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Molecular Breeding of Yeasts for Production of Useful Compounds: Novel Methods of Transformation and New Vector Systems

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Introduction

In the Research Institute for Food Science at Kyoto University we have been able to manipulate genes and breed useful strains of *E. coli* and *S. cerevisiae*, using recombinant DNA (recDNA) techniques. Using genetically engineered cells, we have succeeded in the production of compounds such as ATP, ethanol and glutathione in significant quantities. Some of these processes have been described elsewhere (Kimura, 1985, 1986).

This article covers the following points: (1) novel methods for the transformation of intact yeast cells without making protoplasts; (2) production of ATP and ethanol by genetically engineered cells harbouring genes controlling hexokinase or glucokinase activity; (3) new plasmid vectors for wild and/or industrially useful strains of yeast.

One of the problems in yeast genetics has been that of finding an easy and rapid transformation method for intact yeast cells. We have developed a method, based upon treatment of yeast cells with alkali metal ions, which we have named the KU (Kyoto University) method (Ito *et al.*, 1983; Ito, Murata and Kimura, 1984; Kimura, 1986). This is superior in many ways to the traditional protoplast method and, by this technique, several genes (e.g. hexokinase II: EC 2.7.1.1 and glucokinase: EC 2.7.1.2) have been cloned from yeasts (Fukuda *et al.*, 1984). Genetically engineered cells containing these genes produced more ATP and/or ethanol (Yamaguchi *et al.*, 1984).

ATP: 5′-adenosine triphosphate; CDP-choline: cytidine 5′-diphosphate-choline; F/G: ratio of phosphorylating velocity for fructose & glucose by enzyme; FBP: fructose 1,6-bisphosphate; MG: methylglyoxal; MT: metallothioneins; PEG: polyethyleneglycol.

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An alternative method for yeast transformation using an electric field pulse has been developed recently (Hashimoto *et al.*, 1985).

Another problem in yeast genetics is the host vector system. Usually an auxotrophic mutant is required as a host cell for gene cloning studies. The introduction of a new gene on a hybrid plasmid is generally detected by complementation of the auxotrophic markers by a wild-type allele carried on the vector. However, treatment of the host cell with mutagens may damage normal, useful metabolic pathways. Our object has been the breeding of wild and/or industrially useful yeasts without damaging useful pathways. We have recently succeeded in the construction of hybrid plasmids, which carry genes for resistance to methylglyoxal and certain metals and which can be introduced as markers into wild or industrially useful strains without damaging them as host cells. The hybrid plasmids have been used to create new strains able to produce more ATP or ethanol than the original strains.

A novel method for the transformation of intact yeast cells without the use of protoplasts

KU METHOD

The genetic transformation of yeast with plasmid DNA was first demonstrated by Hinnen, Hicks and Fink (1978), who introduced plasmid DNA into protoplasts with the aid of polyethylene glycol (PEG) and calcium (the protoplast method). However, the preparation of the protoplasts and their subsequent regeneration of cell walls in a solid medium was tedious and time consuming. Furthermore, protoplasts often showed a low regeneration efficiency. It was therefore desirable to develop a more convenient method of yeast transformation. In our studies on the phosphorylation of various nucleotides by yeast cells, we showed that treatment of yeast cells with Triton X-100, a non-ionic detergent, permitted incorporation of various extracellular substances into the cells (Kimura, Arima and Murata, 1981). This suggested that yeast cells, when treated with Triton X-100 or other detergents, might take up plasmid DNA: we therefore studied the uptake of plasmid DNA by intact yeast cells treated with various agents (Ito *et al.*, 1983). Several alkali metal ions such as Li^+, Na^+, K^+, Cs^+, and Rb^+ were effective in this respect. Using intact yeast cells treated with these monovalent cations, Ito *et al.* (1983) reported that high transformation efficiencies could be attained, in the presence of PEG, which were comparable with those achieved by the protoplast method. Additionally, 2-mercaptoethanol and LiSCN (H. Hashimoto and A. Kimura, unpublished data) were effective for yeast transformation. The optimal procedure for yeast transformation is shown in *Figure 1a*. The KU method has been further simplified recently by the simultaneous addition of some of the agents, and the omission of the heat-shock process (Kimura, 1985). This rapid process, the KUR method (*Figure 1b*), is very useful and time saving, although the yield of transformants may occasionally decrease.

