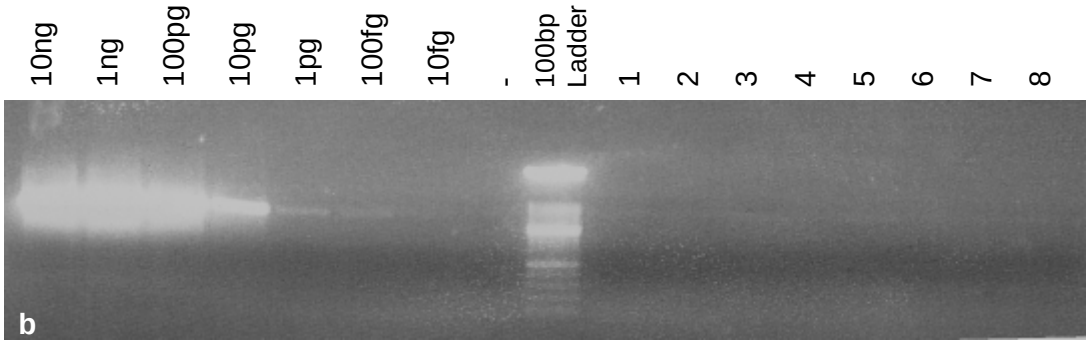


Figure 4.22 Representative PCR screens of fermenting supernatants from day 10 for spiked plasmid DNA stability; **(a)** MS300 and **(b)** filter sterilized grape must. 1 – 8 represent the screened filtered fermentation supernatants (1, 3, 5 and 7 were Vin13 fermentations spiked with pYES2-KanMX/LacZ and 2, 4, 6 and 8 were unspiked Vin13 controls) with 1 & 5 from the standard fermentation set, 2 & 6 from the increased ethanol set, 3 & 7 from the increased OD_{600nm} set and 4 & 8 from the heat shock set. 10ng-10fg represents the template dilution range, screened in 2% fermenting media background taken from the standard Vin13 fermentations set on the same day as each of the samples 1 – 8. – represents the negative control.

The data in Figure 4.22 demonstrates how the intensity of the ~1000bp PCR product, obtained by using primer LacZ(f) and LacZ(r2), decreases as the template concentration decreases, reflecting the sensitivity of each individual screen. The absence of a PCR product in the negative lane indicates the PCR screen to be contamination free. YPD (10% glucose) was omitted from all repeat fermentations, being less representative of true wine making conditions. Figure 4.22 (a) demonstrates how all spiked MS300 fermentations contained intact DNA detectable at ~1000bp throughout the duration of alcoholic fermentation. Figure 4.22 (b), however, shows that the spiked DNA could not be detected in the corresponding filtered grape must fermentations, even though the PCR sensitivity had been established to detect as few as 100fg DNA per screen. Bearing in mind that the 5 positive isolates were obtained from this filtered grape must fermentation set, these data suggest that the DNA was indeed present (manually spiked), but may not be able to serve as template in a PCR screen. A possible explanation is that the spiked DNA is bound to substances present within the fermenting filtered grape must, rendering it physically inaccessible to primer binding or Taq polymerase attachment. The presence of these substances may also contribute to the successful uptake of the DNA, since the only transformation events were observed in real grape must and not in MS300. Table 4.1 summarizes the stability data obtained from this experiment.

Table 4.1 Summary of spiked pYES2-KanMX/LacZ plasmid DNA stability determined through PCR analysis

	MS300		Grape must		YPD (10% glucose)	
Stress	Day*	Sensitivity	Day*	Sensitivity	Day*	Sensitivity
Standard fermentation	18	100fg	n/d	100fg	22	100fg
Increased Ethanol	10	1pg	n/d	100fg	22	10fg
Increased OD_{600nm}	18	100fg	n/d	100fg	22	100fg
Heat Shocked	18	100fg	n/d	100fg	22	100fg

*** last day on which DNA was detected**
n/d – not detected

4.3.2 Detection of the uptake and functional expression of spiked linear DNA by *S. cerevisiae* during alcoholic fermentation

When cells undergo autolysis, the leakage of cellular content is accompanied by the release of nuclear content. This released DNA however, is subsequently subjected to various degrading factors, including R.Es (42). The consequence is total genomic DNA being reduced to smaller linear fragments. These fragments may vary in size, however, only those carrying functional copies of genes (or functional parts thereof) can be identified as HGT events. Research has shown that the size and sequence homology present in such fragments play significant roles in successful integration into new host genomes (7). In order to determine whether the DNA type plays a role in the frequency of such uptake events or the accessibility thereof within the various fermenting media types under investigation, this study was expanded to assess the probability of the uptake of linear DNA fragments obtained through PCR amplification. This linear DNA consisted of the KanMX cassette, capable of conferring G418 resistance to strains containing a functional copy. Such linear DNA would require integration into the *S. cerevisiae* Vin13 genome in order to propagate and persist within the population, implying that a form of illegitimate recombination would be the likeliest route of success for such an event (8). This study investigated the possible occurrence of such a scenario by performing fermentations with Vin13 spiked with linear copies of the KanMX at predetermined time points whilst continuously exposing such fermenting cultures to the stress conditions discussed earlier.

4.3.2.1 Plasmid functionality and selective conditions

In order to establish functionality of the PCR amplified linear KanMX DNA, this cassette was subcloned into YEp351-SMR, generating YEp351-SMR/KanMX. This plasmid was then transformed into Vin13 to yield Vin13YEp351-SMR/KanMX which was plated on selective YPD agar supplemented with 100µg/ml SMM. In order to demonstrate that selective conditions were sufficiently stringent to detect what was expected to be low natural transformation frequencies, screening techniques were assessed for their ability to detect a single transformed cell within 1 000 000 cfus. Transformed cells of strain Vin13YEp351-SMR/KanMX were co-cultured with untransformed Vin13 at various ratios on YPGal agar supplemented with 100µg/ml G418 (Figure 4.23).

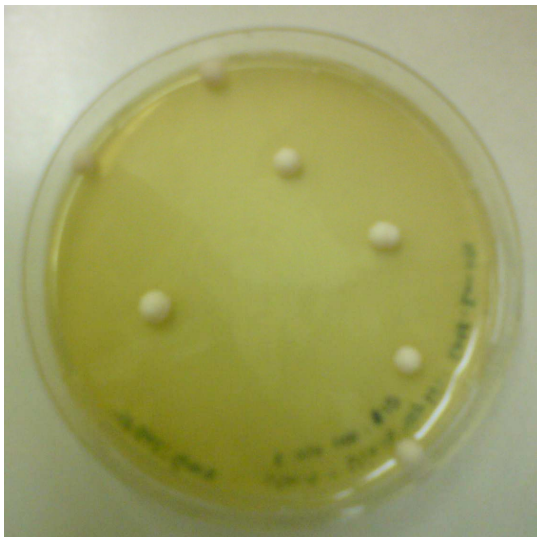


Figure 4.23 Vin13YEp351-SMR/KanMX co-cultured with untransformed Vin13 at a ratio of 10:1 000 000 on YPD agar supplemented with 100µg/ml G418.

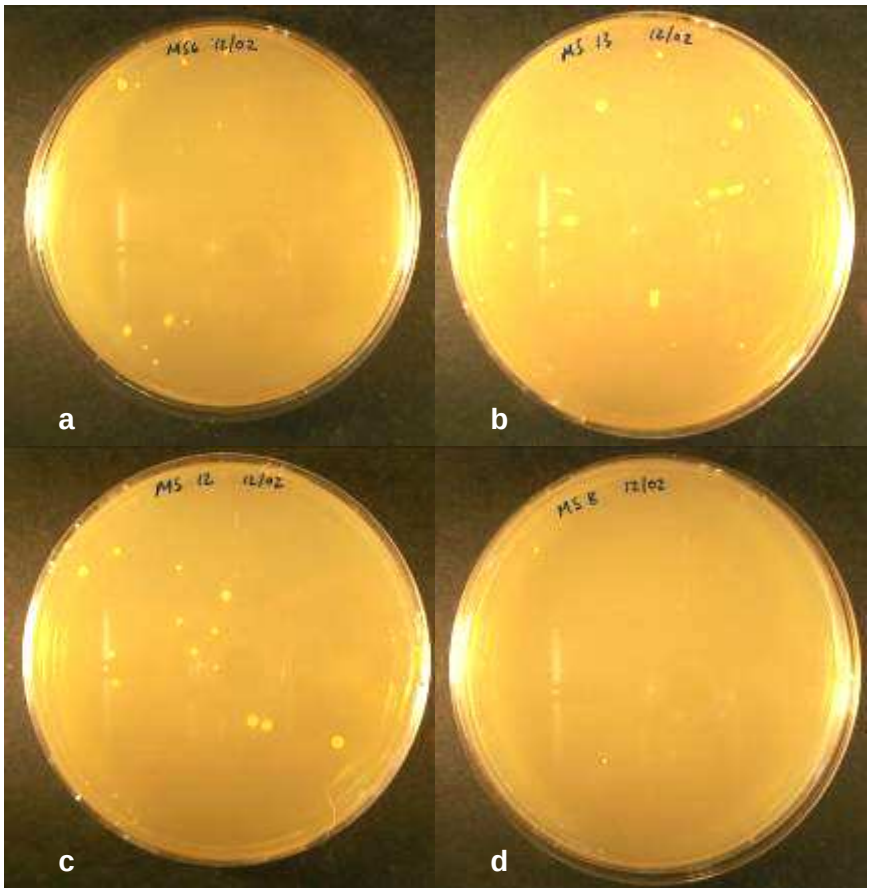
The results obtained indicated that selective conditions were sufficiently sensitive to detect ~10 transformed cells (displaying G418 resistance) among ~1 000 000 untransformed/susceptible co-cultured Vin13 cells (Figure 4.23).

