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Genetic Manipulation of Commercial Yeast Strains

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Introduction

Yeasts of the genus *Saccharomyces* are among the most cultivated micro-organisms exploited by man. A graphic record of baking and brewing was made on the wall of a Fifth Dynasty Egyptian tomb dating from about 2400 BC. This brewing process used malted barley to provide fermentable substrates, but the yeast must have originated from contamination of raw materials (and eventually brewing equipment) and the air. Beer brewing and consumption was a well-established practice in Western Europe in 55 BC when Pliny noted the production of intoxicating beverages from barley. Strains of *Saccharomyces* are also used in baking, distilling and the production of fermented foods such as soy sauce. Yeasts from different genera are used in the treatment of spent sulphite liquor from paper manufacture (*Candida utilis* and *C. tropicalis*), the treatment of whey from cheese manufacture (*Kluyveromyces fragilis* and *K. marxianus*) and the production of single-cell protein from alkanes (*Yarrowia lipolytica*). Many of the commercially employed yeasts have no sexual phase to their life cycles (e.g. *Candida* spp.) or produce very few viable sexual spores (e.g. brewing strains of *Saccharomyces cerevisiae*). In consequence, these strains may be genetically modified only by the techniques of mutation, transformation, cell hybridization (independent of sexual fusion) and protoplast fusion. Those strains of *Saccharomyces* yielding good spore viabilities, and other ascosporogenous yeasts, are also amenable to genetical manipulation by more conventional sexual processes.

This review will concentrate on the manipulation of commercial yeasts with particular reference to brewing strains. Many of the problems en-

Abbreviations: ARS, autonomously replicating sequence; CEN, yeast centromeric sequence; ER, endoplasmic reticulum; GOI, gene of interest.

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countered are in common with those found when biotechnologists attempt to manipulate genetically defined (laboratory) strains of *S. cerevisiae* and to obtain expression of foreign DNA and secretion of foreign proteins. A review of these procedures has been given by Kingsman *et al.* (1985).

From the microbiological point of view, the fundamentals of the practice of brewery fermentation have evolved little since the demonstration by Pasteur in 1876 that fermentation required the participation of living organisms, and that of Hansen in 1888, that yeast could be isolated and propagated in pure culture. However, the technology of brewing and, in particular, the use of procedures and processing aids to improve product quality and production efficiency have evolved very significantly over the same period. From the point of view of the genetic engineer, the most interesting developments have been in the use of enzymes as processing aids to compensate for the inability of brewing yeasts to perform certain tasks. For example, amyloglucosidase is used in some products to convert carbohydrate, not fermentable by brewing yeast, to a fermentable form and the plant protease papain is used to hydrolyse protein, which is not attacked by brewing yeast, and to prevent the formation of haze. In addition, since the time of Pasteur and Hansen, the biochemistry of fermentation has been elucidated and yeast has been developed as an organism for genetic studies. This latter development owes much to the pioneering work of Winge (1935) on the life-cycle of *S. cerevisiae*, and of Lindegren and Lindegren (1943) on mating types. A very comprehensive genetic map of *S. cerevisiae* (an indispensable tool for the genetic engineer) is available (Mortimer and Schild, 1980). The demonstration of the suitability of yeast for use with recombinant DNA techniques (Hinnen, Hicks and Fink, 1978) originally developed in *Escherichia coli*, has led to renewed interest in the genetical manipulation of commercial yeast strains. These advances are seen as increasing the opportunities for modifying existing commercial strains to introduce novel characteristics. It is not surprising that, given its susceptibility to both classic and new genetical manipulation procedures and its acceptability for commercial fermentation processes using well-established technologies, *S. cerevisiae* is rapidly becoming a host of choice for use in the pharmaceutical and medical fields to produce non-yeast products.

The situation with regard to the brewing industry is rather different. The industry produces a well-characterized product by an essentially traditional process (*Figure 1*). The nature of the brewing process embodies certain unique constraints when compared with the use of, for example, yeast in baking, distilling or wine production. Thus:

1. The raw material used is principally an enzymic digest of malted barley produced by mashing and the source of the enzymes is the barley malt, although in some instances enzymes of fungal or bacterial origin may be used as process aids;
2. In most modern brewing processes, specialized brewing yeasts are maintained in a laboratory, cultured and transferred to specialized propagation equipment to produce the amount needed to inoculate a small fermenter.

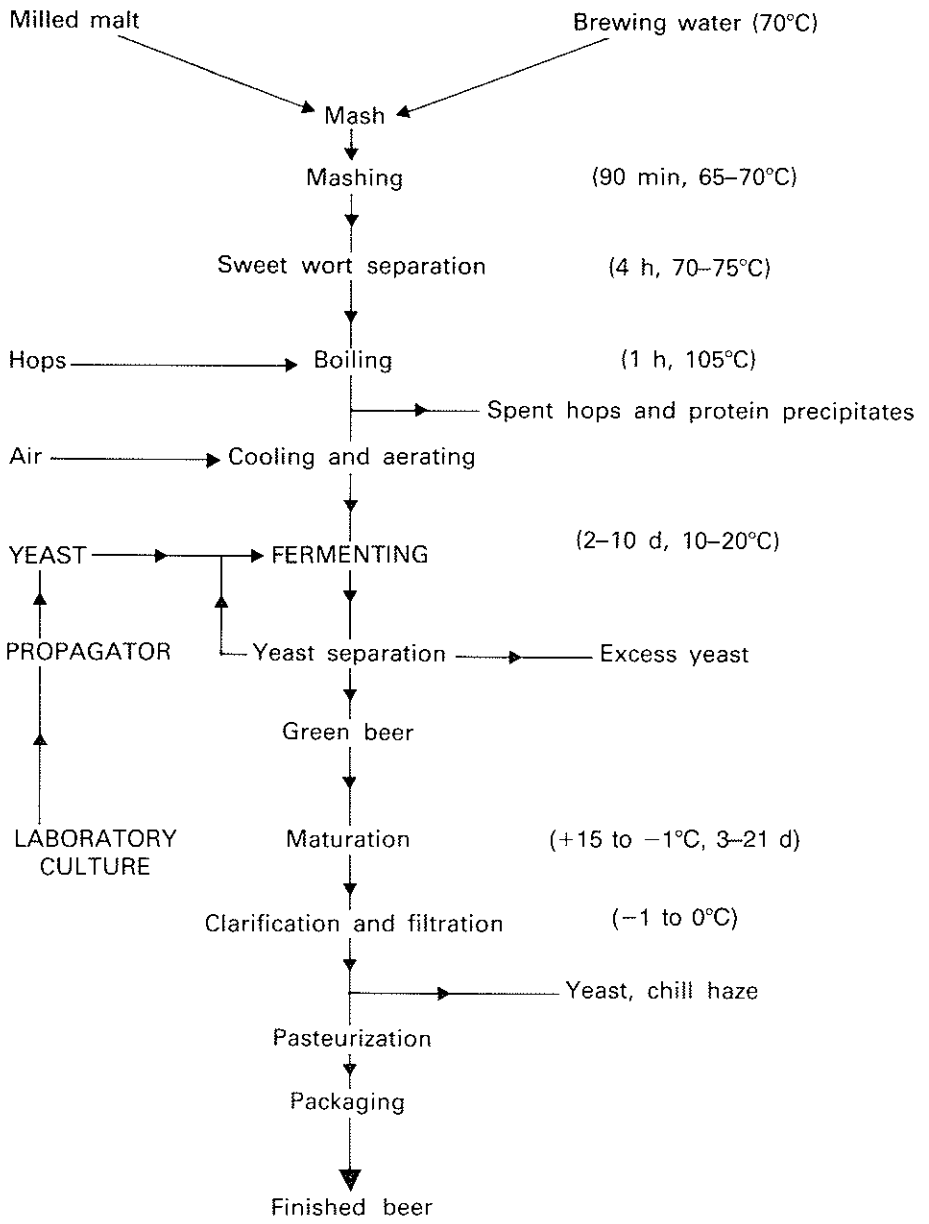


Figure 1. Flow diagram of the brewing process and associated yeast management. Typical times and temperatures for the key operations are indicated in parentheses.

The frequency of this process varies but is kept to a minimum;

3. A proportion of the yeast generated during fermentation is conserved and used to inoculate (pitch) subsequent fermentations. A brewery fermentation of 1000 hectolitres (611 UK barrels) of wort requires some 300 kg (660 lb) wet weight of yeast;
4. Maturation, embodying flavour changes, and stabilization (precipitation of chill haze) are needed.

Genetic manipulation of brewing yeasts may be considered as producing process improvements, more flexibility in choice of raw materials or new products (*Table 1*). In the first two categories the primary concern will be to achieve the objectives without changing the flavour and aroma of the final product. This in itself may prove extremely difficult because the flavour and aroma of beer depend upon the nature and concentration of a large number of minor metabolites of yeast metabolism (e.g. esters, higher alcohols). In addition to flavour, other desirable attributes of brewing yeast must be retained: the yeast must, for example, show adequate utilization of amino acids and sugars with the concomitant production of, and tolerance to, alcohol; cell growth, although inevitable, should be minimized; the flocculent nature of the strain must be retained to expedite the removal of yeast at the end of fermentation, and the strain must be genetically stable, thus ensuring consistent product quality. Commenting in 1938 on genetic change to brewing yeast, Winge stated 'undefined demands cannot be answered to order' (Stewart, 1978).

With these various constraints in mind, *Table 1* indicates those possible improvements to brewing strains which have been, or still are, the subject of active research.

Improvements to the process include lowering the time taken for fermentation, by obtaining yeasts which ferment faster or will withstand higher temperatures; yeasts with a high tolerance to osmotic pressure and alcohol would be capable of fermenting more concentrated substrate, thus increasing productivity (the final product would be diluted to the correct composition); yeast showing a smaller increase in yeast mass (growth rate) during fer-

Table 1. Possible improvements to brewing yeast.

Improvements producing		
Process improvements	Flexible use of raw materials	New products
Rate of fermentation	Hydrolysis of starch	Low carbohydrate
Temperature optimum	Hydrolysis of cellulose	Low alcohol
Osmotic tolerance	Utilization of lactose	Specific flavours
Alcohol tolerance		
Growth rate		
Infection proofing		
Hydrolysis of protein		
Hydrolysis of β-glucan		
Low diacetyl production		

mentation would yield more alcohol from the substrate; infection-proofed yeast would inhibit spoilage organisms and enable more fermentation cycles to be undertaken (less need to have recourse to a propagation cycle) and would enhance the biological stability of the product; yeasts able to secrete protease and β-glucanase would remove haze-forming precursors and reduce the time needed for maturation and stabilization; the formation of diacetyl is an undesirable characteristic of some brewing processes and maturation time would be reduced if yeast unable to produce this compound were available.

Flexible use of raw materials may bring about the replacement of expensive malt carbohydrate with cheaper sources. Engineering the yeast to secrete amylolytic enzymes and cellulases would permit the use of cellulose and starches (provided that they could be extracted in soluble form) from a variety of sources and would remove the dependence of the process on malt enzymes. Similarly, conferring the ability to use lactose on brewing strains would enable whey to be used as a substrate.