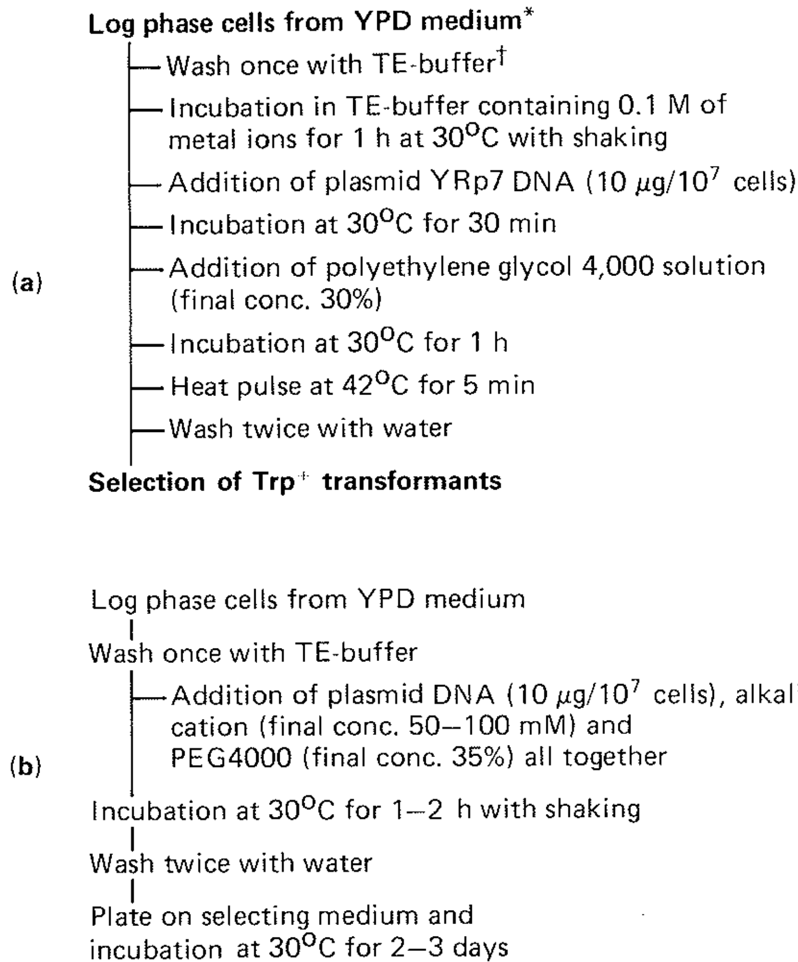

Figure 1. (a) KU method for transformation of intact yeast cells treated with metal ions (Kimura, 1985). **Saccharomyces cerevisiae* D13-1A (a *his*3-532 *trp* 1 *gal* 1);† TE buffer, 10 mM Tris-HCl buffer (pH 7·5) containing 1·0 mM EDTA. (b) KUR method of yeast transformation with alkali cations (Kimura, 1985).

The KU method has many advantages, which are summarized as follows.

1. There is no need to prepare protoplasts;
2. The organism need not be embedded. As transformants can be spread on the surface of agar plates, it is very easy to isolate or replica-plate them;
3. In the KU method, transformants can be isolated in 2–3 days, whereas regeneration of cell walls often takes 5–7 days in the protoplast method;
4. The fusion of protoplasts occurs very easily, so that transformants are sometimes polyploid, which is not desirable. In contrast, no polyploid cells can arise in the KU method;

5. Because protoplasts are fragile and particularly sensitive to drugs, it is difficult to recover transformants by selecting for resistance to various drugs. In the KU method, transformants *can* be selected in this way;
6. Protoplasts can be formed only from those yeasts which are susceptible to lytic enzymes, whereas the KU method can be applied to yeasts which are not susceptible to lytic enzymes;
7. Competent cells treated with lithium compounds can be preserved longer than protoplasts which are fragile;
8. In the KU method, transformants can sometimes be selected on the basis of the size of colonies formed on agar surfaces; this technique cannot be applied in the protoplast method, as regenerated colonies are variable in size and shape, depending on how deep they are embedded in the agar.

Full details of the KU method have been given in a recent review (Kimura, 1986).

ELECTROINJECTION OF PLASMID DNA

Neumann *et al.* (1982) recently reported that the uptake of plasmid DNA into mouse lyoma cells was increased by electric field pulses. Shivarova *et al.* (1983) also reported that the transformation frequency of *Bacillus cereus* protoplasts with plasmid DNA was increased by electric field effects in the presence of PEG.

Recently, plasmid DNA (YEp13) was introduced into intact yeast cells by electric field pulses (Hashimoto *et al.*, 1985) and the electrical conditions for uptake were optimized. As shown in *Figure 2*, the number of surviving cells decreased (curve 1), while the number of transformants (from *leu2* to Leu$^+$) increased (curve 2) with increasing electric field pulse intensity. This pattern is similar to that obtained by the KU method with lithium acetate, although the mechanism for the introduction of DNA into the cells through cell walls and membranes is not completely understood. However, the transformation frequency obtained by the electroinjection method is still lower than that by the KU method with alkali cations (Ito *et al.*, 1983) or thiol compounds (Ito, Murata and Kimura, 1984).

As far as we are aware, the report by Hashimoto *et al.* (1985) was the first to show that intact yeast cells can be transformed with plasmid DNA by electric field pulses. Electroinjection provides a useful method for transformation of intact yeast cells, especially of those from which protoplasts can not be prepared because their cell walls are not susceptible to cell-wall-degrading enzymes. It is possible that electroinjection may offer a new, simple procedure for transforming intact prokaryotic and eukaryotic cells, including plant cells, with foreign DNA, thus allowing the rapid production of transformants.