4.3.2.2 Fermentation trials

All stressed and control fermentations proceeded as described earlier, during which time culture growth curves and fermentation through CO_2 production was monitored (data not shown). Fermentations were performed in 20ml fermentation medium volumes (MS300 and 0,45µm filter sterilized grape must) spiked with ~5µg purified KanMX PCR product per spiking event. YPD (10% glucose) was omitted as a fermentation media.

4.3.2.3 Selective screening for uptake and expression of spiked linear DNA

All fermentations were continuously sampled and screened for the presence of Vin13 cells that would express the phenotypic traits associated with the KanMX cassette. The initial screening process occurred by plating samples on YPD agar supplemented with 100µg/ml G418. Representative examples of these screening procedures are shown in Figure 4.24.



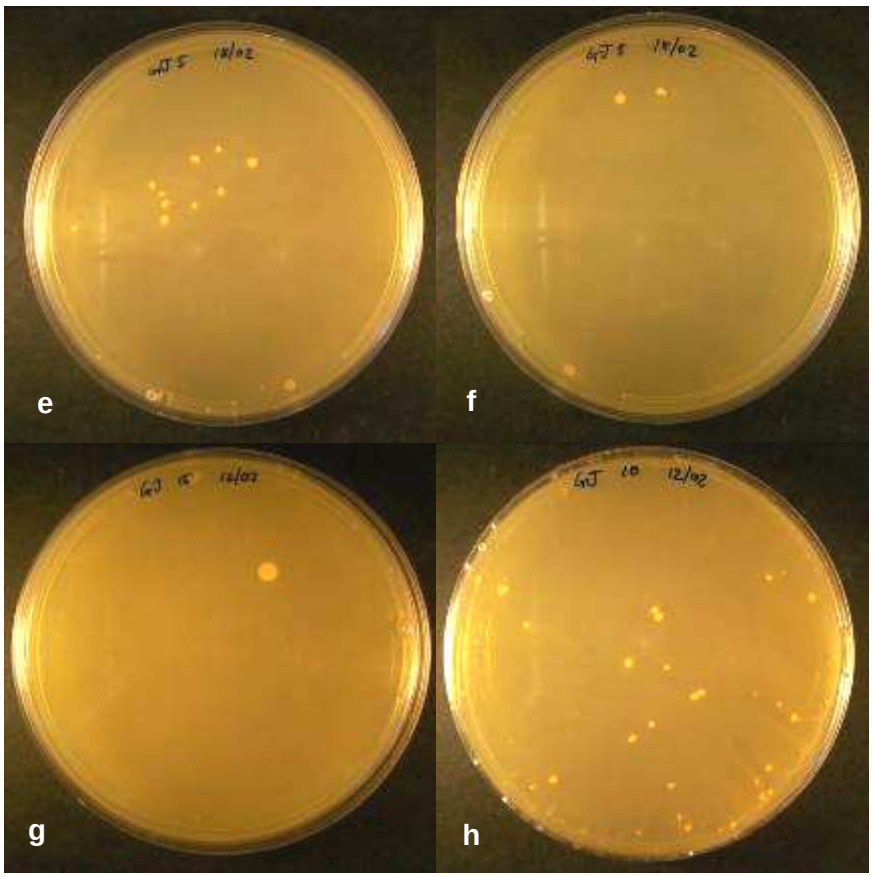


Figure 4.24 Representative Vin13 fermentation (spiked with KanMX linear DNA) samples plated on YPD agar supplemented with 100µg/ml G418. In each sample, ~1% of the total fermentation cultures were plated. Samples were taken on day7 from the MS300 fermentations **(a)** standard fermentation, **(b)** increased OD_{600nm}, **(c)** increased ethanol and **(d)** heat shocked sets; on day 13 from the filtered grape must fermentations **(e)** increased OD_{600nm}, **(f)** increased ethanol, **(g)** heat shocked from day 7 and **(h)** standard fermentation set from day 7.

Based on the sensitivity established in Figure 4.23, it was assumed that if cells had accepted and were functionally expressing the spiked KanMX cassette, they would be observed as the only G418 resistant and thus viable cells among all plated untransformed and susceptible Vin13 cells. The growth of several individual colonies was observed on these plates. However, to verify whether such growth corresponds to a true DNA uptake event of or from spontaneous acquisition of resistance (18), the resistant colonies were further investigated in subsequent PCR screens.

4.3.2.4 PCR screening

Putative positive cfus for the acceptance of spiked linear KanMX DNA were screened for the presence of the KanMX cassette through PCR analysis. However, all results were negative, suggesting that resistance was acquired through spontaneous mutation (18) rather than by uptake and subsequent expression of the spiked linear KanMX cassette DNA (data not shown). These data may reflect on a structure function of the DNA possibly affecting its ability to be accepted and integrated when considering the previously observed uptake and integration of linearized spiked plasmid DNA.

4.3.2.5 Determining the stability of spiked linear DNA during alcoholic fermentation

As before, this study included the additional facet of determining the stability of DNA within the surrounding fermenting media. The stability of the spiked linear KanMX cassette DNA was

therefore determined through PCR screening, using the nested primers discussed in Table 3.6. The results obtained from representatives of these PCR screens are shown in Figure 4.25

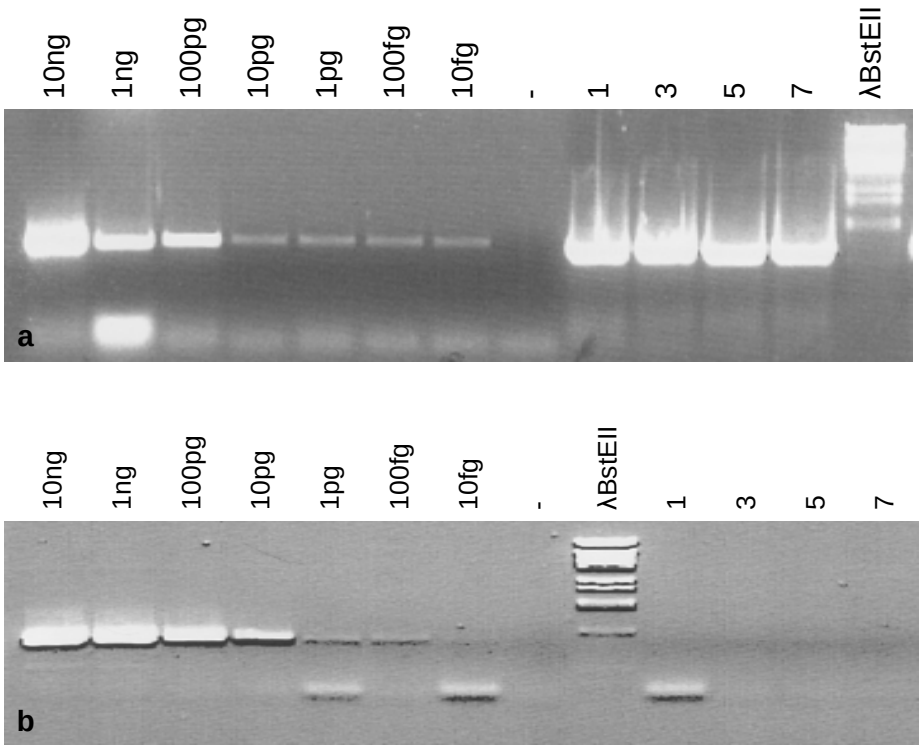


Figure 4.25 Representative PCR screens using primers pYES-KanMX(f), pYES2-KanMX(r1) and pYES2-KanMX(r2) for stability of spiked linear DNA within fermenting media from day 10; **(a)** MS300 and **(b)** filter sterilized grape must. 1, 3, 5 and 7 represent the Vin13 fermentations spiked with linear KanMX cassette DNA with 1 sampled from the standard fermentation, 3 from the increased ethanol fermentation, 5 from the increased OD_{600nm} fermentation and 7 from the heat shocked fermentation. 10ng-10fg represents the template dilution range, screened in 2% fermenting media background taken from the standard Vin13 fermentations set on the same day as each of the samples 1, 3, 5 & 7. – represents the negative control, indicating no PCR contamination.