Genetic manipulation of brewers' yeast could be used to produce new products: for example, conferring the ability to secrete amyloglucosidase enables much of the residual carbohydrate in beer to be converted to fermentable sugar, which in turn is converted to ethanol; thus the overall carbohydrate content of the beer is lowered (so-called 'low cal' or 'lite' beers). Similar advantages would accrue from the production of strains secreting β-glucanase. Low-alcohol beers could, in theory, be produced by engineering the yeast to prevent it utilizing the major wort-fermentable carbohydrate (maltose), and beers with specifically enhanced flavours may be produced by the appropriate enhancement or restriction of yeast metabolic processes.

To date there are no reports of any genetically engineered brewing strains being used. The areas under most active investigation have been the incorporation of β-glucanase activity from *Bacillus* spp. (Cantwell *et al.*, 1985; Hinchcliffe, 1985), incorporation of dextrinase activity (Freeman, 1981; Tubb *et al.*, 1981; Stewart, Panchal and Russell, 1983) and infection-proofing (Young, 1981, 1983a,b; Hammond and Eckersley, 1984). In addition, preliminary reports have been made on alcohol tolerance (Korhola, 1983), hydrogen sulphide production (Takahashi, Hojito and Sakai, 1980) and diketone production (Holmberg, 1984).

Although genetic engineering offers great potential in these areas of brewing, many of the objectives may be achieved by other technically simpler and more economic means, e.g. by the use of exogenous enzymes or selection of other naturally occurring yeast strains. Furthermore, the products produced by genetically engineered yeasts may need to be subjected to rigorous (and expensive) testing before the strains are acceptable and thus the financial gains to be made from the production of such products must be significant.

The desirable characteristics of the yeasts used in other industries have been reviewed by Johnston and Oberman (1979), Rose (1979) and Spencer and Spencer (1983). For example, distillers' yeast would be capable of using

dextrins and starch and ferment mashes with high initial sugar concentration at elevated temperature. Wine yeasts would produce rapid fermentation with low foam production and low levels of organic acids, hydrogen sulphide and sulphurous acid in the product. As in brewing, there is scope for modifying yeast to produce specific flavour changes. Yeast used in baking would show a high level of invertase activity and a high level of enzymes of the glycolytic pathway. Benefit would be obtained from using baking yeasts which are capable of synthesizing enzymes and coenzymes under the essentially anaerobic conditions which develop in the dough. Acid tolerance of baking strains would also assist production processes which use acidified baking aids.

Food yeasts such as *Candida scotii* and *Candida tropicalis* have been modified to be more susceptible to enzymatic digestion and to contain enhanced levels of amino acids such as aspartate, glutamate and alanine, thus giving higher yields of a more valuable product.

Other industrial applications of yeasts such as the large-scale production of enzymes, other macromolecules or foreign proteins could also be improved and new processes evolved by genetic manipulation of appropriate strains.

Strain modification by selection

All programmes aimed at the heritable improvement of any organism rely on the recognition or production of genetic variation followed by its exploitation. The majority of techniques applied to laboratory yeast strains may also be applied to industrial strains, but with varying degrees of difficulty: this is because many commercial yeast strains display homothallism, polyploidy and aneuploidy, poor mating ability, a low frequency of sporulation and low spore viability. Although these problems can be overcome, a breeding programme based solely on mutation, selection and cross-breeding is a tedious and often fruitless process. Some advances have been made in this direction and are described below. General references to the techniques cited here can be found in Sherman, Fink and Lawrence (1974) and Mortimer and Hawthorne (1969). Similarly, detailed descriptions of many of the procedures in molecular cloning are given by Maniatis, Fritsch and Sambrook (1982).

SELECTION

Despite Hansen's isolation of pure cultures, a complete loss of variation in brewing strains did not ensue. These strains clearly displayed a degree of genetic heterozygosity that was probably increased by mitotic recombination during vegetative growth. Hansen (1888) himself exploited this in a search for strains with improved sporulation characteristics, and selection based upon such naturally occurring intrastrain variation has yielded non-foaming strains of sake wine yeast (Ouchi and Akiyama, 1971). Strains of rum yeast that yield increased levels of ethanol have also been isolated. Azevedo, Tavares and Melo-Cruz (1978) proposed the application of biometrical and quantitative genetic principles, currently employed in classical plant breeding,

to the improvement of cell-mass production in *S. uvarum*. These authors were able to partition the genetic and environmental variation statistically in their experiments and thus to choose the most appropriate selection procedure to maximize cell dry weight. They found that they were able to improve their strains by as much as 50% by selection between colonies of individual strains. Although this relatively under-utilized approach may well make dramatic initial improvements in quantitative traits, there is likely to be a rapidly acquired limit to the genetic gains possible. Furthermore, this technique is of little use for strain improvement where it is desirable to maintain or improve several parameters of commercial relevance. However, the use of selection indices (Williams, 1962) to improve multiple correlated traits that are weighted according to their economic importance could overcome this. In view of the wide variation, in brewing yeast strains, for certain characteristics such as ethanol tolerance, maltase activity, flocculation and the production of various flavour components, scope probably exists for the clonal selection of the optimal variant in such traits.

MUTATION SELECTION

The application of mutagenesis to extend the pre-existing natural variation has been responsible for the plethora of information available on laboratory yeast genetics. The use of agents such as ultraviolet irradiation or ethyl methane sulphonate on industrial yeasts has not been as successful. The mutations are frequently recessive and thus are expressed in commercial polyploid strains only when the effects of the unmutated alleles are lost. Mechanisms having this effect, such as mutation, gene conversion or mitotic recombination, frequently cause further rearrangements of the genome, resulting in other unwanted changes. The haphazard nature of mutation, which can simultaneously lead to improvement in certain traits as well as to debilitation of other characteristics, is the major disadvantage of this approach. However, mutations in certain biosynthetic pathways have been produced in commercial strains by such methods. Mutants resistant to thia-isoleucine produce beer containing more D-amyl alcohol than the parental strain (Kielland-Brandt, Petersen and Mikkelsen, 1979). These strains had a threonine deaminase that was relatively insensitive to feedback inhibition by L-isoleucine. Several higher alcohols are derived from the same biochemical pathway: the insensitivity of the pathway to isoleucine in the growth medium therefore leads to elevated levels of all of these flavour components during fermentation. Conversely, a leucine auxotroph was derived from a widely used wine yeast strain. It lacked isopropylmalate dehydratase (EC 4.2.1.33) and produced a wine with 50% less isoamyl alcohol than the original strain (Rous, Kunkee and Snow, 1983). Similarly, cysteine auxotrophs derived by mutagenesis of brewing yeast produced very low levels of hydrogen sulphide in beer (S. W. Molzahn, cited by Johnston and Oberman, 1979). Brewing yeast strains have been shown to harbour several recessive auxotrophic mutations (Delgado and Conde-Zurita, 1983); these were revealed by chromosome loss (induced by benomyl treatment) of the dominant prototrophic

alleles. This could prove to be a useful method of isolating new variants of commercial strains for further genetic manipulation. Generally, however, although mutation undoubtedly will continue to yield considerable information about biochemical processes, its direct use for strain improvement is limited.

The use of recombinant DNA techniques and integrative transformation to generate site-specific mutations and to reintroduce them to the yeast genome are considered later in this review.

Strain modification by hybridization

Winge and Laustsen (1938) were the first to produce new yeast types by recombination of existing variation between commercial strains. Johnston (1965) also attempted the production of new hybrids by conventional cross-breeding. These and other workers all encountered the problems mentioned previously, related to the life cycle of commercial strains. Because of this, repeated backcrossing of a derived strain to its original parent to remove undesirable characteristics is both tedious and inefficient. Gjermansen and Sigsgaard (1981) have demonstrated, however, that for certain commercial strains it is possible to segregate the nuclear genome of brewers' yeast into spores and recombine them by mating to produce hybrids that make acceptable beer. Anderson and Martin (1975) isolated mating derivatives of commercial yeasts and examined the hybrids obtained by crossing them. The hybrids displayed intermediate properties when compared with the parental strains, a result which would be expected for characteristics under polygenic control, e.g. fermentation rate. Hybridization has similarly been used to obtain novel baking yeast strains (Harrison, 1971). The application of the principles currently employed in classical plant breeding in the choice of the appropriate crossing scheme would introduce an element of control to this approach, but in general the process is rather imprecise, primarily because undesirable characteristics are as likely to be combined in a new hybrid as desirable ones.

RARE MATING AND SPHAEROPLAST FUSION

Two techniques have been employed to overcome the relative inability of commercial yeast strains to mate and have thus extended the range of crosses that can be performed. Respiratory-deficient auxotroph rare mating (Gunge and Nakatomi, 1972; Spencer and Spencer, 1977) involves the crossing of commercial strains lacking the ability to grow on glycerol or ethanol (because of a defective form or lack of mitochondrial DNA) with auxotrophic strains having normal respiratory characteristics. The hybridization product, although rare, is easily detected by its ability to grow on medium lacking amino acids but containing glycerol or ethanol as the sole carbon source. The hybrid so obtained can then be encouraged to sporulate and the segregants tested for their brewing performance. Tubb *et al.* (1981) used this method to construct dextrin-fermenting derivatives of *S. uvarum*/*S. dias-*

taticus hybrids that produced beer lacking the phenolic off-flavour which is characteristic of the beer produced using *S. diastaticus*. The genes responsible for dextrin utilization and phenolic off-flavour from *S. diastaticus* were found to segregate independently in a Mendelian manner and so were separated during meiosis.

The incomplete enzymatic removal of yeast cell walls and the polyethylene glycol-induced fusion of the resulting sphaeroplasts has been used to produce hybrids between what were previously non-mating commercial yeast strains (Hockney and Freeman, 1979; Stewart, Panchal and Russell, 1983). In general, sphaeroplast fusion serves only to remove any natural barriers to hybridization: the products are still likely to combine both desirable and undesirable characteristics of the parents. Some fusion products have been obtained that show potential in the production of high levels of ethanol (Seki *et al.*, 1983). This method also greatly increases the size of the 'gene pool' that may be exploited, as interspecific and intergeneric fusions are possible. Thus, stable fusion hybrids have been obtained between *S. diastaticus* and *S. rosei*, and between *Candida pseudotropicalis* and *Pichia membranaefaciens*. Conversely, some hybrids have been unstable, tending to revert and therefore to resemble one or other of the original parents. In the case of fusion of *S. diastaticus* and *Hansenula capsulata*, after several generations the hybrids gradually came to resemble the *H. capsulata* parent (Spencer *et al.*, 1983). The existence of some form of incompatibility system like that operating in other fungi could be involved and the fusion partners may have to be chosen with care.