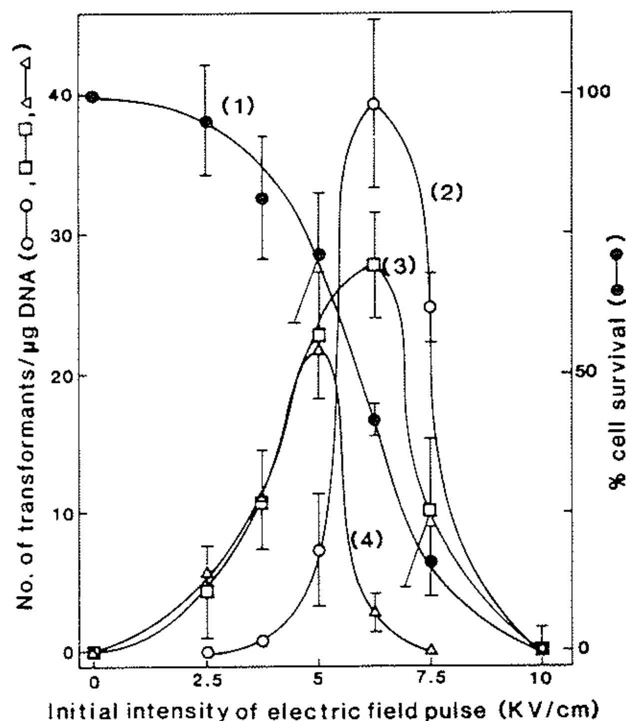

Figure 2. Percentage cell survival and number of transformants per µg DNA plotted against the initial intensity of the electric field pulse. The single pulses were applied by an electric discharge method with a capacitance of 1 µF (curves 1 and 2), 2 µF (curve 3) and 4 µF (curve 4). The bar represents the standard deviation of three experiments (Hashimoto *et al.*, 1985). (1): surviving cells; (2), (3), (4): transformants.

Construction and improvement of a microbial bioreactor (or biocatalyst) system for the production of useful compounds

OUTLINE OF BIOREACTOR OR BIOCATALYST SYSTEM

A microbial bioreactor or biocatalyst system using dried yeast cells has been constructed to produce various useful compounds (Kimura, 1982). As shown in *Figure 3*, it consists of two processes: an energy (ATP)-regenerating process and a substrate-converting (transformation) process. The ATP which is regenerated in the former is used in the latter to produce useful compounds.

The detailed mechanism of this bioreactor system is as follows. The ATP is generated through the glycolytic pathway. This was shown by the use of respiration-deficient cells of *S. cerevisiae* which could carry out this process in the absence of mitochondrial function (Kimura *et al.*, 1976). The system is supplemented with a high amount of inorganic phosphate, which at the beginning of the reaction inhibits the activity of hexokinase (EC 2.7.1.1), the first enzyme of the glycolytic pathway (Umemura, Fukuda and Kimura, 1982). Thus glucose can not be used as an energy source until sufficient ATP

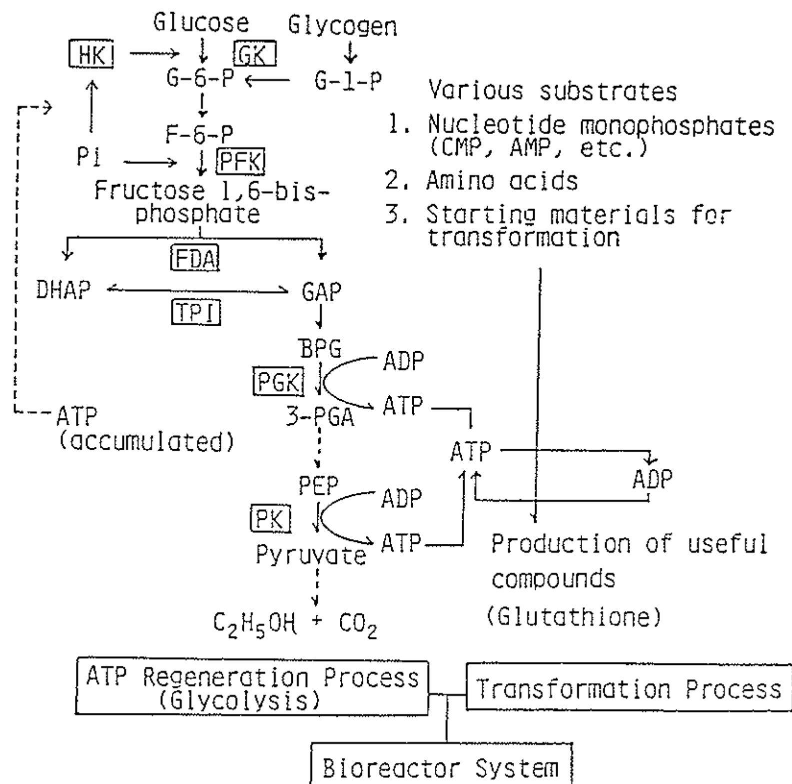

Figure 3. Mechanism of bioreactor or biocatalyst system. – – – –: release of inhibition.