Data shown in Figure 4.25 demonstrates how the intensity of the PCR product decreases as the amount of template decreases, indicating the sensitivity of each individual screen. The absence of a PCR in the negative control indicates that the PCR screen was free from any contamination. Figure 4.25 (a) demonstrates how all spiked fermentations contained intact DNA detectable at ~1000bp with primers pYES2-KanMX(f) and pYES2-KanMX(r2) throughout the course of alcoholic fermentation. Figure 4.25 (b), however, demonstrates that the spiked DNA could not be detected in the corresponding filtered grape must fermentations with primers pYES2-KanMX-(f) and pYES2-KanMX(r1), yielding a product of ~500bp, even though the PCR sensitivity had been established to detect as few as 100fg DNA per screen. As discussed earlier, a possible reason for this could be that the DNA is bound to factors present in the filtered grape must, rendering bound DNA sterically inaccessible to either primers or Taq polymerase or both. The summarized results for plasmid stability can be found in Table 4.2.

Table 4.2 Summary of spiked linear KanMX cassette DNA stability determined through PCR analysis

	MS300		Grape must	
Stress	Day*	Sensitivity	Day*	Sensitivity
Standard fermentation	62	100pg	n/d	100pg
Increased Ethanol	20	100pg	n/d	100pg
Increased OD_{600nm}	62	100pg	n/d	100pg
Heat Shocked	62	100pg	n/d	100pg

*** last day on which DNA was detected**
n/d – not detected

4.4 DETECTION OF WHOLE PLASMID TRANSFER BETWEEN *S. cerevisiae* Vin13 STRAINS DURING ALCOHOLIC FERMENTATION

The previous two sections independently investigated the possibility of two steps that, taken together, would allow for a HGT event, i.e. firstly the release of DNA from a donor cell and secondly, the subsequent uptake and expression of such donated DNA from within the environment into a new host. The data presented here strongly suggest that such transfers can occur during wine fermentation, since DNA release, while not demonstrated in this work, is certainly taking place (42), and DNA uptake was directly observed in our project. To assess whether such an event of transfer between cells can be experimentally monitored, two Vin13 strains transformed with different plasmids containing different markers were co-cultivated during wine fermentation. In addition, this evaluation can possibly also provide an indication whether HGT events can occur by means of direct transfer between yeast cells, perhaps based on direct cell-to-cell interactions. Indeed, this theoretical scenario would protect transferring DNA from damaging or degrading factors within the environment, allowing for the safe passage of intact DNA from one cell to another. If circular plasmid DNA could be transferred unharmed, this might indeed suggest that such events, that would not involve the release of free DNA, may occur in yeast. This section therefore monitors whether transfers involving whole plasmids between uniquely transformed Vin13 strains during alcoholic fermentation could be detected. As with the previous sections, the rationale here was to drive the system to a theoretical threshold optimal for the occurrence of such HGT events by subjecting the fermentations to various stress conditions.

4.4.1 Plasmid functionality and selective conditions

The previously established Vin13pYES2-KaNMX/LacZ strain functionality was established by selective plating on YPGal agar supplemented with 100µg/ml G418 and followed by an X-gal overlay assay (Figure 4.26a). Similarly, Vin13YEp351-SMR/LacZ functionality was established by plating on selective YPD agar supplemented with 100µg/ml SMM and followed by an X-gal overlay assay (Figure 4.26b).

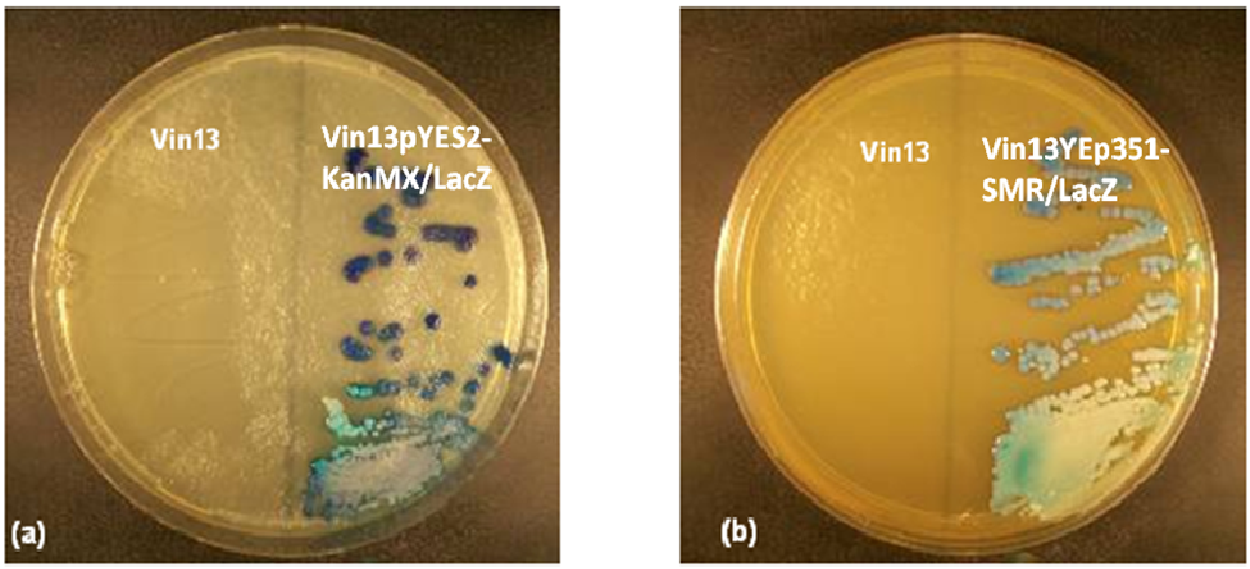


Figure 4.26 (a) Functionality of Vin13pYES2-KanMX/LacZ was confirmed by plated on YPGal agar supplemented with 100µg/ml G418 and **(b)** Vin13YEp351-SMR/LacZ plated on YPGal agar supplemented with 100µg/ml SMM. Vin13 was co-cultured as a control.

The data presented in Figure 4.26 indicates that selective conditions were sufficient to detect G418 resistant Vin13pYES2-KanMX/LacZ and SMM resistant Vin13YEp351-SMR/LacZ whilst simultaneously inhibiting growth of susceptible untransformed Vin13. In addition, the blue colour change of both transformed Vin13 strains indicated functional expression and activity of β-galactosidase from the LacZ cassette.

In order to demonstrate that selective conditions were sufficiently stringent to detect what was expected to be low natural transformation frequencies, screening techniques were assessed for their ability to detect a single transformed cell within 1 000 000 cfus. Vin13 was thus co-transformed with both plasmids pYES2-KanMX/LacZ and YEp351-SMR/LacZ and co-cultured with untransformed Vin13 at various ratios and cultures were plated on YPGal agar supplemented with 100µg/ml G418 and 100µg/ml SMM (Figure 4.27).

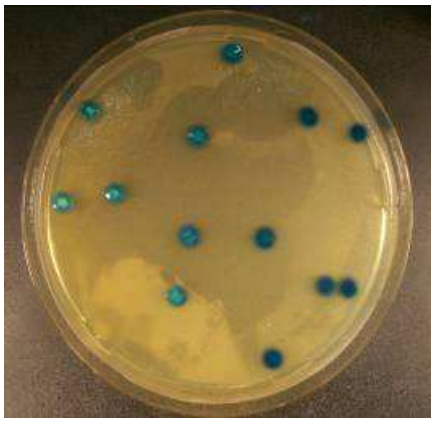


Figure 4.27 Vin13 co-transformed with YEp351-SMR/KanMX and YEp351-SMR/LacZ co-cultured with untransformed Vin13 at a ratio of 10:1 000 000 on YPD agar supplemented with 100µg/ml G418 and 100µg/ml SMM.