Sphaeroplast fusion may have greater relevance in the genetic manipulation of yeast genera that have no sexual phase to their life cycle (e.g. *Candida* or *Torulopsis*; Fowell, 1969). This technique has been utilized in attempts to develop a cyclic parasexual system in *C. albicans* based on fusion of differentially marked strains and the subsequent gradual reduction in ploidy of this 'tetraploid' ($2n \times 2n \rightarrow 4n \rightarrow 2n$; Poulter *et al.*, 1982). Some success has been obtained with respect to the generation of unstable hybrids that are resistant to the clinical antifungal agent 5-fluorocytosine (Whelan *et al.*, 1985). Similar approaches to species of industrial importance (e.g. *C. utilis*) may yet follow. As many of these yeasts are involved in the production of single-cell protein, any method that increases the variation present in a strain will ultimately lead to strains that yield a higher cell mass.

CYTODUCTION AND SINGLE CHROMOSOME TRANSFER

A greater degree of specificity in a hybridization programme has been introduced by the use of *kar1-1* mutation (defective in karyogamy; Conde and Fink, 1976). The *KAR1* gene product is intrinsically involved in the function of the spindle pole body in *S. cerevisiae* (Rose and Fink, 1986). Inevitably, therefore, similar mutations could be isolated and exploited in other yeast genera of industrial importance. When present in either parent, this mutation impedes nuclear fusion after conjugation. The initial result of such a cross gives a high yield of progeny with the nuclear genotype of one

parent and the cytoplasmic elements of both parents: thus, hybrids containing the mitochondrial genome of one parent and the nuclear complement of the other have been constructed (Conde-Zurita and Mascourt-Suarez, 1981). This phenomenon of cytoduction has also been used to transfer other cytoplasmic elements, such as the 2 μm plasmid (Livingston, 1977) and its derivatives (Kielland-Brandt *et al.*, 1979) as well as to confer anticontaminant properties on commercial strains by the transfer of the double-stranded RNA, killer factor (Ouchi *et al.*, 1979; Young, 1981) (*Figure 2*). This plasmid encodes the production of, and immunity to, a toxin known to kill many wild yeasts. Unfortunately brewing yeast lacks killer factor and so industrial fermentations can be contaminated, resulting in fouled beer. Young (1983a,b) demonstrated that a commercial yeast strain engineered to produce this zymocide, by cytoduction of the killer factor, was resistant to contamination by certain wild yeasts and produced beer indistinguishable from that made by the parental strain.

It has been shown that, at low frequencies, whole chromosomes can be transferred. Nilsson-Tillgren *et al.* (1980) obtained transfer of chromosome III by constructing a cross of a canavanine-resistant adenine- and histidine (*his4*)-requiring haploid *S. cerevisiae* with a prototrophic haploid *S. cerevisiae* of opposite mating type. Because of the presence of the *kar1-1* mutation in the first parent, the transfer of chromosome III (carrying the *HIS4* gene) was selected by the ability of such clones to grow on minimal medium containing adenine and canavanine. The auxotrophic parent would not grow, because of its requirement for histidine, while the other parent and the true diploid were selected against on the basis of canavanine sensitivity (canavanine resistance is recessive). Subsequent genetic analysis indicated that few, if any, other chromosomes were transferred and that the incoming chromosome III was apparently intact.

The significance of single chromosome transfer was indicated by Nilsson-Tillgren *et al.* (1981), who crossed chromosome III of a commercial *S. carlsbergensis* strain into a laboratory *S. cerevisiae*. Although the brewing yeast chromosome was functionally indistinguishable from chromosome III of laboratory strains, it showed distinct structural differences. Restriction endonuclease digestion, agarose electrophoresis and hybridization of these fragments to a radioactively labelled copy of the *HIS4* gene indicated distinct differences in the *HIS4* region of the two chromosomes. Similarly, chromosome V has been shown to differ between *S. carlsbergensis* and *S. cerevisiae* (Kielland-Brandt *et al.*, 1983). This approach has obvious potential in the genetic characterization of whole chromosomes and could be used to transfer single chromosomes to commercial strains. However, the effect of other, unknown, genes also carried on the chromosome could alter other characteristics of the strain; the use of this approach in strain improvement is therefore limited.

Strain modification by DNA transformation

The addition of exogenous DNA to yeast and its subsequent incorporation into the genetic framework of the cell, resulting in the acquisition of a novel

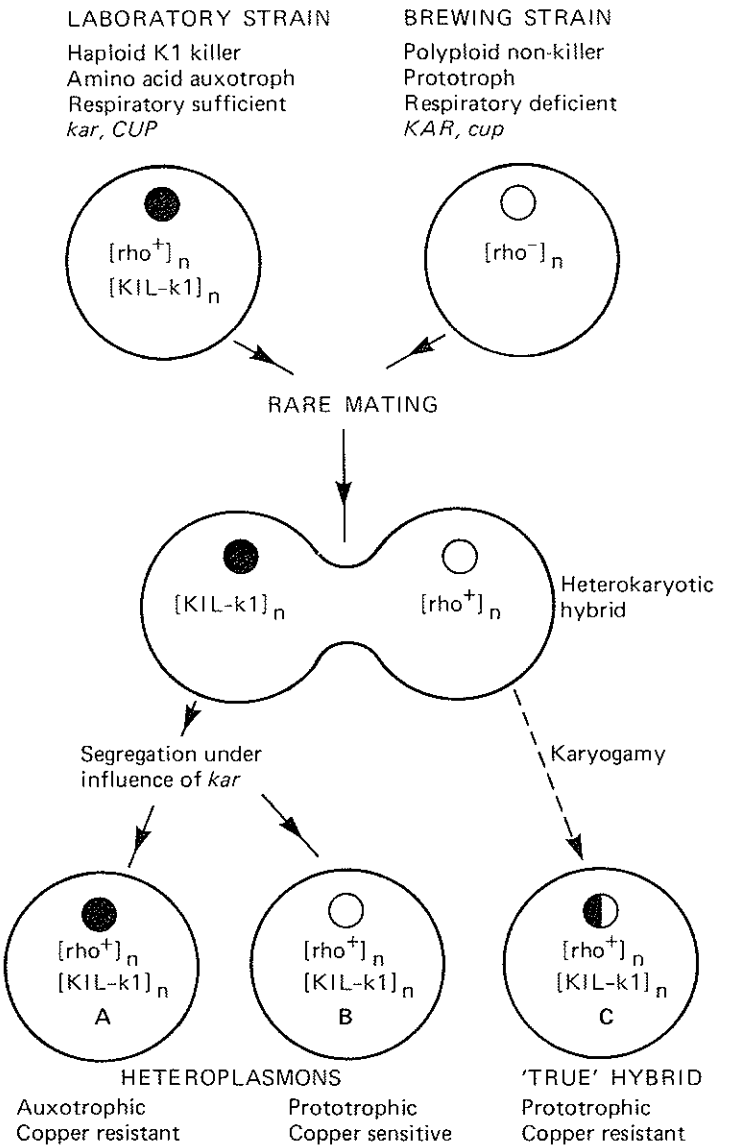


Figure 2. Genetical manipulation of brewers' yeast to yield an infection-proofed strain. Rare-mating and cytoduction in the presence of *kar* (Young, 1981). Cytoplasmic determinants: $[rho^+]_n$ = functional mitochondria; $[rho^-]_n$ = petite mutants (non-functional or no mitochondria); [Kil-k1] = killer dsRNA species. Nuclear alleles: *kar* = defective in karyogamy; *cup* = copper sensitive; *CUP* = copper resistant. ● = nucleus of laboratory strain; ○ = nucleus of brewing strain; ◐ = hybrid nucleus of hybrid strain. Following a hybridization event (rare mating), the heterokaryon segregates, under the influence of *kar*, the two parental nuclear genotypes with mixed cytoplasms (Heteroplasmons A and B). Selection on minimal medium plus glycerol ensures that the auxotrophic laboratory-strain and respiratory-deficient brewing-strain parents fail to grow. Heteroplasmon A is auxotrophic and therefore cannot grow. Heteroplasmon B may be distinguished from the true hybrid by its sensitivity to copper. Heteroplasmon B contains an unchanged brewing yeast nuclear genotype but now harbours the killer character dsRNA species and expresses killer phenotype. The secreted killer factor (zymocide) kills contaminating yeast species.

characteristic, is termed transformation. As the nature of the transforming DNA is under the control of the experimenter, the potential exists to change commercial strains with a degree of precision previously unattainable (*see also* Chapter 2 of this volume).

The isolation of *S. cerevisiae* genes with the ability to complement *E. coli* mutations led to the development of the vector pYeLeu10 carrying a yeast *LEU2* gene on an *E. coli* ColE1 plasmid (Ratzkin and Carbon, 1977). Treatment of sphaeroplasts from a leucine-requiring *S. cerevisiae* strain (*leu2* double mutant) with this DNA, in the presence of polyethylene glycol and calcium ions, resulted in their conversion to leucine prototrophy at a low frequency (Hinnen, Hicks and Fink, 1978). Subsequently this approach has been varied enormously, primarily in terms of the nature of the DNA used. Recently a method of transforming intact yeast cells using lithium or caesium ions has been described (Ito *et al.*, 1983). It has the advantage of being quicker and not requiring sphaeroplast formation or regeneration in agar; however it does not yield as high transformation frequencies for certain types of DNA as the sphaeroplasting method.

To be incorporated into the heritable components of the yeast cell, the transforming DNA normally suffers one of two fates: either it is maintained as a self-replicating plasmid, physically separate from the endogenous yeast chromosomes, or it must integrate into the chromosome and thus be maintained by the functions thereon. This categorization is not all-encompassing, as certain autonomously replicating plasmids also have the potential to integrate at low frequencies.

INTEGRATION INTO THE YEAST CHROMOSOME

Which of these two alternatives occur, is determined by the particular sequences on the transforming DNA. Most exogenous DNA is unable to replicate autonomously in yeast cells. The DNA can be propagated, however, if it recombines with homologous sequences in the host genome. The first report of yeast transformation, mentioned previously and involving pYeLeu10, resulted from integration of this plasmid by homologous recombination at the *leu2* mutant locus on chromosome III (Hinnen, Hicks and Fink, 1978). The integration either involved a single crossover and was additive, resulting in a tandem duplication of the mutant and wild-type alleles, or it was substitutive, involving the replacement of the mutant allele by the incoming *LEU2* gene by a double crossover or gene conversion. This type of transformation is not restricted to DNA encompassing the *LEU2* gene: vectors of the YIp type (yeast integrating plasmid) carrying the *HIS3* and *URA3* (Struhl *et al.*, 1979) genes have been shown to integrate at the appropriate loci by similar mechanisms.