has accumulated in the reaction mixture. Initially, ATP is produced from the glycogen that had accumulated in the cells during their previous growth. As phosphorylase (EC 2.4.1.1) is not inhibited by phosphate, it is able to hydrolyse glycogen gradually to produce glucose-6-phosphate (G6P). The G6P thus generated is further metabolized by the glycolytic pathway to generate ATP. When the ATP level reaches a critical concentration, the inhibition of hexokinase by phosphate is overcome; more glucose is then phosphorylated and metabolized, and more ATP generated. Thus, the ATP generation system operates automatically and at accelerating rates (Umemura, Fukuda and Kimura, 1982). The amount of glycogen present in the cell therefore has an important role at the beginning of the reaction, and cells without glycogen cannot carry out the reaction. This mechanism was elucidated by the use of a petite-negative yeast, *Hansenula jadinii* (Kimura and Okuda, 1976; Kimura, Okuda and Fukuda, 1979; Kimura *et al.*, 1980; Kimura, 1982; Umemura, Fukuda and Kimura, 1982). Aerobically cultured cells of *H. jadinii*, which did not contain glycogen, did not increase the ATP level. They could not release the inhibitory effect of phosphate on hexokinase and could not carry out the whole reaction. On the other hand, anaerobically cultured cells of *H. jadinii* (Kimura and Okuda, 1976; Kimura *et al.*, 1980),

which contained a large amount of glycogen, could carry out the reaction efficiently (Umemura, Fukuda and Kimura, 1982). This mechanism was illustrated by computer simulation (Asada *et al.*, 1981). The calculated time-dependent concentrations of glucose, FBP, adenosine and its nucleotides (AMP and ATP) were in good agreement with experimental values, both qualitatively and quantitatively.

By the use of this microbial (yeast) bioreactor or biocatalyst system, various useful compounds have been biosynthesized such as CDP-choline and various sugar nucleotides. We have tried to improve this bioreactor using recombinant DNA techniques. To produce useful compounds efficiently, the ATP-generating process needs to be coupled with a substrate-converting system, which would vary according to the kind of compound desired (*Figure 3*). Therefore, the ATP-generating process, as a common process, should be as efficient as the substrate-converting process. An example is the production of glutathione (Kimura, 1985, 1986). As outlined below, by cloning the genes for glucose phosphorylation, the first step in the glycolytic pathway, the ATP-regenerating process was improved and, as a result, an efficient biocatalyst system was constructed (Fukuda *et al.*, 1983; Yamaguchi *et al.*, 1984).

CLONING GENES FOR GLUCOSE PHOSPHORYLATION BY THE KU METHOD (LITHIUM ACETATE METHOD)

In trying to clone genes for glucose-phosphorylating enzymes (Fukuda *et al.*, 1983), we found that the introduction of these genes into *E. coli* or *S. cerevisiae* resulted in an improvement in ATP and ethanol production (Fukuda *et al.*, 1984; Yamaguchi *et al.*, 1984). *S. cerevisiae* has three glucose-phosphorylating enzymes: hexokinases I, II and glucokinase. We have attempted to clone the genes, *Hxk 1* and *2*, and *GLK1*, which encode these enzymes. A deficient *S. cerevisiae* strain SY215 (α, *leu 2–3, leu 2–112, trp 1, his 2* and/or *3, hxk 1, hxk 2, glk 1*), was constructed which had a low glucose-phosphorylating activity and a slow growth rate on both glucose and fructose. The SY215 host strain was then transformed, by the KU method, with a yeast genomic library of hybrid plasmids constructed by the ligation of *Sau*3A partially digested DNA from a wild-type strain (DKD-5D-H) into the *Bam*HI site of the vector YEp13. From 80 000 Leu^+ transformants, 11 were identified as fast-growing colonies. We thought that these might contain the genes of interest because complementation of either the glucokinase or hexokinase deficiency should enable cells to metabolize the glucose in the medium and so increase their growth rate. Transformants with this phenotype could not have been isolated by the protoplast transformation method because with embedded cells the difference in colony size does not necessarily reflect the difference in cell growth rate.

From these 11 transformants, four (T_1–T_4) were chosen and the hybrid plasmids were isolated and re-introduced into two strains of yeast (SY215 and DKD-5D-H).

Table 1 shows the sugar-phosphorylating activity of the yeast strains with and without the hybrid plasmids. Depending on the particular combination of sugar-phosphorylating genes that they carried, the yeast strains showed a characteristic F/G value (ratio of phosphorylating velocity for fructose and glucose): 2.89 for hexokinase I, 1.76 for hexokinase II, and 0.12 for glucokinase, respectively (*see* data for strains DKD-5D-H, P1T8C, P2T22D, D308, D308.3 and SY215, in *Table 1*). An increase in both glucose- and fructose-phosphorylating activity was observed in transformants with T2 and T4, whereas the transformants T1 and T3 showed an increase only in glucose-phosphorylating activity (*Table 1*). These results suggested that the hybrid plasmids in T2 and T4 carried the hexokinase II gene, and the plasmids in T1 and T3 carried the glucokinase gene. In addition, extracts of these transformants were found to contain glucokinase (T1 and T3) and hexokinase (T2 and T4) activities that migrated with the same mobility as the purified enzymes during polyacrylamide gel electrophoresis.