The results obtained indicated that selective conditions were sufficiently sensitive to detect ~10 transformed cells (displaying simultaneous resistance to G418 and SMM in combination with β-

galactosidase activity from the LacZ cassette) among ~1 000 000 untransformed/susceptible co-cultured Vin13 cells.

4.4.2 Fermentation trials

All stressed and control fermentations proceeded as described, during which time culture growth curves and fermentation through CO_2 production was monitored (data not shown). Co-inoculated (Vin13pYES2-KanMX/LacZ and Vin13YEp351-SMR/LacZ) fermentations were performed in 20ml fermentation medium volumes (MS300 and 0,45µm filter sterilized grape must

4.4.3 Selective screening for co-transformation

All fermentation sets were continuously sampled and screened for the presence of Vin13 cells that would express the phenotypic traits associated the presence of both plasmid pYES2-KanMX/LacZ and YEp351-SMR/LacZ simultaneously. The initial screening process occurred by plating samples on YPGal agar supplemented with 100µg/ml G418 and 100µg/ml SMM and initially yielded numerous putative positive cfus. Considering the scenario where two adjacent colonies, one containing pYES2-KanMX/LacZ and the other containing YEp351-SMR/LacZ, were to be found in close enough proximity to one another to create a combined protective environment against simultaneous antibiotic selection, a false positive result would appear as one colony displaying the combined phenotype of both plasmids. Successive re-culturing steps were therefore performed in order to ensure that single colonies were obtained, displaying the combined phenotype and thus eliminating such potential errors. 5 Putative positive cfus were subsequently selected from the following fermentation sets for continued research:

- 70 – MS300 / Heat shocked fermentation / Day 3
- 95 – Grape must / Standard fermentation / Day 9
- 108 - Grape must / Heat shocked fermentation / Day 9
- 109 - Grape must / Heat shocked fermentation / Day 9
- 113 – Grape must / Standard fermentation / Day 9

Collective culturing on selective media of these 5 isolates can be seen in Figure 4.28.

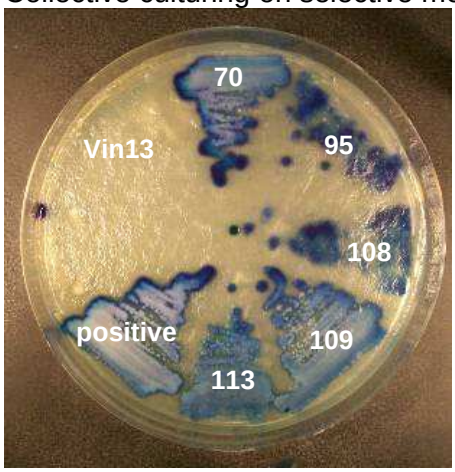


Figure 4.28 Putatively positive whole plasmid transfer isolates (70, 95, 108, 109 and 113) cultured on YPD agar supplemented with 100µg/ml G418 and 100µg/ml SMM.

Results observed in Figure 4.28 indicate that isolates 70, 95, 108, 109 and 113 are resistant to both SMM and G418 simultaneously, suggesting the presence of both plasmids YEp351-SMR/LacZ and pYES2-KanMX/LacZ. In addition, the colour change of these isolates to blue

following an X-gal overlay assay indicates that the LacZ cassette is also functionally expressed from either or both these plasmids. Untransformed Vin13 was co-cultured but showed no growth as a result of its susceptibility to both SMM and G418, indicating true selective conditions of this screen.

4.4.4 PCR screening

In order to ensure that these 5 isolates displaying simultaneous SMM and G418 resistance were a consequence of plasmid transfer as opposed to having acquired resistance through spontaneous mutations, colony PCR screens with primers KanMX(f) & KanMX(r) (Figure 4.29a) and ILV2-F & ILV2-R (Figure 4.29b) followed, confirming the presence of both the KanMX and ILV2 genetic elements.

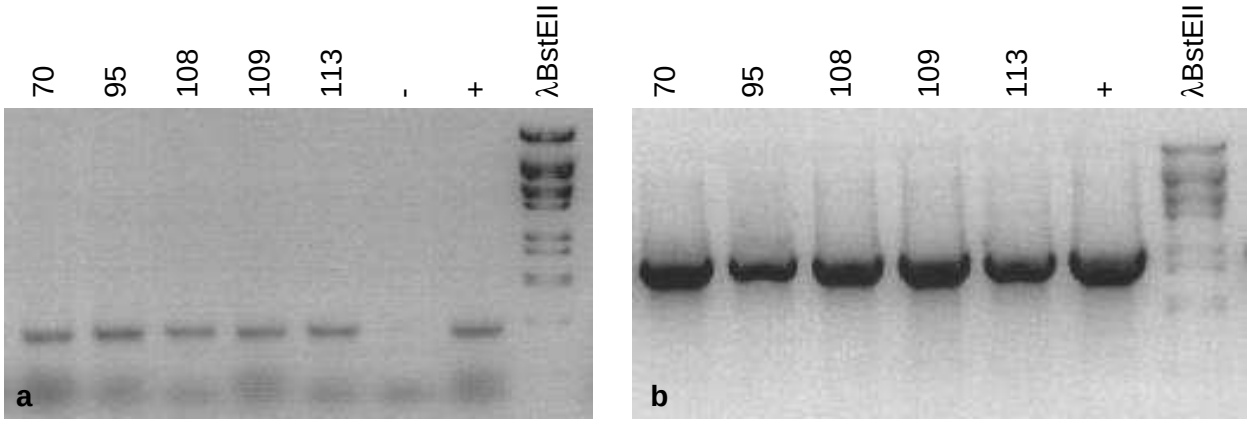


Figure 4.29 PCR screening of 5 putative positive whole plasmid transfer isolates using primer sets **(a)** KanMX(f) and KanMX(r) for detection of the KanMX cassette from plasmid pYES2-KanMX/LacZ and **(b)** ILV2-F and ILV2-R for the detection of the SMR cassette from plasmid YEp351-SMR/LacZ. Cfus were used as templates for all PCR screens, including a Vin13pYES2-KanMX/LacZ cfu as positive control in (a) and a Vin13YEp351-SMR/LacZ cfu as positive control in (b). The – represents the negative control included in both screens (only demonstrated for the KanMX screen here), where only ddH_2O was used as template.

These data indicated that the KanMX orf and the SMR cassette was detectable through PCR screening, suggesting that these 5 isolates contain both plasmids pYES2-KanMX/LacZ and YEp351-SMR/LacZ. The absence of a PCR product in the negative control indicated that the PCR screen was free of any contamination.

4.4.5 Plasmid extraction from putative whole plasmid transferred Vin13 isolates and subsequent culturing in DH5α

To verify the presence of both plasmids YEp351-SMR/LacZ and pYES2-KanMX/LacZ simultaneously in each of these isolates, plasmid DNA was extracted from each of these 5 Vin13 isolates individually and transformed into *E.coli* DH5α. For each of these 5 isolates, the resulting transformed DH5α cfus would represent a combined pool of DH5α pYES2-KanMX/LacZ and DH5α YEp351-SMR/LacZ, assuming that both plasmids were present in the total plasmid extractions. Cfus were continuously screened through colony PCR, using primers KanMX(f) & KanMX(r) and ILV2-F & ILV2-R, until a representative of each DH5α pYES2-KanMX/LacZ and DH5α YEp351-SMR/LacZ were obtained from each pool (data not shown). These data verified the presence of both plasmids in isolates 95, 108, 109 and 113. Although

performed in duplicate, the inability to obtain DH5α transformants from plasmid DNA extracted from isolate 70 could be accounted to a technical limitation, either during the extraction or transformation process. An additional observation at this point was the abundance of DH5α transformants containing plasmid pYES2-KanMX/LacZ as compared to those containing YEp351-SMR/LacZ. This can be explained by differences in plasmid extraction efficiency or transformation efficiency. Indeed, plasmids are well known to display significantly different behaviour in this regard, and the finding can therefore not be taken as an indication that more copies of one of the plasmids may have been present in the initial yeast strain. Isolates representing each one of these plasmids from each of the pools were subsequently selected for plasmid DNA extraction which was further subjected to verification through R.E digestion (Figure 4.30).