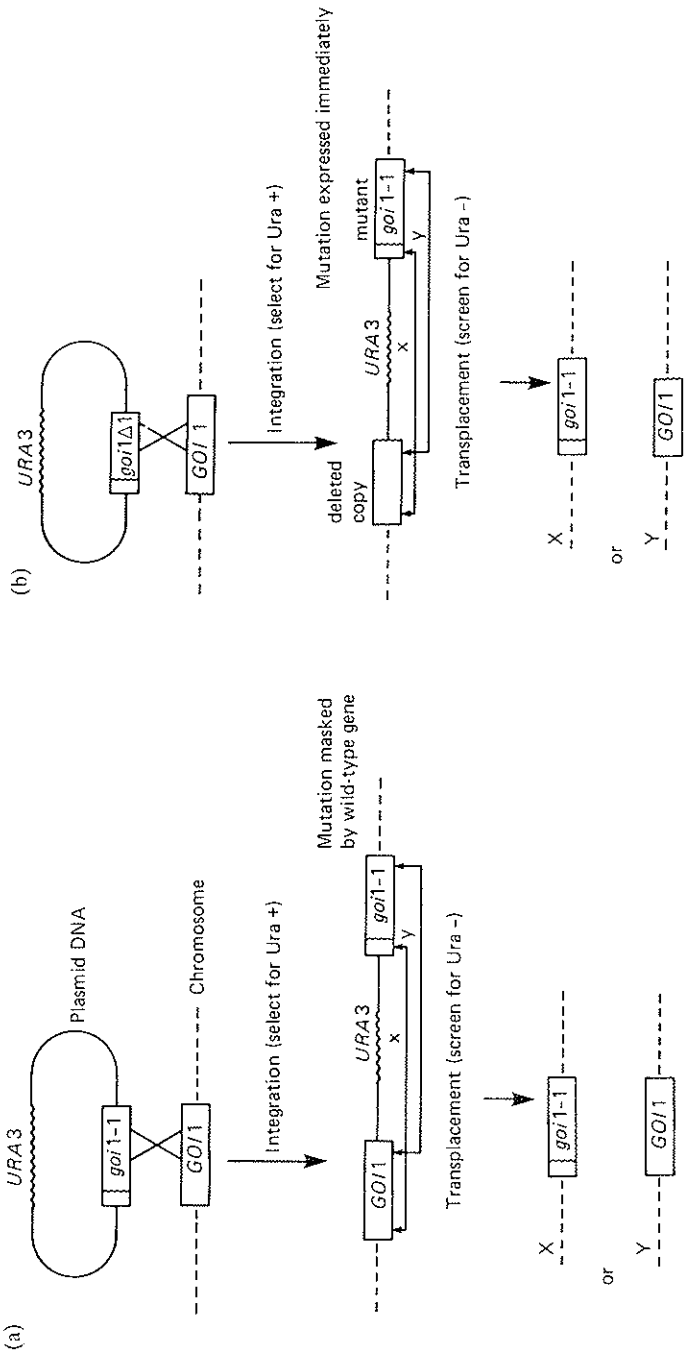
Surprisingly, pYeLeu10 also integrated at other points in the yeast genome. This was subsequently found to result from the presence of a Ty1–17 sequence adjacent to the *LEU2* gene. Ty1–17 is structurally related to the Ty1 transposable element, present at numerous locations in the haploid yeast genome; thus integration of pYeLeu10 also occurred via homology with this

sequence (Kingsman *et al.*, 1981). Plasmids containing two or more yeast genes (e.g. *LEU2* and *URA3*) have the potential to integrate at both chromosomal loci; however, transformants most frequently arise that have all the incoming DNA integrated at one or other of the loci involved. The genetic linkage of plasmid-borne markers was used to good effect in the mapping of the ribosomal RNA genes by integrating a plasmid containing *LEU2* and rDNA into the genomic tandem repeat of the latter. This enabled the rDNA locus to be 'tagged' with *LEU2* and facilitated mapping by standard procedures (Petes, 1980). In fact, the ability of newly isolated genes to integrate at the previously mapped chromosomal locus is often used as verification that the gene cloned is the one desired, and not an extragenic suppressor.

Integrative transformation occurs at the very low frequency of 1–10 transformants/μg DNA, but can be made more efficient by linearization of the plasmid with a restriction endonuclease. The location of the double-strand cut generated by endonuclease treatment determines where the plasmid integrates, because of the recombinogenic nature of free DNA termini. Thus a complex plasmid containing several yeast genes can be directed to integrate at a single site in the genome by cutting it within the appropriate region (Orr-Weaver, Szostak and Rothstein, 1981).

Integrative transformation has been utilized for the disruption (yielding a null phenotype) or site-specific mutagenesis of several chromosomal genes (e.g. actin; Shortle, Haber and Botstein, 1982). Both approaches rely on the initial cloning of the gene of interest (*GOI1*), its *in vitro* manipulation and the integration of the altered DNA sequence at the genomic locus involved (*see Figure 3*). The precision of the process is such that individual genes involved in any industrial process can be eliminated if they are deleterious, or altered to give their products novel properties such as thermal stability (or sensitivity), pH tolerance or constitutive expression. This has the clear advantage over conventional mutagenesis that it is essentially non-random and unlikely to involve any other genes (for a comprehensive review, *see* Botstein and Shortle, 1985).

Gene disruptions or replacements can be performed in several ways in one or two steps. Scherer and Davis (1979) pioneered the transplacement method or two-step disruption (*Figure 3a*). This involves the introduction of a copy of the wild-type gene (*goi1–1*), modified *in vitro*, by homologous integration at the chromosomal locus. This results in a tandem duplication of the gene of interest: the mutant and wild-type copies separated by vector sequences. Subsequently, after several generations of non-selective growth, a second recombination event between the unstable duplicated regions at each side of the vector occurs and is detected by the loss of the selectable marker. In such cases, the excision of the wild-type sequences leaves the mutant gene in the chromosome (*Figure 3a*, X) and excision of the mutant sequences (*Figure 3a*, Y) leaves a wild-type gene in the chromosome. A variation of this approach, which does not require transplacement, involves the integration of a mutated gene that is also deleted at one end (*Figure 3b*; Holm *et al.*, 1985). This results in a tandem duplication where only the


(a)
URA3
Plasmid DNA
goi1-1
GOI1
Chromosome
Integration (select for Ura+)
Mutation masked by wild-type gene
URA3
x
y
GOI1
goi1-1
Transplacement (screen for Ura-)
X
goi1-1
or
Y
GOI1
(b)
URA3
goi1Δ1
GOI1
Integration (select for Ura+)
Mutation expressed immediately
deleted copy
URA3
x
y
mutant
goi1-1
Transplacement (screen for Ura-)
X
goi1-1
or
Y
GOI1

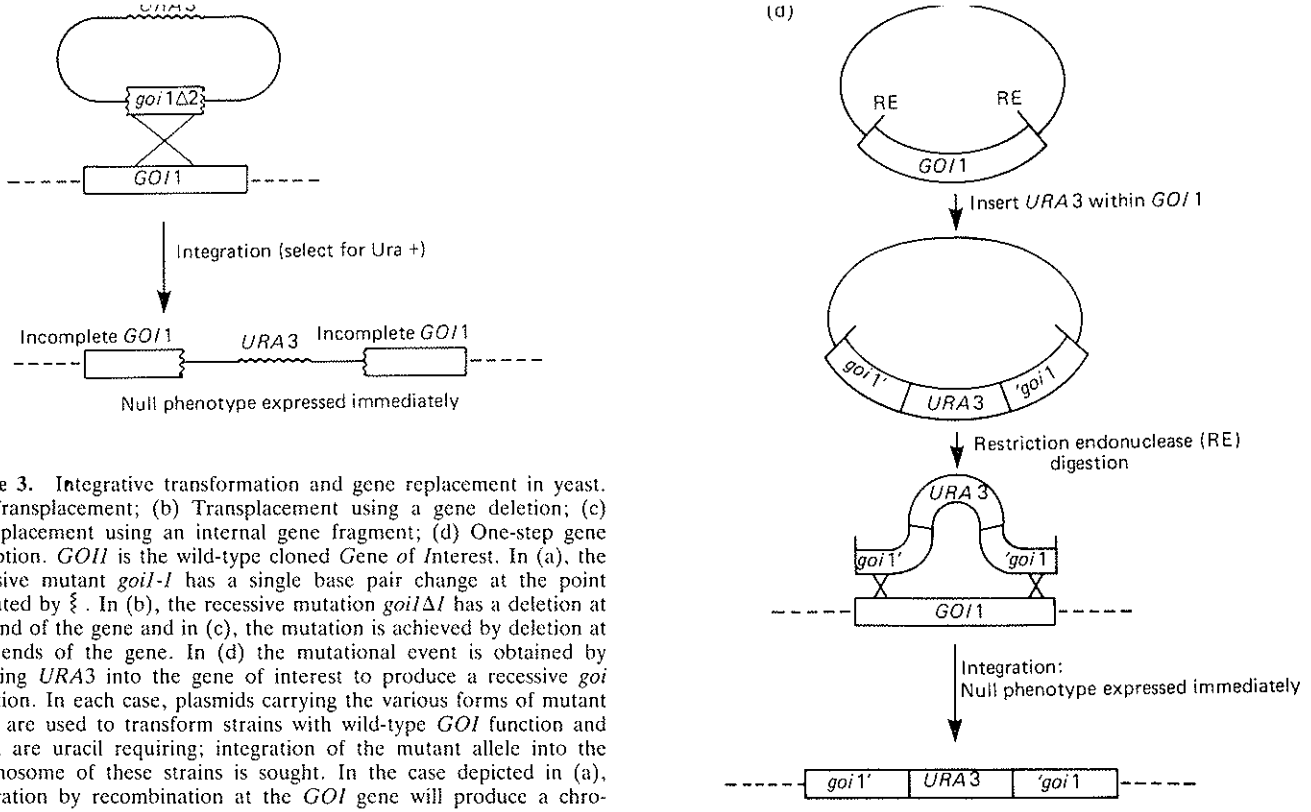


Figure 3. Integrative transformation and gene replacement in yeast. (a) Transplacement; (b) Transplacement using a gene deletion; (c) Transplacement using an internal gene fragment; (d) One-step gene disruption. *GOI1* is the wild-type cloned *G*ene *o*f *I*nterest. In (a), the recessive mutant *goi1-1* has a single base pair change at the point indicated by ⚡. In (b), the recessive mutation *goi1Δ1* has a deletion at one end of the gene and in (c), the mutation is achieved by deletion at both ends of the gene. In (d) the mutational event is obtained by inserting *URA3* into the gene of interest to produce a recessive *goi* mutation. In each case, plasmids carrying the various forms of mutant allele are used to transform strains with wild-type *GOI* function and which are uracil requiring; integration of the mutant allele into the chromosome of these strains is sought. In the case depicted in (a), integration by recombination at the *GOI* gene will produce a chromosome carrying *GOI1* and *goi1* separated by *URA3*; integrated clones will be URA$^+$ and can be selected for. In the absence of selective pressure, transplacement events may be selected for by looking for URA$^-$ clones. Recombination between *GOI1* and the point mutation *goi1* will leave either a functional allele (crossover event x) or a mutant allele (crossover event y) indicated by ↑——↑ and the resultant alternative chromosome arrangements (X or Y) are depicted at the bottom of (a). In the case of (b), the process is the same except that, unlike case (a), the mutant allele can be detected before the transplacement event because the use of a deletion mutant prevents the restoration of the wild-type allele (which in (a) masks the presence of the recessive mutation). Transplacement can occur in the absence of selective pressure giving clones containing the mutant allele *goi1-1* (X) and a deleted copy of *GOI* (Y, which of course is also a non-functional mutant). Single-step processes are depicted in (c) and (d): in both cases the integration events result in the mutant phenotype (null phenotype, no functional *GOI*) being expressed immediately. The process depicted in (d) generates a more stable product. In *Figure 3* as a whole the initial crossovers leading to integration events are indicated by X. In (a) and (b), x and y denote the crossovers (and X and Y the products of the crossovers) leading to the excision of the wild-type and mutant sequences respectively. – – – – – represents the yeast chromosome and *URA3* is the *S. cerevisiae* gene encoding uracil prototrophy in a *ura3* host. The manipulations shown in (c) and (d) yield null mutations which are lethal and are therefore usually carried out in diploid strains to ensure survivors.

mutated allele is intact. Thus, recessive mutations are expressed immediately (*Figure 3b*, X) because of the lack of an intact functional wild-type gene (*Figure 3b*, Y). As before, this unstable configuration can undergo a second recombination if the selective constraint is removed.