Restriction maps of the hybrid plasmids isolated from transformants T1–T4 were constructed. Although both T2 and T4 showed the same F/G values, indicating the presence of the hexokinase II gene (*Table 1*), the hybrid plasmids they contained were of different sizes due to the insertion of different-sized DNA fragments into the vector. However, comparison of the restriction maps revealed that the DNA inserts were derived from the same chromosomal region because they contained a common pattern of endonuclease recognition sequences. A similar result was obtained for the plasmids, carrying the presumptive glucokinase gene, which were recovered from transformants T1 and T3. *Figure 4* shows the circular restriction maps of plasmid pYH1 (hexokinase) which was recovered from transformant T2, and plasmid pYG1 (glucokinase) which was isolated from transformant T1.

Table 1. Glucose- and fructose-phosphorylating activities in transformants (Fukuda *et al.*, 1984).

		Sugar-phosphorylating activity (U/mg-protein)		
Strain	Genotype	Glucose	Fructose	F/G*
DKD-5D-H	Wild type	0·356	0·513	1·44
P1T8C	*HXK1, hxk2, glk1*	0·147	0·425	2·89
P2T22D	*hxk1, HXK2, glk1*	0.175	0·308	1·76
D308	*hxk1, hxk2, GLK1*	0·102	0·013	0·12
D308.3	*hxk1, hxk2, glk1*	0·001	0·001	—†
SY215	*hxk1, hxk2, glk1*	0·001	0·001	—
SY215/T1		0·425	0·009	0·02
SY215/T2		0·592	0·950	1·62
SY215/T3		0·484	0·012	0·02
SY215/T4		0·680	1·159	1·70
DKD-5D-H/T1		0·848	0·502	0·59
DKD-5D-H/T2		0·736	1·323	1·79
DKD-5D-H/T3		0·813	0·547	0·67
DKD-5D-H/T4		0·606	1·273	2·10

* F/G was defined as velocity of phosphorylation of fructose relative to that of glucose.
† Both activities were zero.

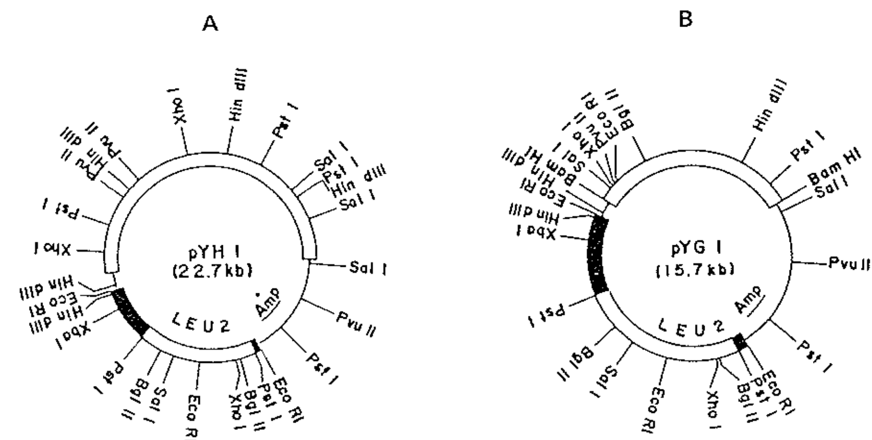

Figure 4. A. Circular restriction map of pYH1: B. Circular restriction map of pYG1. DNAs of hybrid plasmids pYH1 and pYG1 were digested with various restriction endonucleases and the sizes of linear fragments generated were determined by agarose gel electrophoresis. The restriction sites are drawn to scale on a circular map. —— plasmid pBR322; ■ yeast 2 μ DNA; □ chromosomal fragment of *S. cerevisiae* (Fukuda *et al.*, 1984).

Plasmid pYH1 contains a 12 kb fragment of DNA and plasmid pYG1 contains a 5 kb fragment of DNA (*Figure 4*). The restriction maps of these fragments were found to be different, confirming that they were derived from different regions of the yeast genome.

PRODUCTION OF ATP AND ETHANOL BY GENETICALLY ENGINEERED YEAST CELLS

In the microbial biocatalyst, the glycolytic pathway is a potent ATP-generating process through which ADP is phosphorylated to ATP by the utilization of glucose and other energy sources. The yeast cells DKD-5D-H harbouring hybrid plasmids carrying the hexokinase gene (pYH1) or glucokinase gene (pYG1) showed an approximately two- to threefold increase in glucose-phosphorylating activity compared with that of wild-type cells without the plasmids (T2 and T1 in *Table 1*). After being dried, the yeast cells containing these hybrid plasmids (pYH1 or pYG1) were used for the bioreactor or biocatalyst system, and showed a higher ATP-producing activity (five- to sixfold; *Figure 5*) and/or ethanol-producing activity, than cells without plasmids (Fukuda *et al.*, 1984). They consumed glucose more rapidly, using the enhanced glycolytic pathway, than the strain without the plasmid. This result indicated that a higher level of glucose-phosphorylating activity caused by the cloned genes of hexokinase II or glucokinase accelerated the accumulation of FBP and further promoted the conversion of adenosine into ATP.