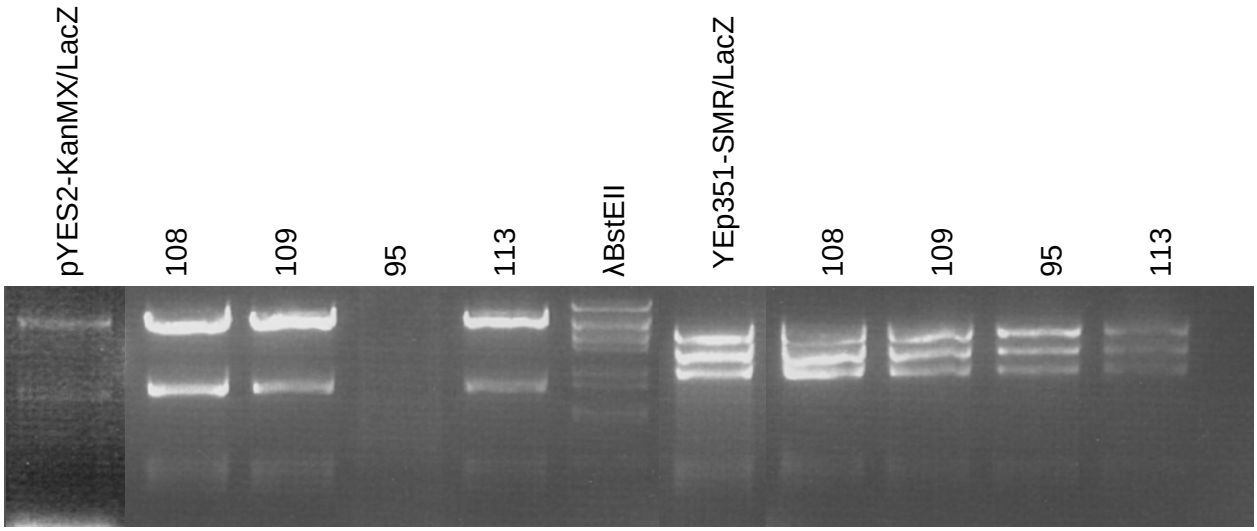


Figure 4.30 R.E digestion verification of plasmids isolated from putatively positive HGT of whole plasmid between Vin13 strains during co-inoculated fermentation.

The R.E. digestion data shown in Figure 4.30 confirm that plasmid DNA collectively obtained from isolates 108, 109 and 113 following co-inoculated fermentation in filtered grape must, contained two plasmids, showing banding patterns corresponding to that observed for pYES2-KanMX/LacZ digested with HpaI/BamHI R.E. as well as YEp351-SMR/LacZ digested with HpaI/BamHI R.E.

4.4.6 CHEF analysis

As confirmation that all isolates found to have undergone whole plasmid transfer events were indeed *S. cerevisiae* Vin13 in origin, total DNA extracts were subjected to karyotyping through CHEF analysis (Figure 4.31) (33).

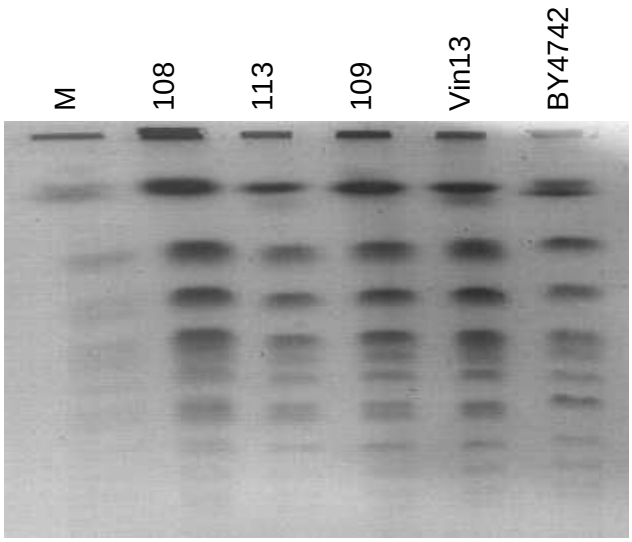


Figure 4.31 Karyotyping through CHEF analysis as additional confirmation that all isolates were Vin13 in origin; M = *S. cerevisiae* marker, *S.cerevisiae* strains Vin13 and BY4742 were included as positive and negative controls respectively.

The results obtained from the CHEF karyotyping indicated each of the isolates 108, 113 and 109 to have a identical banding patterns to that observed for Vin13, confirming their background to be *S. cerevisiae* strain Vin13.

This direct observation of whole plasmid transfer between Vin13 strains showed stable maintenance of the plasmid structure as opposed to the illegitimate recombination and subsequent integration observed in the spiked DNA experiments (See 4.3.1). However, similar to the integration results, these HGT events were found to occur at extremely low numbers when considering the total number of cells present in the fermenting cultures. A possible explanation to why the plasmid DNA involved in these HGT were not degraded during exposure to the supernatant (42) may suggest that these plasmids were in fact not exposed to the supernatant. This might therefore imply that these HGT events occurred through a mechanism involving direct cell to cell contact, which would allow for whole intact plasmid transfer from one cell to another (37).

4.5 ASSESSING THE OCCURRENCE OF HGT WITHIN A BIOFILM

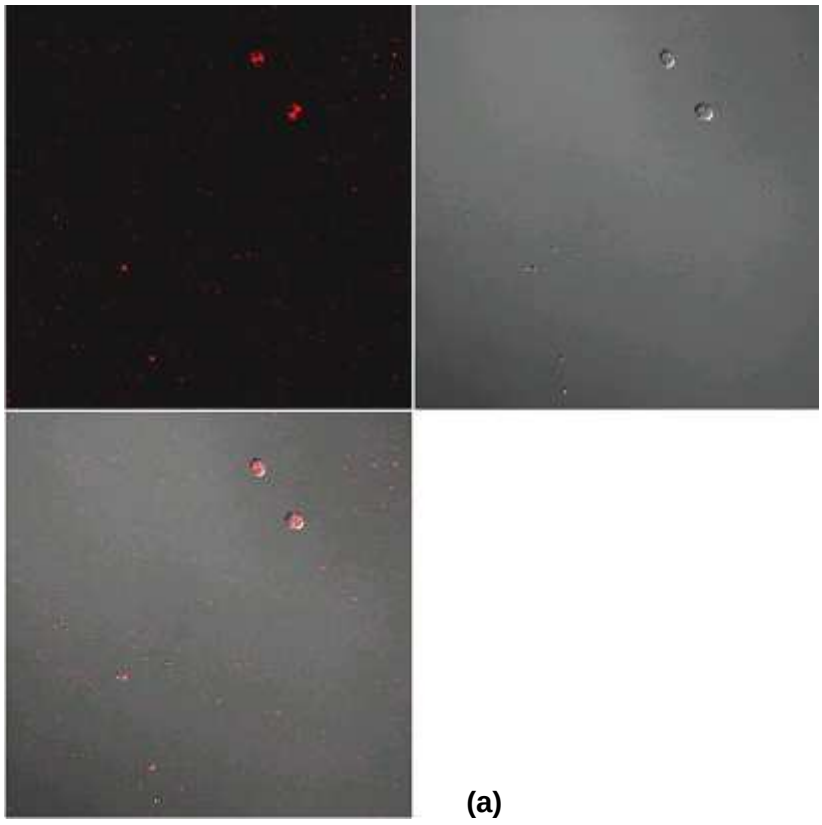
Biofilms are by definition a structure of microorganisms of a homo- or heterogenous composition, attached to living or inert surfaces, one consisting of free floating cells, referred to as planktonic cells, and the other consisting of cells densely attached to each other and the biofilm surface substance (4,5,26). This whole structure is maintained by a matrix consisting of largely variable extracellular polymeric substances (EPS), defined in composition by the nature of the microbial community itself (6). Biofilms are thus capable of sustained proliferation in countless environments; inadequate cleaning of the fermentation and waste effluent equipment in wineries present scenarios directly relevant to this study (11). In principle, these biofilm structures serve as an ideal environment for the occurrence of HGT where, once autolysis occurs, the EPS could potentially act as a net, capturing and immobilizing released DNA (10). Whether or not agents present in this highly variable EPS structure will serve to degrade or protect such extra cellular DNA remains to be determined. Nevertheless, assuming such degradation does occur, fractionated DNA may still be containing functional genetic information.

If naturally competent microorganisms were to be present in the viable fraction of the biofilm community, the close physical proximity thereof provides an ideal opportunity for an HGT event (34).