Integration of a gene deleted at both ends results in a tandem duplication where neither gene copy is active (*Figure 3c*; Shortle, Haber and Botstein, 1982). This results in the immediate expression of a null phenotype. Alternatively, a highly stable null mutation can be generated by selection for the double crossover in one step (*Figure 3d*; Rothstein, 1983). In this case, a selectable marker such as *URA3* is inserted within the cloned gene. The disrupted gene is then liberated *in vitro* from the vector DNA by restriction enzyme digestion such that the selectable marker is bounded by only *GOI1* DNA sequences (i.e. *goi1'-URA3-'goi1*; a minimum of 300 base pairs of homology with the target site is needed). The digested DNA is then used to transform a *ura3-GOI1* strain of yeast; selection is for uracil prototrophy. The desired transformants are strains that are simultaneously URA and goi.

Many of these manipulations, particularly those that generate null phenotypes, are lethal in haploid cells. However, as the integration events are rare, transformation of a diploid results in a heterozygous genotype and such strains are usually viable. The lethality of the disruption then becomes apparent only after meiosis where it segregates in a Mendelian manner (i.e. 2:2).

A corollary to this technique facilitates the isolation and ultimate analysis of mutant alleles of cloned genes (Stiles, 1983). The technique relies on the homologous integration of a portion of a cloned wild-type gene and vector sequences (normally pBR322) at the mutant locus. The yeast transformants (harbouring a duplication of the wild-type sequence and the mutant allele) are easily identified by the presence of the selectable marker which will show tight genetic linkage to the mutant phenotype. The yeast DNA is then cut with a restriction endonuclease that does not cut either the vector, the gene of interest, or those sequences between them. The DNA fragments are then ligated under dilute conditions that favour intramolecular events, and are used to transform *E. coli*. By selection for the marker originally on the vector, the mutant allele as well as some flanking yeast DNA is 'rescued'. The amount of flanking DNA recovered can be extended if a partial restriction digest is performed (again with an enzyme that does not cut the vector or 'target' sequence). In this way, naturally occurring mutations could be isolated and then transferred to commercial strains by integration at the wild-type locus without affecting any other genes.

Integrative transformation has also been demonstrated in non-*Saccharomyces* yeasts such as *Pichia pastoris* (Brust *et al.*, 1986) and *Yarrowia lipolytica* (Davidow *et al.*, 1985) and has led to the development of a recombinant DNA system in these organisms. Initially, genes complementing *S. cerevisiae* mutations such as *leu2, ura3* and *his4* were isolated and shown to integrate also at the equivalent loci in their original hosts. These genes were then cloned into pBR322 to form integrative vectors and used in the isolation of more industrially relevant genes such as exocellular proteases

(*Y. lipolytica*; L. S. Davidow, personal communication), alcohol oxidase and ARS sequences (*P. pastoris*; Brust *et al.*, 1986 and Cregg *et al.*, 1986, respectively). Similarly, we have recently cloned a gene from *Kluyveromyces drosophilarum* that complements a *his3* mutation of *S. cerevisiae* (Sturley, 1985). The techniques cited previously, involving gene disruption and mutagenesis, will undoubtedly soon be applied to these yeasts.

AUTONOMOUS REPLICATION

Yeast replicating plasmids

A much higher frequency of transformation of yeast can be obtained by the inclusion of certain DNA sequences into vectors. Plasmids constructed in the same way as the YIp types, carrying the *TRP1* (Struhl *et al.*, 1979) or *ARG4* (Hsiao and Carbon, 1979) alleles were found to transform yeast at a frequency of 500–5000 colonies/μg of DNA. The vector DNA within the cell was of a closed circular type. Further work on the *TRP1* plasmid (YRp7—yeast replicating plasmid 7) indicated that sequences close to the *TRP1* gene, rather than the gene itself, were responsible for this elevated frequency. Such autonomously replicating sequences (ARS) have been identified elsewhere in the genome and have been estimated to occur at a frequency of once every 32 kb of genomic DNA (Chan and Tye, 1980). A putative consensus sequence for these ARS elements of T(or A)TTTATA(or G)TTTA(or T) has been identified (for review, *see* Williamson, 1985). Furthermore, ARS sequences from *C. utilis* (Hsu *et al.*, 1983), *Schizosaccharomyces pombe* (Beach and Nurse, 1981), *K. lactis* (Das and Hollenberg, 1982), *P. pastoris* (Cregg *et al.*, 1986) and *Yarrowia (Saccharomycopsis) lipolytica* (D. M. Ogrydziak, personal communication) have been isolated by virtue of their ability to promote autonomous replication in *S. cerevisiae*. This has given further credence to the notion that ARS elements may be origins of DNA replication, although this has been demonstrated only indirectly *in vivo* at their normal location (i.e. on the chromosome) or in some cases in their original hosts. Plasmids of this type are normally present in multiple copies (3–10 copies per cell; Struhl *et al.*, 1979) and do not segregate normally at mitosis or meiosis. Thus they are very unstable and are easily lost from transformed cells.

Yeast centromere plasmids

In *S. cerevisiae*, this instability can be rectified by combining ARS elements with yeast centromeric sequences (CEN; Clarke and Carbon, 1980). Such plasmids (termed YCp—yeast centromere plasmid) transform yeast with efficiency similar to that of YRp plasmids but are present as a single copy of a covalently closed circular molecule. They are stably maintained during mitosis and segregate in a Mendelian fashion during meiosis (i.e. 2:2). The CEN fragment presumably offers the plasmid a potential kinetochore or spindle-binding site. To date, at least five centromeres (*CEN3, CEN4,*

CEN5, CEN6 and *CEN11*) derived from different chromosomes (III, IV, V, VI, and XI respectively; for review, *see* Carbon, 1984) have been extensively characterized. More recently, Hieter *et al.* (1985b) have isolated an additional five centromeres. In general, they share some interesting properties: for instance, centromere sequences are interchangeable (i.e. *CEN11* can replace *CEN3* on chromosome III) but species-specific (they do not function in *S. pombe* or *K. lactis*). The degree of stability that they impart to a plasmid is clearly related to the plasmid size and sequence. Thus a YRp plasmid stabilized by the addition of a *CEN3* region shows a higher frequency of non-disjunction than a 55 kb linear *CEN3* minichromosome containing yeast telomeric ends. This in turn is several orders of magnitude less stable than chromosome III itself (Murray and Szostak, 1983a). A consensus sequence has been established consisting of a central 82–89 bp A+T-rich (93–94% AT) region, designated CDEII, bounded by a conserved 8 bp region (CDEI, in *CEN3* GTCACATG) and a highly conserved 25 bp sequence (CDEIII, in *CEN3*, TGTTTTTGATTTCCGAAGTTAAAA). The orientation of this whole unit, in relation to flanking sequences, is irrelevant with respect to its ability to stabilize ARS-based plasmids or chromosomes. Generally, changes in the consensus regions tend to impair centromere function: in particular, deletion or alteration of the CCGAA sequence of element III totally inactivates *CEN3*. This region is conserved in *S. pombe* centromeres and is a strong candidate for a microtubule binding site, but it is clearly not sufficient for complete centromere function. In addition, consensus element II appears to be required to hold sister chromatids together during the first meiotic division (Carbon *et al.*, 1986).

Yeast episomal plasmids

The combination of portions, or all, of the indigenous yeast 2 μm plasmid with a selectable yeast gene such as *LEU2*, has yielded vectors of the YEp type (yeast episomal plasmid). These transform yeast very efficiently at a frequency of 5000–20 000 colonies per μg of DNA (Beggs, 1978; Struhl *et al.*, 1979), are more stable during mitosis and meiosis than YRp plasmids and are present in transformed cells as covalently closed circles at an average copy number of approximately 40 per cell. Although no biological role has been ascribed to the yeast 2 μm plasmid, considerable information exists about its replication and maintenance. The plasmid is a 6318 base pair (bp), covalently closed circular, double-stranded DNA molecule consisting of two unique sequences of 2744 bp and 2346 bp separated by two 599 bp inverted repeats (*Figure 4*). The origin of replication (or 2 μm *ARS*) extends from within one of the inverted repeats and must be retained by all 2 μm-derived vectors (Kojo, Greenberg and Sugino, 1981). Intramolecular site-specific recombination between the inverted repeats leads to the presence of two isomers, A and B, in roughly equimolar concentrations within the cell. The recombination is mediated by the *FLP* gene product acting in *trans*. Thus, chimaeric plasmids lacking this region will undergo intramolecular recombination only if the strain already harbours a resident 2 μm plasmid (a so-

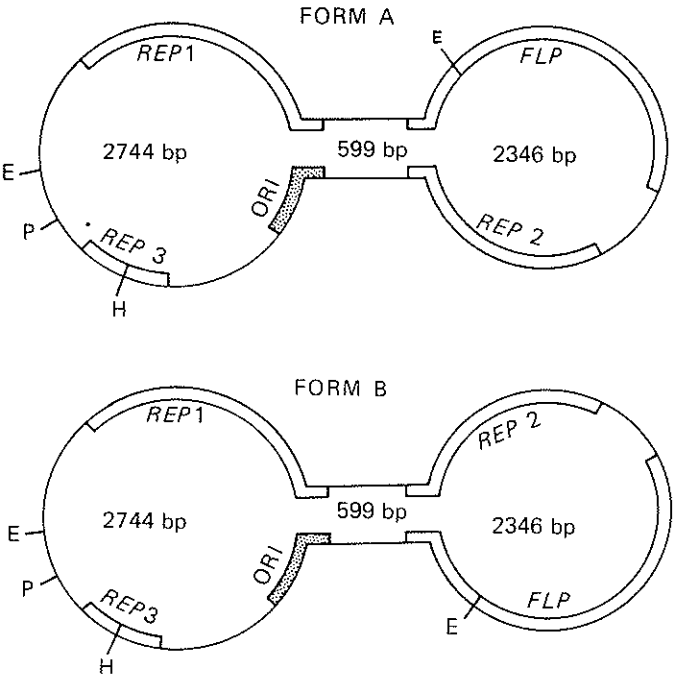


Figure 4. *S. cerevisiae* 2 μm plasmid (after Broach, 983). E, H and P denote restriction endonuclease recognition sites for *Eco*RI, *Hpa* I and *Pst* I respectively. ORI is the origin of replication. *REP*1, 2, 3 and *FLP* are explained in the text.