Thus, improvement of the glycolytic pathway in yeast cells by the introduction of hybrid plasmids carrying the glucose-phosphorylating genes seems

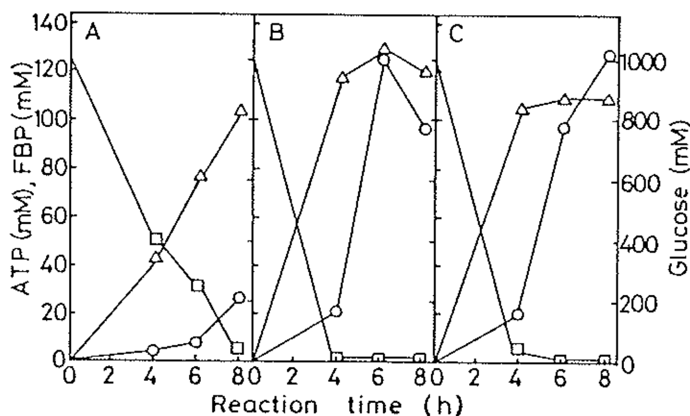

Figure 5. Comparison of ATP-producing activity in yeast cells with or without pYH1 or pYG1. A: DKD-5D-H; B: DKD-5D-H/pYH1; C: DKD-5D-H/pYG1; -□-; glucose; -△- FBP; -○-; ATP (Fukuda *et al.*, 1984).

to be promising for the construction of a more economical ATP regeneration process in the biocatalyst system.

Construction of new host–vector systems to improve wild or industrially used yeast strains

OUTLINE

A commonly used route for the introduction of new genes into microbial cells by gene cloning is, first, to isolate an auxotrophic mutant and then to use it as a host for a vector which can complement the deficiency. This method is simple and convenient when a mutation is lethal under appropriate growth conditions However, it is sometimes difficult (especially in polyploid strains) to isolate a derivative which is mutant for a special enzyme. In addition, the mutagenesis procedure might affect genes other than the intended one, so that the metabolic pathways of the host cell are seriously disturbed and it cannot be used for actual industrial production. It is necessary and important, therefore, to develop plasmids that can be introduced into wild-type or industrially used prototrophic strains and so can be used as vectors for the genes of commercial interest. For this purpose good dominant markers are required, which distinguish transformants from their wild-type host cells. We have chosen resistance to high amounts of methylglyoxal (MG) and certain metals as these are agents to which most yeast cells are usually sensitive. It is known that wild-type yeast cells do contain biochemical detoxification systems which can metabolize these chemicals and confer resistance to low concentrations. We therefore reasoned that if the level of the enzymes involved in these processes could be increased, then the cells should become resistant to higher concentrations. One way that

overproduction of a protein can be brought about is by increasing the dosage of the structural gene, for example by cloning it on to a multicopy vector. We therefore thought that it should be possible to isolate genes encoding enzymes involved in these resistance mechanisms by the selection of clones able to grow on media containing the compounds at concentrations normally inhibitory to yeast growth, after transformation of the cells with a yeast gene library constructed on a multicopy plasmid vector. Once they have been isolated, the hybrid plasmids could be used as vectors for the introduction of foreign DNA into any sensitive strain of yeast by use of the resistance selection. As described below, we have been able to isolate *S. cerevisiae* genes encoding enzymes involved in resistance to methylglyoxal and certain metal ions by an approach based on this rationale (Murata *et al.*, 1985a).

CLONING OF GENES FOR RESISTANCE TO METHYLGLYOXAL

The metabolism of methylglyoxal and mechanism of cell resistance

Methylglyoxal (MG) is a toxic metabolite usually found in all organisms at extremely low concentration and is thought to be a regulator of cell division: for example, MG can arrest the growth of *E. coli* at millimolar concentrations. It is synthesized via a bypass of the glycolytic pathway and/or aminoacetone cycle. As the compound is toxic, the synthetic and degradative pathways must be strictly controlled to avoid overaccumulation of MG in the cell. In *E. coli* cells, this is achieved by inhibition of methylglyoxal synthase (EC 4.2.99.11), which catalyses the synthesis of MG from dihydroxyacetone phosphate (*Figure 6*) (Murata *et al.*, 1985b).

Several enzymatic reactions are believed to be involved in the degradation of MG. Among them the glyoxalase system, consisting of glyoxalase I (lactoylglutathione lyase; EC 4.4.1.5) and glyoxalase II (hydroxyacylglutathione hydrolase; EC 3.1.2.6) (*Figure 6*), is thought to have an important role, not only in the degradation of MG, but also in conferring resistance to MG. These reactions have been shown to transform MG or its derivatives into lactate or corresponding α-hydroxy acids in the presence of glutathione in a wide variety of organisms. Thus the biosynthetic and degradative pathways of MG metabolism have been studied in detail (Murata *et al.*, 1985a,b, 1986).