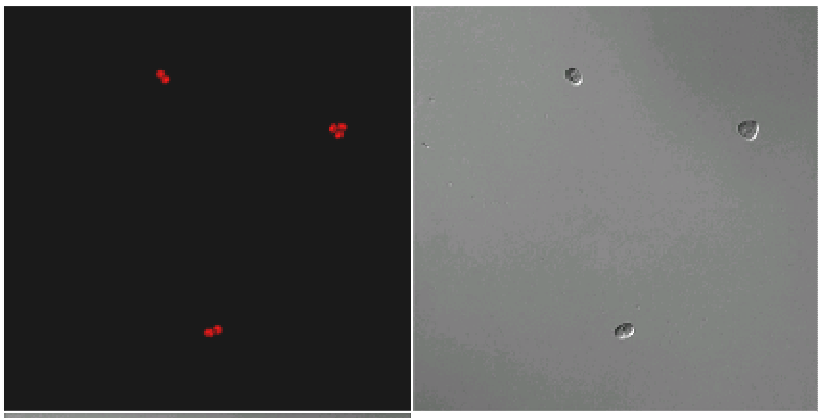
The initial focus of this study was therefore to visualize the release of DNA within a pure culture *S. cerevisiae* Vin13 biofilm by means of CLSM imaging, using various combinations of molecular probes selected to target cell walls and nuclear contents specifically. This strategy would allow visualization of a damaged cell wall and subsequent release of nuclear content, providing proof of concept of released DNA being immobilized within the EPS of a biofilm structure. The following section provides an overview of some preliminary data.

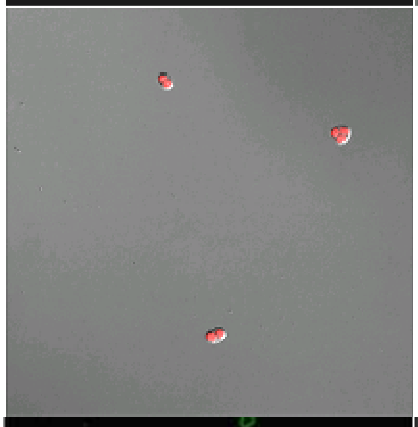
4.5.1 Optimizing molecular probes

In order to identify the optimal molecular probe combinations for visualization of Vin13 cell wall and nuclear content simultaneously through CLSM imaging, overnight Vin13 cultures, prepared in both 10% YPD and 1% YPD, were spotted on microscopic glass slides and stained with various molecular probes followed by CLSM visualization. Figure 4.32 demonstrates these stained cultures with the various probes discussed in Table 3.8.

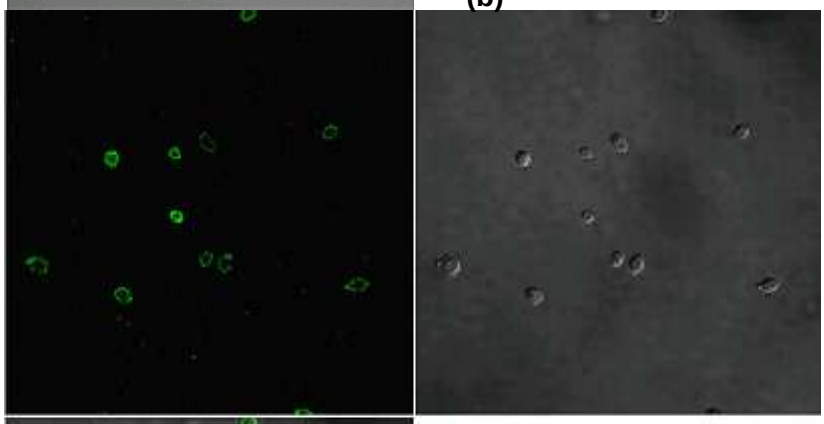


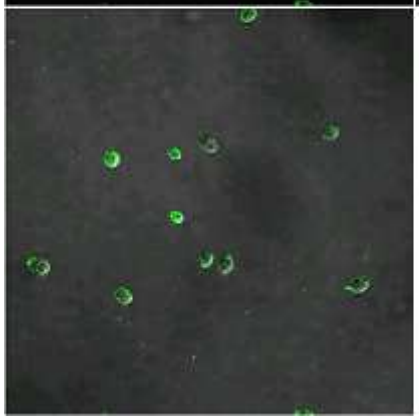
(a)



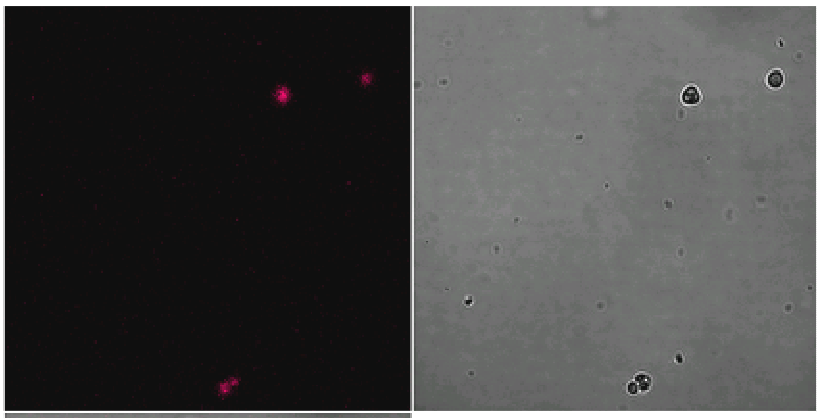


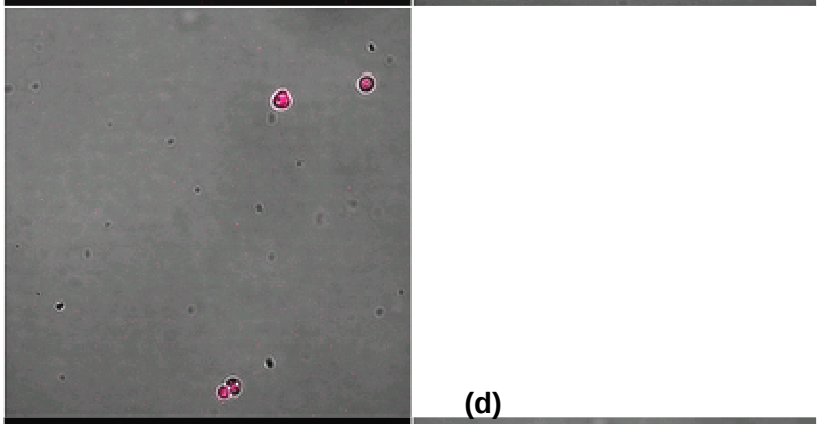
(b)



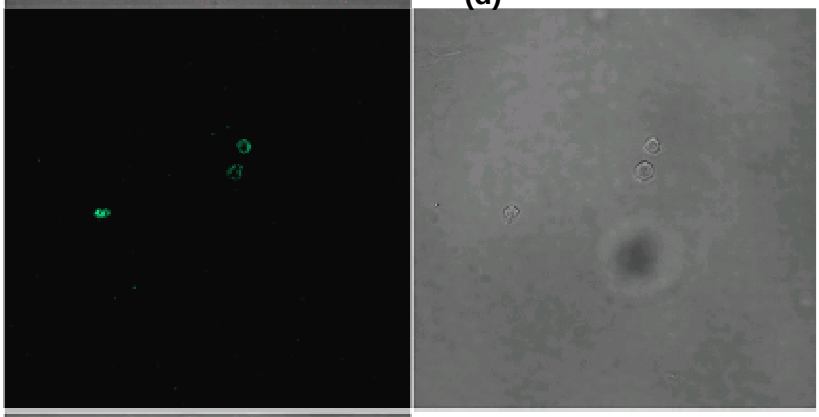


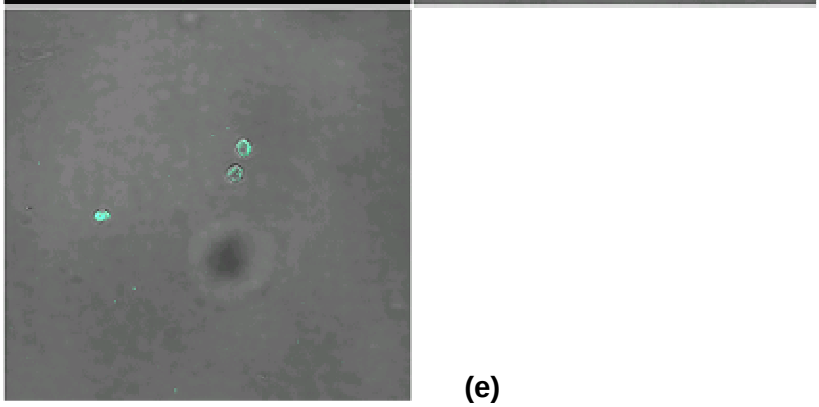
(c)