called [Cir$^+$] strain). It has also been postulated (A. W. Murray and J. W. Szostak, personal communication) that this recombination is responsible for the amplification process involved in 2 μm copy number control. During DNA replication, a crossover could occur between the inverted repeats such that one replication fork chases the other (instead of proceeding in opposite directions and joining up at the end of DNA synthesis). 'Run-away' plasmid replication would probably be lethal and would be prevented by a second recombination between the newly formed inverted repeats as well as an interaction with host-encoded functions. Plasmids containing only the 2 μm *ars* are present at much lower copy number in populations of strains lacking the 2 μm plasmid (i.e. [Cir^0] strains) than in [Cir$^+$] strains. Clearly, some *trans*-acting functions of 2 μm DNA, separate from the origin of replication, are involved in the maintenance of segregation of these plasmids. Two such genes have been identified, *REP1* and *REP2*, while a third region, *REP3*, acting in *cis* to the 2 μm *ars*, has been implicated as being the site through which *REP1* and *REP2* gene products exert their effect (Jayaram, Li and Broach, 1983). Pedigree analysis (Murray and Szostak, 1983b) of strains carrying these plasmids has indicated that the *REP* loci are involved primarily in segregation. By observing plasmid segregation in individual cell lineages,

it was clear that ARS and 2 μm *ARS, REP3* plasmids displayed a strong bias to segregate to the mother cell at mitosis. Only in the presence of CEN sequences or the endogenous 2 μm plasmid (i.e. the *REP1* and *REP2* gene products) was their partitioning uniform and therefore essentially stable within the cell population. Interestingly, CEN regions are epistatic to the 2 μm plasmid copy number control system (if one exists). Hybrid 2 μm/CEN vectors exist in only one or two copies per cell and are stable during mitosis and meiosis (Tschumper and Carbon, 1983). Presumably this reflects both the toxic effect of excessive numbers of CEN sequences in the cell (cells containing five extra centromeres show high levels of general chromosome non-disjunction, ultimately leading to cell death; Carbon *et al.*, 1986) and the absence of any segregational bias. Although *REP3* and CEN sequences are functionally similar, they share no DNA sequence homology: they could act by different mechanisms or be recognized by different proteins.

Very few chromosomal genes have been identified that are specifically involved in the maintenance of 2 μm DNA. An exception is the *NIB* locus (Holm, 1982). Lesions in this gene result in a 'nibbled' colony morphology in [Cir^+] strains due to a preponderance of abnormally large, mortal cells containing excessive levels of 2 μm DNA. The author proposed that *NIB* acts antagonistically to the amplification process and maintains the 2 μm copy number at tolerable levels. However, such a mechanism must be sensitive to the physiological status and requirements of the cell. For example, vectors such as pJDB219 (Beggs, 1978) are able to amplify to a copy number of 200 on medium lacking leucine. This plasmid contains the *leu2-d* allele which expresses only 5% of wild-type β-isopropylmalate dehydrogenase activity and so a high copy number is required to overcome this deficiency (Erhart and Hollenberg, 1983). This in turn results in the curing from the cell of extraneous (i.e. wild-type) 2 μm plasmids (Dobson, Futcher and Cox, 1980).

All the vectors cited above are 'shuttle' plasmids in that, in addition to the yeast sequences, they all carry *E. coli* plasmid replicons and markers (*see Figure 5*). Generally, these are derived from pBR322 (Bolivar *et al.*, 1977) and harbour the ColE1 origin of replication and genes specifying resistance to various antibiotics. The scope therefore exists for the selection, amplification and purification of these plasmids in *E. coli*, before transformation of yeast. Furthermore, within the antibiotic resistance genes are unique restriction endonuclease sites which facilitate the cloning and manipulation of foreign DNA. Vectors lacking bacterial sequences have been used in the transformation of yeast. These have consisted of 2 μm plasmid fragments ligated to yeast markers such as the *HIS4* gene (Kielland-Brandt *et al.*, 1979) or the use of total native yeast DNA (Jansen *et al.*, 1979). In addition, the TRP1 R1 plasmid (formed by circularizing the 1.4 kb *TRP1/ARS1* fragment; Zakian and Scott, 1982) is a very stable high-copy yeast plasmid. However, its use in strain improvement could be limited if these properties result from its small size. Alternatively, the stability of this vector could be due to the absence of certain pBR322 sequences which are deleterious or toxic to the yeast cell. The removal of these sequences could result in increased stability for any plasmid and therefore would be worth while in an industrial context.

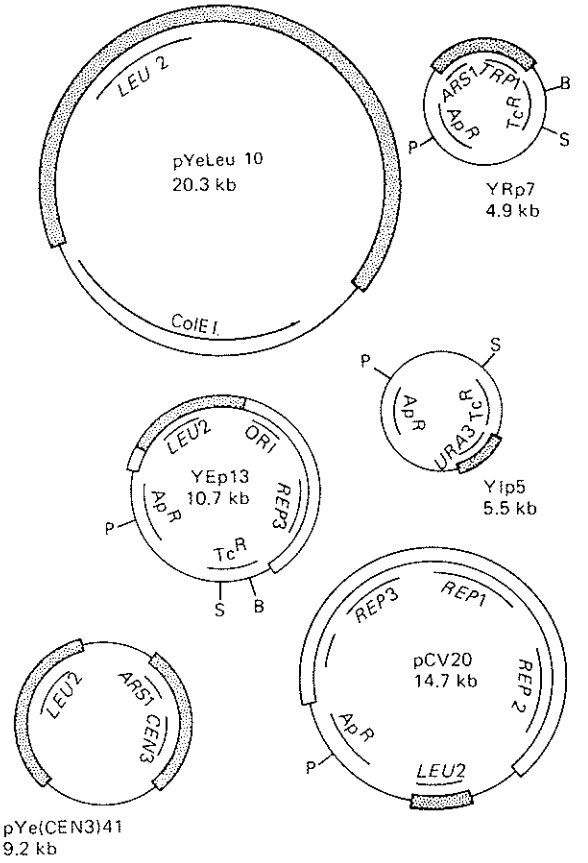


Figure 5. Yeast–*E. coli* shuttle vectors. P, S and B indicate unique restriction endonuclease recognition sites for *Pst* I, *Sal* I and *Bam* HI respectively. Single lines indicate sequences derived from pBR322 (or Col E1 in pYeLeu10). Shaded double lines represent yeast chromosomal DNA sequences. Double lines represent yeast 2 μm plasmid sequences. Ap^R = ampicillin resistance; Tc^R = tetracycline resistance; ColE1 = colicin resistance; ORI = 2 μm plasmid origin of replication. Gene designations are given in the text.

Such an approach has the added advantage that the involvement of *E. coli* DNA sequences or the passage of the DNA through a bacterial host is avoided. This might be appropriate in the breeding of strains used to produce beverages for human consumption.

Expression, linear and promoter plasmids

Parent, Fenimore and Bostian (1985) have extended the YIp, YRp, YEp and YCp nomenclature for yeast plasmids (Botstein and Davis, 1982). This includes expression plasmids (YXp) that have transcriptional promoters and terminators, and occasionally sequences that direct post-translational pro-

cessing and secretion. These have obvious applications for heterologous gene expression and are dealt with at a later point. Plasmids of the YLp type (yeast linear plasmid; Murray and Szostak, 1983a) contain sequences (C–A rich) that function as telomeres in yeast and are modified by the addition of several hundred base pairs of a (C_{1-3}-A) repeat unit (Szostak *et al.*, 1986). Yeast promoter plasmids (YPp) have easily assayed protein-coding sequences (e.g. β-galactosidase; Casadaban *et al.*, 1983) that permit the fusion and analysis of homologous and heterologous promoters.

Selection of vectors for use with commercial yeasts

The use of recombinant DNA techniques has obvious, if unproven, advantages in the production of improved commercial strains. As the source of transforming DNA is virtually unlimited, almost any change in a yeast strain can be accomplished precisely. Some caution concerning the choice of the vector is advisable. As stability of the novel characteristic during fermentation and in subculturing for subsequent fermentations is of prime importance, plasmids of the YRp type are unlikely to be of use. 2 μm-containing YEp plasmids, although considerably more stable, may integrate at low frequencies into yeast chromosomes (Falco and Botstein, 1983). The regions around these integrations frequently break, resulting in chromosomal rearrangement or loss. Furthermore, many industrial strains possess endogenous 2 μm plasmids (Tubb, 1980) and recombination between these and the incoming hybrid plasmid could result in loss of the newly acquired characteristic. However, Walmsley, Gardner and Oliver (1983) have shown that loss of 2 μm-based plasmids, especially in the case of pJDB219 (Beggs, 1978), from continuously cultured cells is not exponential and that pJDB219 at least is relatively stable. In the initial stages of a breeding programme, the isolation and cloning of genes is most efficiently achieved by the use of high-copy stable yeast vectors that can be selected and propagated in *E. coli* and that will accept relatively large foreign DNA inserts. Vectors combining yeast and *E. coli* markers and replicons with the cohesive (*COS*) ends of λ bacteriophage can be joined to large foreign DNA fragments (about 30 kilobase pairs of DNA is possible). Cosmids are renowned for their instability (Hohn and Hinnen, 1980), but if selection can be accomplished in *E. coli*, use of such vectors considerably reduces the number of colonies to be screened. In general, however, 2 μm-based YEp vectors are favoured in such endeavours. For high-copy stable propagation of hybrid 2 μm plasmids in yeast, the origin of replication and the *REP3* gene are prerequisites. Furthermore, the *REP1* and *REP2* gene products must be provided, either by the chimaera itself or by endogenous 2 μm plasmids. Thus, vectors such as YEp13 (Broach, Strathern and Hicks, 1979) can be used only with [Cir^+] hosts whereas plasmids like pCV20 (Broach, 1983) possess the whole 2 μm sequence and will be maintained equally well in [Cir^0] and [Cir^+] strains.

The presence of plasmids in the osmotolerant yeasts *Zygosaccharomyces rouxii, Z. bailii* and *Z. bisporus* (Toh-e, Tada and Oshima, 1982), that show homology with 2 μm could form the basis of a similar vector system in these

organisms. Similarly, linear DNA killer plasmids have been identified in *K. lactis* (Gunge *et al.*, 1981). However, at present the development of a broad host-range vector system for yeast remains elusive.