It was Szent-Gyorgyi who first suggested the possible role of MG in the regulation of cell division (Egyud and Szent-Gyorgyi, 1965; Szent-Gyorgyi, 1965). Like other α-ketoaldehydes, MG interacts with NH_2- or SH- groups, which may react with polyamines and which function in cell division (Russell and McVicker, 1972). Szent-Gyorgyi and his colleages found that cell division was inhibited at low concentrations of these chemicals. He also suggested the formation of a complex, designated 'retine', which he was able partially to purify and show to be an α-ketoaldehyde. If α-ketoaldehydes are really involved in the regulation of cell division, the glyoxalase system could also be involved in the regulation of cell division and release of cells from the inhibitory action of MG and its derivatives. Using *cdc* (cell division cycle)

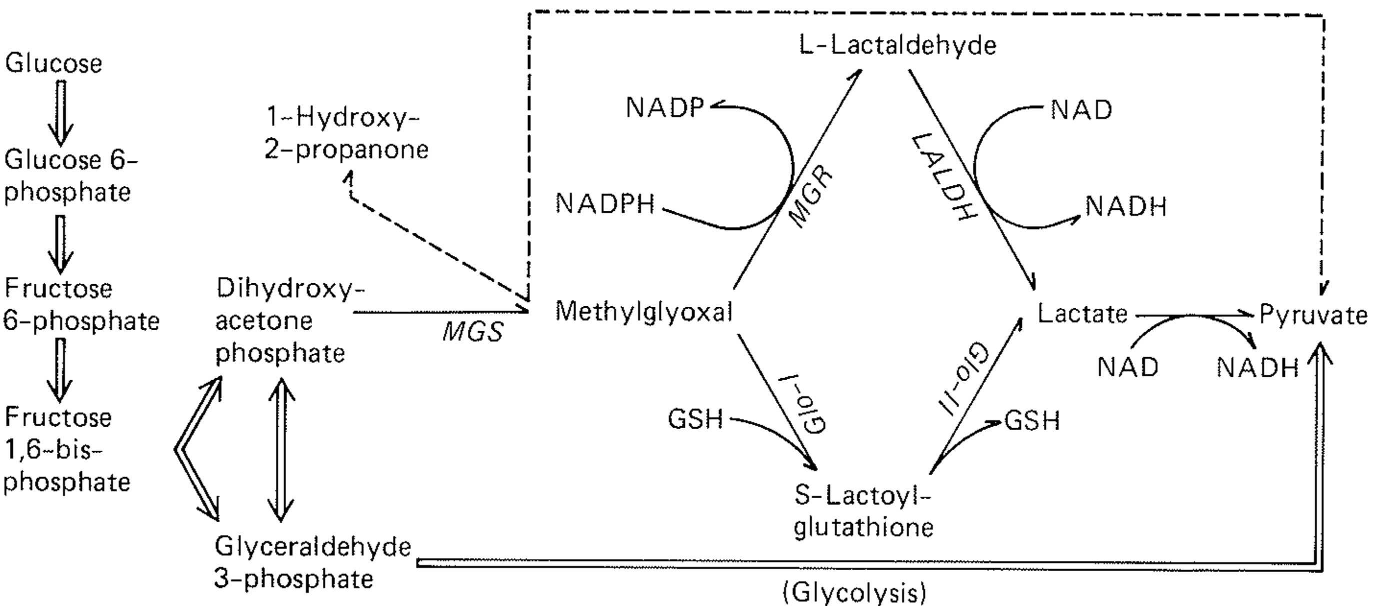

Figure 6. Glycolytic bypass of *Saccharomyces cerevisiae*. MGS: methylglyoxal synthase; *Glo-I*: glyoxalase I; *Glo-II*: glyoxalase II; MGR: methylglyoxal reductase; LALDH: L-lactaldehyde dehydrogenase (Murata *et al.*, 1986).

mutants of yeasts, Dudani and co-workers demonstrated an important role of the glyoxalase system (glyoxalases I and II), as an indicator of cell growth (Dudani, Srivastava and Prasad, 1984).

It is possible that the unregulated growth of cancer cells may be attributable in part to the inactivation of α-ketoaldehydes by the glyoxalase system. For this reason, glyoxalase system indicators have been thought to hold promise in screening for anticancer agents. The carcinostatic activities of various glyoxal derivatives towards certain tumours and leukaemias support this possibility.

Cloning of a gene responsible for resistance to methylglyoxal (MG)

A recombinant plasmid which was able to confer resistance to MG was isolated from a yeast gene library constructed by cloning genomic DNA fragments of strain DKD-5D-H into the multicopy vector YEp13. The recombinant plasmid pYMG14 was identified by its ability to permit cells of a normally sensitive strain to grow in the presence of a high concentration (5.0 mM) of MG (Murata *et al.*, 1985a). We found that this phenotype could be conferred on a range of unrelated strains including industrial polyploids, by introduction of the plasmid (Murata *et al.*, 1985a). The molecular size of the hybrid plasmid pYMG14 was 19·5 kb, indicating the insertion of a chromosomal DNA fragment with a molecular size of 8·8 kb. The restriction map of pYMG14 is shown in *Figure 7*.

Effects and implications of the introduction of the MG-resistance plasmid into strains of yeast and E. coli

Yeast cells carrying pYMG14 showed a marked resistance not only to MG but also to heavy metal ions and to some organic chemicals (*Figure 8*). Resistance to tetramethylthiuram disulphide (TMTD) and iodoacetamide

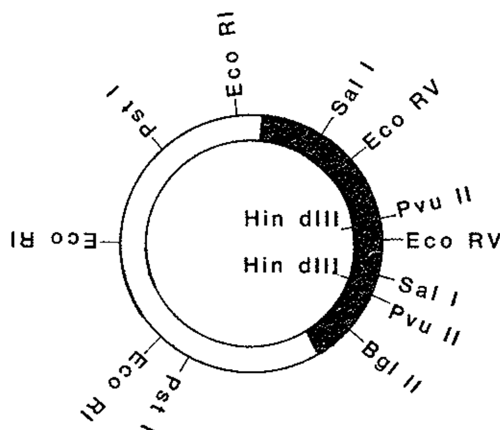

Figure 7. Physical map of pYMG14 carrying the gene for methylglyoxal resistance □YEp13 (10·7 kb); ■DNA fragments (8·8 kb), (Murata *et al.*, 1985a).