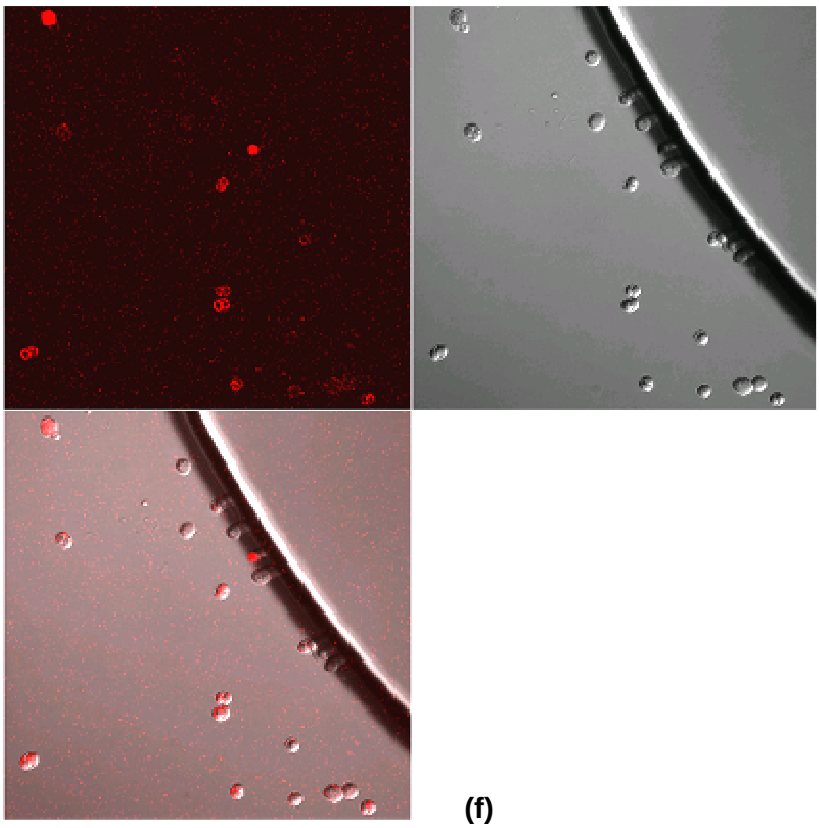


(d)





(e)



(f)

Figure 4.32 CLSM images of Vin13 cultured overnight in 1% YPD spotted on glass slides and stained with **(a)** nile red, **(b)** acridine orange, **(c)** concanavalin-A, flourescein conjugate, **(d)** ethidium bromide, **(e)** syto 9 and **(f)** propidium iodide.

These preliminary Vin13 CLSM images demonstrated indicated nile red (a lipid probe) to stain the cell wall unsatisfactory. Similarly, acridine orange and ethidium bromide (both nucleic acid probes) seemed to stain nuclear content diffusely and syto9 and propidium iodide (both nucleic acid probes) appeared to stain the cell wall preferentially. These data show that these molecular probes proved unsatisfactory. In addition, these images demonstrated how the 1% YPD cultures experienced nutrient depleted stress, resulting in sporulation, as observed by the ascospore formation in Figures 4.36 a, b, d and f. From these results, it was that for the purpose of CLSM visualization, concanavalin-A, flourescein conjugate as a cell wall stain and Draq5™ as nuclear probes gave the most relevant results (data not shown).

4.5.2 Stained biofilm visualization through CLSM imaging

Having identified concanavalin-A, flourescein conjugate and Draq 5™ as the best combination for the purposes of this study, Vin13 pure culture biofilms were established on the base of a flowcell manufactured from Perspex and sealed from above with a glass microscopic slide, however, the dynamics of the flowcell design limited microscopic visualization. The flowcell was thus adjusted to consist of a Perspex frame, creating a chamber that was sealed from above and below with glass microscopic slides. This would allow for biofilm establishment on the basal glass slide and CLSM visualization could occur by inverting the flowcell, if necessary. It was noted, however, that upon inversion, the biofilm would detach from the glass slides, only retaining partial attachment in clumps or batches (Figure 4.33).

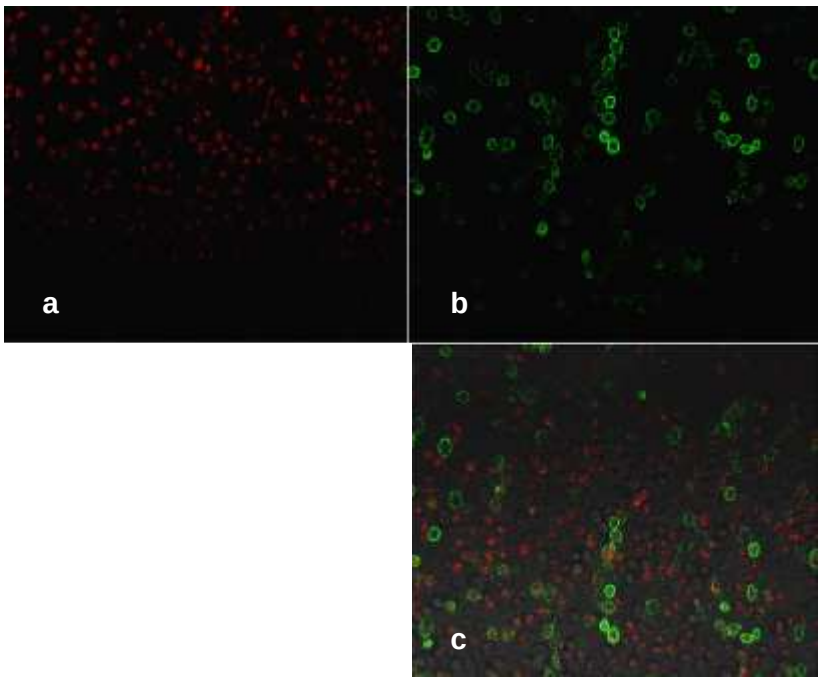


Figure 4.33 Vin13 pure culture biofilms stained with **(a)** Draq5™ (nucleic acid probe), **(b)** concanavalin-A, flourescein conjugate (cell-surface carbohydrate probe) and **(c)** Draq5™ and concanavalin-A, flourescein conjugate in combination.

The two molecular probes (concanavalin-A, flourescein and Draq5™) in combination proved to yield best results, staining their respective targets in such a manner that cell wall and nucleus could be clearly distinguished from each other. Prior to CLSM visualization, these flowcells had been inverted, resulting in near complete detachment and dissolution of the biofilm structure, retaining only small clusters of cells remaining attached to the original basal glass microscopic slide, as demonstrated in Figure 4.33. Furthermore, these results indicated that staining was not sufficiently sensitive to identify compromised cell wall and subsequent nuclear content leakage, as was the original aim of this experiment.

4.5.3 Screening for uptake and functional expression of spiked DNA in a biofilm

In order to still incorporate the biofilm aspect within the scope of the study, the focus was shifted from visualizing DNA leakage from biofilm-bound Vin13 cells through CLSM imaging to detecting uptake events of free DNA potentially having been trapped within the EPS matrix. This approach would also allow for controlled concentration presence of extracellular DNA by spiking at desired concentrations. This was done by establishing a Vin13 pure culture biofilm whilst continuously feeding a media supply containing multi-copy plasmid pYES2-KanMX/LacZ DNA at a predetermined concentration of 5µg/ml.

However, upon selective plating of the harvested mature Vin13 biofilm on YPGal agar supplemented with 100µg/ml G418, no growth was observed, indicating that no spiked pYES2-KanMX/LacZ had been accepted by the Vin13 biofilm cells (data not shown).

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Chapter 5

General discussion and conclusion

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5.1 CONCLUDING REMARKS AND PERSPECTIVES

Saccharomyces cerevisiae has been extensively applied as a model organism for countless purposes, allowing for the growth and development of widespread biotechnological applications. However, to date, no direct study to assess the occurrence of and/or elucidate the mechanisms involved in horizontal gene transfer (HGT) within this organism has been published. The field of HGT has gained much momentum over the past several years; a Pubmed Central search for research published related to HGT indicated 12 publications for 1990, 113 publications for 2000, 362 publications for 2009 and a staggering total of 3104 publications for the last decade as opposed to the 358 publications for the previous decade (http://www.ncbi.nlm.nih.gov/sites/entrez?db=pmc). Besides the significant implications of HGT for many evolutionary studies, this growing interest in HGT can also be attributed to the controversy surrounding the use of GMO's and fears related to the risks associated with "runaway genes". When considering the approach taken during the design of the afore mentioned biotechnological applications, particularly with regards to the development of commercially applicable microbial strains, one has to acknowledge the possibility of this "runaway gene" scenario, specifically because these strains may have been intentionally designed to have advantageous properties over their unmodified counterparts, potentially providing a natural selective advantage.