Although integration of the cloned gene using YIp vectors offers yet more stability, the point of integration may result in deletions or insertional mutations in unknown genes that are essential for brewing performance. Although it is a matter of trial and error, the precision of integration (particularly after linearization of the plasmid) may be sufficient to achieve consistent retention of the characteristic without affecting other parameters. At present, centromere-containing plasmids may be the best choice for the production of stable commercial yeast strains. Such vectors have the added advantage that any toxic effect due to high copy number of the cloned gene is avoided. Ultimately, however, the construction of artificial chromosomes involving cloned genes and promoters, *ARS* sequences, centromeres and telomeres, of a similar size to existing yeast chromosomes, could be the solution (Murray and Szostak, 1983b).

One major problem concerning the use of recombinant DNA techniques with commercial yeast strains is that the majority of vectors carry genes complementing auxotrophic mutations, whereas the yeasts themselves are prototrophic. A solution to this has been to mutate commercial strains, for example to leucine auxotrophy (*leu2*), and then to transform them using *LEU2*-based vectors (von Wettstein, 1983). Alternatively, vectors carrying dominant markers such as copper resistance (*CUP1*; Fogel and Welch, 1982) have been used to transform commercial brewers' yeast (Henderson, Cox and Tubb, 1983).

There are few examples of the use of transformation to improve industrial strains. Barney, Jansen and Helbert (1980) added purified *S. diastaticus* DNA to *S. cerevisiae* sphaeroplasts and obtained dextrin-utilizing transformants. Genes involved in the isoleucine–valine biosynthetic pathway, which also leads to the production of higher alcohols and diacetyl, have been isolated from *S. cerevisiae* (von Wettstein, 1983). In particular, the *ILV1* gene (threonine deaminase) has been cloned on a multicopy 2 μm shuttle vector. The gene has been sequenced (M. C. Kielland-Brandt, personal communication) with a view to altering it by site-directed mutagenesis and returning it to commercial strains using integrating vectors. Thus a strain without threonine deaminase would result, producing less vicinal diketones (e.g. pentane-2,3-dione) in beer. When this is combined with a similar approach to the *ILV2* gene, a strain could be developed that would produce beer lacking the off-flavours associated with diacetyl (Holmberg, 1984). The *DEX1* gene of *S. diastaticus*, which is responsible for dextrin utilization, has been cloned on the 2 μm-based vector pJDB207 (Beggs, 1981), and recently has been transferred to commercial strains by use of *CUP1*-based vectors (J. R. Johnston, personal communication). Similarly, a gene for the decarboxylation of malic acid to lactic acid has been cloned from *Lactobacillus delbrueckii* and expressed in *E. coli* and laboratory yeast. This gene product could enable commercial wine yeasts to carry out a secondary fermentation, which traditionally is achieved by addition of the appropriate bacterial species after the primary yeast fermentation (Williams *et al.*, 1984).

Production of foreign proteins by yeast

As noted by Spencer and Spencer (1983) and by Kingsman *et al.* (1985), the use of yeast as a production vehicle for foreign proteins has several distinct advantages over the use of *E. coli*. There is a wealth of knowledge concerning the industrial handling of yeast and the organism itself, as a waste product, has potential for use as animal feed. In addition, as yeast will glycosylate many proteins, the final product is more likely to resemble closely the original foreign molecule. *S. cerevisiae* also shares many transcriptional, translational and post-translational characteristics in common with higher eukaryotes and may therefore be a more suitable host for the expression of proteins from these organisms. Moreover, yeast readily secretes several proteins and, by judicious choice of vector DNA sequences, the potential exists to cause yeast to secrete virtually any protein. Because of the reducing intracellular environment, many proteins such as calf prochymosin (Smith *et al.*, 1985) are both insoluble and incapable of activation when produced solely in the cytoplasm of micro-organisms. However, when such proteins are secreted, disulphide bonding appears to be normal and proteins capable of activation can be retrieved from the culture supernatant with ease. Although there is a great deal of information concerning the genetics and molecular biology of *S. cerevisiae*, other yeast genera have recently gained favour as suitable production vehicles of foreign proteins. This is primarily because organisms such as *P. pastoris, Y. lipolytica* and *K. lactis* are used in well-characterized and established commercial processes and have a greater capacity to secrete larger proteins than does budding yeast. In this section, we concern ourselves solely with *S. cerevisiae* but the mechanisms and principles described below are likely to be ubiquitous in yeasts.

TRANSCRIPTION AND TRANSLATION

S. cerevisiae has been shown to be an amenable host for the transcription and translation of a variety of heterologous prokaryotic and eukaryotic genes. These have originated from such diverse organisms as *E. coli* (e.g. ampicillinase; Chevallier and Aigle, 1979), *Bacillus sphaericus* (cytosine methylase; Feher, Kiss and Venetianer, 1983), *Dictyostelium discoideum* (a gene complementing the *ura3* mutation of *S. cerevisiae*; Boy-Marcotte and Jacquet, 1982), and *Drosophila melanogaster* (complementing the *ade8* mutation of *S. cerevisiae*; Henikoff *et al.*, 1981). The transcription of these heterologous genes resulted from the possession of non-*Saccharomyces* promoter sequences that were recognized by *S. cerevisiae* RNA polymerase II. Extensive studies of the genes involved in galactose utilization (*GAL1, GAL7* and *GAL10*; Guarente, Yocum and Gifford, 1982), iso-1-cytochrome *c* (*CYC1*; Guarente and Mason, 1983) and histidine biosynthesis (*HIS3* and *HIS4*; Struhl, 1982; Donahue *et al.*, 1983) have identified the DNA elements that are crucial for the regulation and transcription of homologous yeast genes. These sequences have since been utilized in YXp vectors for the expression (sometimes regulated) of foreign genes in *S. cerevisiae*. In general,

mRNA initiation sites are always preceded by one or more TATA boxes. These canonical sequences act as recognition sites for RNA polymerase II and in yeast are approximately 90 bp upstream of the translational start. In contrast to the situation in prokaryotes, transcriptional regulation sequences are typically much larger in *S. cerevisiae* and can be several hundred base pairs upstream of the transcription start point (Guarente, 1984). These upstream activation sites (UASs) interact with regulatory proteins encoded elsewhere in the yeast genome, resulting either in entry of RNA polymerase II at the UAS or in the alteration of the secondary chromatin structure so that the polymerase binds at the TATA box. Negative control sites are distinct from UASs and have been identified in the gene encoding glucose-repressible alcohol dehydrogenase (*ADR2*; Beier and Young, 1982) and in *a*-specific genes repressed by the *MATα2* gene product (for review, *see* Brent, 1985). Repressor proteins bind to these regions and sterically block transcription by interfering with the binding of positive regulators at the UAS or TATA box, or by hindering the movement of the polymerase molecule from the UAS to the startpoint of transcription.

The optimal expression of a gene in yeast will also be affected by DNA sequences that terminate transcription and direct 3′ polyadenylation or 5′ 7-methylguanosine capping of the message. The latter is probably intrinsically associated with the recognition of the TATA box and the initiation of transcription. Zaret and Sherman (1982) have shown that transcription termination may be coupled with polyadenylation and that in an analysis of 15 yeast genes, a poorly conserved tripartite consensus sequence could be involved. In general, 200 bp of DNA spanning the point corresponding to the 3′ end of any yeast polymerase II transcript when fused to the 3′ end of the heterologous gene is probably sufficient to terminate transcription. Hitzeman *et al.* (1981, 1983) used the 3′ end of the *TRP1* gene for this purpose in the expression of the human leukocyte interferon in budding yeast. In contrast, the expression of the rabbit β-globin gene was aberrant in budding yeast (Beggs *et al.*, 1980): transcription termination and polyadenylation occurred at a site within an intron. Despite the presence of an intron splicing system for actin in *S. cerevisiae* (Gallwitz and Sures, 1980), heterologous genes that contain introns have not been expressed correctly in this host. This problem of expression is easily overcome by the generation of cDNA copies of the mRNA molecules derived from intron-containing genes.

The sequences that determine the strength (i.e. the amount of message produced) of promoters from genes such as *GAL10* are not evident from these studies. It is clear that high-level transcription of heterologous genes would be best accomplished by their fusion to the promoters of highly expressed genes. In *S. cerevisiae*, these genes generally encode glycolytic enzymes or other enzymes involved in carbon metabolism. Human leukocyte interferon has been constitutively expressed from the *ADC1* (constitutive alcohol dehydrogenase) promoter (Hitzeman *et al.*, 1981, 1983) and the *PGK1* (inducible phosphoglycerate kinase) promoter has been used to transcribe the gene encoding hepatitis B virus surface antigen (Valenzuela *et al.*,

1982). For commercial production of genetically engineered products and heterologous proteins there is some merit in the use of inducible promoters; these exhibit a high level of transcription in the presence of the inducer. For example, in the presence of galactose, the *GAL10* transcript represents 1% of the total polyadenylated RNA (St John and Davis, 1981) but is undetectable in the absence of this carbon source. This transcriptional 'switch' means that high-level transcription could be induced after the culture has grown to a high cell density. If the gene product of interest is toxic to yeast, this would be particularly advantageous. Under conditions where inorganic phosphate is depleted, the *PHO5* (inducible acid phosphatase) gene is transcribed to yield a message that represents 5% of the total cellular polyadenylated RNA (Oshima, 1982). Thus it is possible to design a medium that would be depleted of inorganic phosphate after a strain harbouring a *PHO5*-based vector had reached a high cell density. At this point, transcription from *PHO5* would start without the need for further manipulation of the medium. In both these systems, the role of the regulatory proteins must also be considered. In particular, transcription of *GAL10* is absolutely dependent on the *GAL4* gene product and its interaction with other components of the system (Oshima, 1982). If these proteins are limiting in the cell (for instance, when the structural gene is on high copy number vectors) then transcription will also be limited. This could be overcome by use of mutants that overexpress the regulatory proteins or by the incorporation of the regulatory genes into multiple copy vectors. In the phosphatase system this may be unnecessary, as single copies of the regulatory genes are sufficient to induce acid phosphatase expression even when the *PHO5* gene is on a multiple copy vector (Rogers, Lemire and Bostian, 1982). Both the galactose and phosphatase systems have been incorporated into expression plasmids (Johnston and Davis, 1984 and Thill *et al.*, 1983, respectively).

The precise mRNA sequences required for efficient translation of an heterologous gene have not been strictly defined. There is no ribosome-binding site in any *S. cerevisiae* mRNA species studied so far. The most proximal AUG codon at the 5′ end of the molecule sets the reading frame and initiates translation. After translation has been terminated at a UAA, UAG or UGA codon, reinitiation at subsequent AUG codons does not occur, in contrast to the situation in prokaryotes (Sherman and Stewart, 1982). Furthermore, *S. cerevisiae* (and, presumably, other commercial yeasts) have a marked degree of preferential codon usage (Bennetzen and Hall, 1982) and the heterologous gene is likely to be better expressed if it reflects this bias.