From the wine industries perspective, this issue has great relevance. The constant consumer driven demand for improved wine yeast strains has led to the development of numerous genetically engineered strains, providing several advantageous traits which impact on wine quality, wine production practices and consumer perceptions. However, prior to the industrial application, the possibility of HGT events involving these genetically engineered strains requires thorough evaluation. This reality was the driving force behind this PhD research project, aimed at establishing the relevance of these concerns within a specific environment by evaluating the occurrence and mechanisms involved in HGT with regards to specifically *S. cerevisiae* strain Vin13 during alcoholic fermentation.

This study revealed several novel and important findings. Firstly, during an investigation of the possibility of DNA being released from genetically modified Vin13, it was shown that no DNA of the investigated size could be detected within the fermenting background with a PCR screen. The detection levels achieved with the optimized PCR procedure were sufficiently sensitive to detect released DNA at levels requiring significantly less than 1% autolysis of the total fermenting culture at any point during the growth phase. The absence of a positive finding can in all likelihood nevertheless be attributed to insufficient sensitivity, since some DNA release can be assumed to take place in any microbiological system. A plausible explanation for the lack of DNA detection is that external factors e.g. nucleases, temperature and pH (9) are responsible for severe degradation of released DNA, consequently yielding fragments smaller than the 500bp detection limit set within this study. Regardless, it is assumed that no significant genetic information can be attributed to such small DNA molecules, placing such releases outside the scope of this study.

A second finding of this study is the remarkable stability of the spiked DNA observed under the MS300 fermentative conditions, returning our attention to the prior discussed scenario of DNA degradation. This stability suggests that degradation of DNA is rather limited in the media, since spiked plasmid DNA of up to ~1000bp was stably maintained for up to 18 days in

fermenting synthetic must (MS300) and up to 22 days in fermenting YPD (containing 10% glucose). Similarly, spiked linear DNA was stably maintained for up to 62 days in fermenting MS300. The spiked DNA, both plasmid and linear, could however not be detected in fermenting grape must within the sensitivity range establish for the MS300 fermentations. We propose that as yet unknown components within this specific fermenting background may have been responsible for binding to the DNA, rendering it physically inaccessible to the PCR amplification technique.One should also consider the possibility of factors yet unknown present within the fermenting grape must, capable of DNA degradation in a fashion similar to that observed for R.Es.

That free floating DNA in grape must may be bound to other molecules ties in with the novel finding that uptake and functional expression of spiked plasmid DNA by fermenting Vin13 cultures only occurred in real grape must fermentations. It is possible that such unknown components may also serve as a carrier molecule for successful transformation of the fermenting cultures, much like the carrier molecules employed during standard laboratory transformation protocols (such as herring sperm DNA) (2).

In addition, the data suggest that spiked plasmid DNA was indeed degraded within the supernatant, since no intact plasmid DNA was detected within the transformed cells. All transformants indeed contained only fragments of the original plasmid. All were integrated at different chromosomal loci. Interestingly, while the chromosomal integration site varied in each of the investigated strains, the integration site within the linear plasmid was restricted to a small section of the original plasmid backbone close to the TEF promoter. While it cannot be excluded that this may be without biological significance and rather reflect the limited size of the sample, it is more likely an indication that this sequence contains an element favouring recombination.

Degradation within the media would have generated linear fragments of varying lengths with varying amounts of critical genetic information. Our analyses show that the KanMX cassette has integrated into chromosomes III and XII of the *S. cerevisiae* genome. The initial Southern blot analysis produced identical banding patterns, suggesting a single integration site for each of these isolates. However, it was later determined through sequence analysis that these integration sites all differed from one another. These results could be accounted to chromosomal translocation events, possibly influenced by the presence of TY elements, resulting in rearrangements subsequent to the initial CHEF analysis (7). These integration sites further revealed no significant sequence homology to the integrated KanMX cassette, implying that integration occurred through a process known as illegitimate recombination, a recombination mechanism which can occur without the need for extended homologous sequences. Research has indicated that as little as 4bp homology was sufficient for illegitimate recombination in *S. cerevisiae* (5). In a study performed in an *Acinetobacter* species it was demonstrated that these short homologous stretches (3-8bp) are capable of serving a recombinational anchor, thus facilitating the illegitimate recombination and subsequent integration of the foreign non-homologous DNA (1). It was also found in the same study that integration into the *Acinetobacter* genome often accompanied deletion of genomic DNA of a similar size to that integrated. Whether or not that occurred in this case remains to be determined. It has been found, however, that the efficiency of homologous recombination is directly proportional to the length of sequence similarity (7). This may then also explain the low number of HGT events observed in this study.

Several cultures initially displayed β-galactosidase activity and/or G418 resistance suggesting the presence of the LacZ and KanMX cassettes, but lost these cassettes in successive generation cycles. This observation can be explained as an initial period of transient expression from what would most likely have been a linearized fragment containing the LacZ or

KanMX cassettes. Such linear fragments, if not integrated into the genome, may persist for a several generations, but will be lost over time. On the other hand, the fragments may have been integrated, but may have contributed to genome instability followed by DNA deletions (3,4,8). Such a scenario would be in line with suggestions that transgenic DNA may in some cases be less stable than normal chromosomal DNA.

The final highly relevant novel finding from this study was the direct observation of whole plasmid transfer between Vin13 strains, showing stable maintenance of the plasmid structure as opposed to the illegitimate recombination and subsequent integration of linearized DNA observed in the spiked DNA experiments. These HGT events also occurred at extremely low numbers in fermenting grape must.

Both findings, the uptake of free DNA and the observation of probable direct transfer events, are of high scientific and industrial relevance since they suggest that DNA transfers may indeed occur during industrial wine fermentations. On day 13, when the first single uptake and integration event was observed, the fermenting culture OD_{600nm} was approximately 4.4 (data not shown), correlating to a total of more or less 1.3×10^{10} cells present in the 100ml fermenting grape must at that specific time point. Having only detected 1 uptake event amongst approximately 1.3×10^{10} cells, the fact remains that, while the observed frequencies were low when considering the number of events compared to the number of cells involved, a single transfer event can in principle present a significant risk. Additionally, the character conferred by the transferred DNA should be taken into account when gauging whether the DNA involved merely poses a risk for HGT or whether it poses a hazardous risk (to humans, animals and the direct ecosystem) if it were to undergo HGT. By analysing the hologenome of the particular environment possibly affected by an uncontrolled HGT event, one can gain an insight as to which genes are already present in that environment, thus posing less of a hazardous risk.

However, there is a possibility that what has been monitored here as a direct transfer of DNA may instead have been the consequence of sporulation and mating during the alcoholic fermentation – otherwise said a rather traditional vertical gene transfer. Microscopic observations of wine fermentations never revealed sporulation events during the early stages of fermentation when some of the transfers were detected. Furthermore, the chromosome patterns of the identified HGT strains were all identical to Vin13, while strains that originate from traditional mating events, even from single parent events, tend to show adjusted chromosomal patterns because of the random assortment of parental chromatides during segregation. This is indeed usually the outcome when strains that are bred from Vin13 as single parent are investigated, since less than 50% of descendents will show a chromosomal pattern identical to the parent (unpublished data). On the other hand, it is obvious that the genetic selection applied here to identify HGT events is of tremendous sensitivity, while single or isolated sporulation events in large populations would not be easily detected. It is obvious that the experimental design in this case was suboptimal, since two different parental strains with clearly distinguishable chromosomal patterns would have allowed an easier differentiation between real HGT and possible low-level sporulation and mating events.

Nevertheless, the fact that free DNA can be taken up during alcoholic fermentation, as clearly demonstrated in the uptake of spiked DNA experiments, strongly suggest that the potential for HGT during such processes is significant. This clearly would have an impact on risk assessment of GM microorganism in wine making and other fermentation processes. In this regard, the concept of the hologenome, or environmental genome, would become a major determinant of potential risk. Indeed, if a gene is present within a given environment, and if HGT has been shown to occur within this environment, the assumption would be that events similar to the targeted GM transfer can and will happen.

Whether our results are reproducible within an industrially applicable setting cannot be addressed at this stage, since no industrially sized fermentation unit that would be approved for the use of GM yeast is currently available in SA. Nevertheless, the data suggest that HGT has the potential to occur within an industrial scenario. Thus, before any claims that HGT poses a significant risk in industrial wine fermentations can be made, further studies similar to this one should be undertaken.

5.2 LITERATURE CITED

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