POST-TRANSLATIONAL MODIFICATION AND SECRETION

Accurate protein transport and localization within the cell results from the interaction between intracellular receptors (such as the signal recognition particle and the docking protein; Walter, Gilmore and Blobel, 1984) and the amino-terminal peptide sequence (or leader) of the protein involved. Proteins destined for secretion or for direction to the vacuole, enter the

endoplasmic reticulum (ER) as nascent polypeptides, from where they are passed on to the Golgi complex either for localization to the vacuole or to secretory vesicles and ultimately the cell surface. In budding yeast, this process has been defined by the temperature-sensitive *sec* mutations originally isolated by Novick and Schekman (1979). At the non-permissive temperature, lesions in these genes result in a cessation of protein transport, due to the intracellular accumulation in organelles such as the ER, Golgi body or secretory vesicles. Thus, strains bearing the *sec7* mutation do not secrete invertase but accumulate it in an aberrant form of the Golgi complex called the Berkeley body. No secretory vesicles have been found in these cells (Novick, Field and Schekman, 1980). The degree of efficiency with which a heterologous protein will traverse this pathway is open to manipulation at the level of the protein itself (i.e. the leader peptide) or by altering host-encoded mediators such as the *SEC* gene products.

Secretion leader sequences are typically short (approximately 20 amino acids) and highly hydrophobic in nature, consistent with their role in membrane anchorage. In many cases, (e.g. invertase; Emr *et al.*, 1983) these sequences are cotranslationally removed during the passage of the protein into the lumen of the ER. However, they are retained in at least two cases; processing of prepro-α-factor and type 1 killer preprotoxin (Julius, Schekman and Thorner, 1984 and Hanes *et al.*, 1986, respectively). For these proteins, the retention of the intact leader may direct the post-translational processing essential for their maturation.

As in animal cells (Struck, Lennary and Brew, 1978), a consensus sequence of asparagine-X-threonine (or serine) has been shown to be modified by the addition of a core $(GlcNAc)_2$-$(Man)_9$-(Glu) glycosyl subunit in the ER of budding yeast (Esmon, Novick and Schekman, 1981). For the majority of proteins such as invertase, further extension of this core subunit then occurs in the Golgi although, for the α-factor and killer factor precursors, this is not the case. Despite the fact that the secreted forms of α-factor and killer factor are not glycosylated, the addition of carbohydrate moieties to their precursors is essential for efficient secretion (treatment of cells with tunicamycin inhibits the synthesis of the glycosyl donor and blocks secretion; Julius, Schekman and Thorner, 1984; Hanes *et al.*, 1986). Glycosylation may be a prerequisite for some or all of the proteolytic cleavages involved in the maturation of these proteins. For example, the *KEX2*-mediated cleavage of α-factor and killer factor occurs after glycosylation of the primary translation product has been completed. Mutants at this locus either fail to secrete killer factor (Bussey *et al.*, 1983), or secrete abnormally large inactive prepro-α-factor because of a deficiency in a calcium-dependent endopeptidase with specificity for cleaving on the carboxyl side of a pair of basic amino acids (namely LysArg; Julius *et al.*, 1984). Similar cleavage sites are common in higher eukaryotes (e.g. in human encephalins) and the existence of a peptidase (similar to mammalian calpains; EC 3.4.22.17) with this type of specificity in budding yeast is encouraging for the production of proteins that require multiple processing events.

With these constraints in mind, numerous strategies can be adopted that will maximize the secretion of any protein. If the protein in its natural host

is secreted in a heavily glycosylated form, vectors based on the invertase (*SUC2*) leader are likely to be the most suitable. For example, Smith *et al.* (1985) used this secretion vehicle in the production of calf prochymosin. They found that, in contrast to use of the *PHO5* or *MFα1* leaders, the *SUC2* leader gave the highest yield of secreted active faithfully glycosylated prochymosin. In this respect, the native prochymosin leader sequence was the least efficient of the sequences tested, presumably because it was not adequately recognized by the *S. cerevisiae* secretion pathway. Furthermore, Hanes *et al.* (1986) have used the *PHO5* leader to direct the secretion of killer factor and found it to be inefficient in this process: the protoxin molecule accumulated within the cell and was secreted at approximately one-quarter of the rate found with use of the natural protoxin leader. Both *MFα1* and killer-toxin leader sequences have potential for the secretion of proteins that require extensive processing. In particular, the former has found favour with many industrial companies. For example, by the inclusion of an artificial *KEX2* processing site downstream of the secretion leader of pp-α-factor, Zsebo *et al.* (1986) were able to secrete high levels of several human proteins (β-endorphin, calcitonin and α-interferon) from yeast.

Whatever the efficiency of the leader sequence involved, there is clearly a host-endowed constraint on the amount of precursor that can be processed and secreted. In the case of *SUC2*-directed secretion of prochymosin, as the secreted protein was glycosylated, the limiting step was not entry into the ER but more likely a Golgi-associated step late in the secretion pathway (Smith *et al.*, 1985). When the *SUC2* leader–prochymosin gene fusion was expressed from a stronger promoter, a fivefold increase in production of intracellular prochymosin led to only a one-tenth increase in its secretion. One approach to overcome this is to mutate the host and select strains that bypass the rate-limiting step and so become supersecretors. Smith *et al.* (1985) isolated at least three complementation groups for supersecretion (*ssi*) that were additive in their effects and on average gave a tenfold higher level of secretion than wild-type strains. These mutants were non-specific with respect to the leader sequences tested and the protein being secreted. The same authors also noted that strains with integrated gene sequences displayed higher secretion efficiencies than strains bearing the same genes on multiple copy vectors.

Conclusions and future prospects

A major restriction to the use of *S. cerevisiae* in the large-scale production of cloned gene products is the absence of highly stable, high copy number vectors. A theoretical yet tangible solution to this is presented in *Figure 6*: this involves the construction of a synthetic chromosome as originally suggested by Murray and Szostak (1983a). The essential features of this system are the presence of several autonomously replicating sequences (*ARS*) to promote DNA replication, a single *CEN* region and two telomere consensus sequences for mitotic and meiotic stability. The chromosome ideally would be the same size as *S. cerevisiae* chromosome III (or at least 137 kb) and

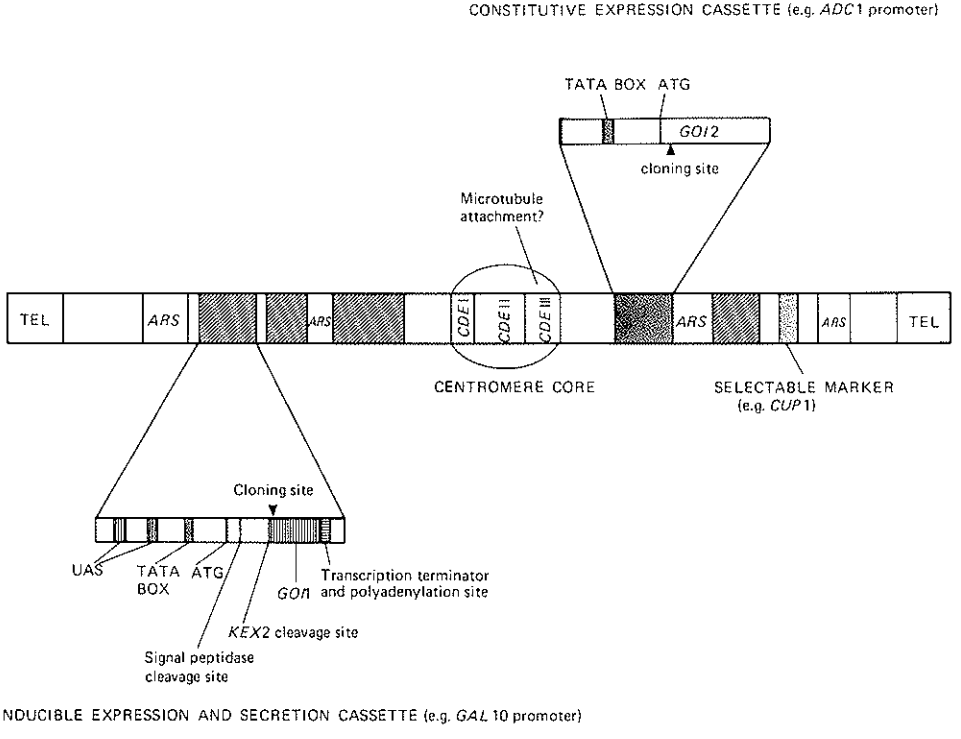


Figure 6. An artificial chromosome for manipulation of commercial strains of *Saccharomyces*. *GOI1* is the gene of interest; *GOI2* could be a regulator of the promoter used for the inducible expression of *GOI1* or a signal peptidase involved in its processing. Consensus sequences defining the telomeres (*TEL*), centromere (*CDE* I, II and III), autonomously replicating sequence (*ARS*) and transcription terminator are described in the text. The shaded regions represent additional expression cassettes which could be identical (thus increasing the copy number of *GOI1*) or different (if for example the chromosome were to be used to construct novel biosynthetic pathways). Several *ARS* units are shown on the assumption that this would maximize the replication of the artificial chromosome; however, a single *ARS* may suffice. Unshaded regions represent non-functional ('stuffer') DNA sequences essential to achieve the correct size to give the unit stability.

metacentric because this would further improve its stability (Hieter *et al.*, 1985a). We have included the *CUP1* gene as a selectable marker which will allow transformation and maintenance in prototrophic commercial strains. Sequences from pBR322 could be included for selection in *E. coli* but would probably be removed prior to the introduction of these vectors into production strains. A multiple tandem repeat of a gene complex makes up the remainder of the vector. This consists of the appropriate promoter elements from a highly expressed gene such as *GAL10* including UASs and a TATA box as well as the transcription terminator and polyadenylation sites from a gene such as *TRP1*. We have also included the 5′ portion of *MFα1* encoding pp-α-factor prior to the *KEX2* cleavage site but this would be removed if the gene product were not to be secreted. Suitable restriction sites would, of course, be included for the fusion of the gene of interest (*GOI1*). The vector could also be developed for the differential expression of several genes by the inclusion of promoters regulated by other effectors. Furthermore, constitutive promoters could also be included for the concerted production of regulatory gene products pertinent to the promoter involved (e.g. *GAL4*). The increased expression of processing enzymes (e.g. the *KEX2* endopeptidase, signal peptidases, etc) that might otherwise be in short supply and so limit secretion could also be included.

Although they are considered in isolation in this review, the genetic techniques of mutation, hybridization (by whatever means), cytoduction and transformation are more likely to be used in conjunction for commercial yeast improvement. Procedures centred around DNA transformation have revolutionized strategies for strain modification but it is difficult to clone unidentified genes. Thus mutation (and subsequent genetic analysis) will persist as an integral part of many breeding programmes: for example, the novel bypass secretion pathways defined by the *sst* mutants described above, could not have been identified by any other means. Furthermore, although recombinant DNA methods are the most precise way of introducing novel traits encoded by a single gene to commercial strains, hybridization remains the most effective method for improving and combining traits under polygenic control.

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