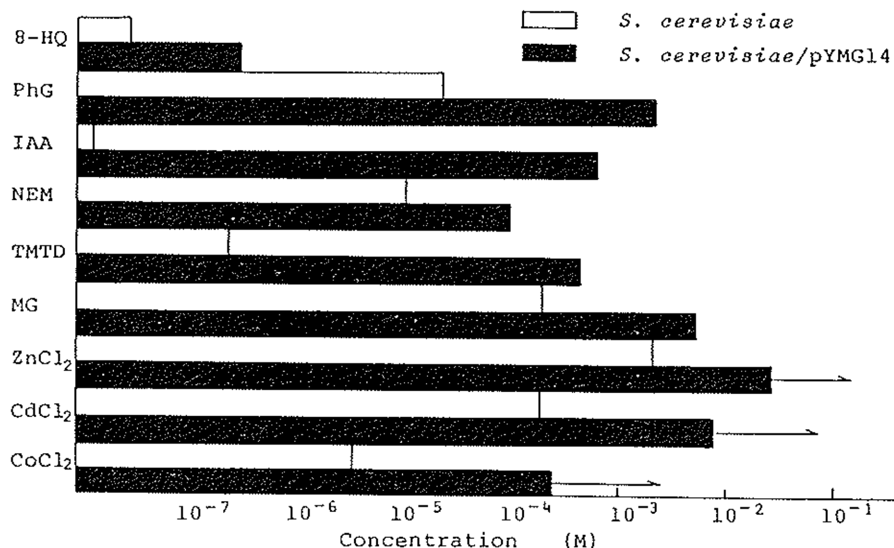

Figure 8. Increased resistance of *S. cerevisiae* with pYMG14 to various toxic chemicals (Murata *et al.*, 1985a).

(IAA) was increased dramatically by the presence of the plasmid. In addition, the plasmid caused increased resistance to Zn^{2+}, Cd^{2+}, Co^{2+}, phenylglyoxal (PhG), *N*-ethylmaleimide (NEM) and 8-hydroxyquinoline (8-HQ) (*Figure 8*). However, the resistance of DKD-5D-H/pYMG14 to Hg^{2+}, *p*-benzoquinone, Sn^{2+} or Rb^{2+} did not alter significantly.

We found that cultures of *S. cerevisiae* DKD-5D-H with pYMG14 accumulated twice as much glutathione in comparison to cultures of DKD-5D-H without pYMG14. This may be important in the expression of higher resistance to toxic compounds, because many of these agents could be neutralized through non-enzymatic interaction with the SH-group of glutathione. Thus, the mechanism leading to the overaccumulation of glutathione by yeast cells with pYMG14 needs to be elucidated.

Recently, a series of interesting compounds (Cadystin A, B etc) were found to be produced in a resistant strain of fission yeast, *Schizosaccharomyces pombe*, when it was cultured in a medium containing cadmium (Murasugi, Wada and Hayashi, 1981a,b). These compounds have the structure (γ-Glu-Cys)$_n$-Gly as shown in *Figure 9* (Kondo *et al.*, 1984). The γ-Glu-Cys-Gly at the *C*-terminus of these compounds is the structure of glutathione itself.

Interestingly, the resistance genes of pYMG14 were also expressed in *E. coli* C600: pYMG14 transformants were able to grow on agar containing MG or $CdCl_2$, whereas the control C600 was unable to do so. This broad-spectrum phenotypic expression of the MG-resistance determinant is an important finding as it suggests that both a eukaryotic cell (yeast) and a prokaryotic cell (*E. coli*) may use a common detoxification mechanism. At

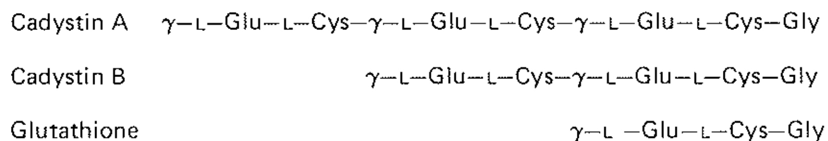

Figure 9. Structures of Cadystin A and B.

present we have no further explanation for the basis of the multiple-resistance phenotype that is conferred by plasmid pYMG14.

However, the resistance phenotype has proved to be extremely useful as a dominant marker for the selection of transformants of wild-type strains of yeast. By using the methylglyoxal resistance phenotype we have successfully introduced plasmid pYMG14 into strains of yeast that are used commercially for baking, brewing and cake manufacture. All of these strains are prototrophic diploids and carry no other genetic marker that can be used for plasmid transformation.

To date, only the yeast killer factor and an antibiotic—gentamycin—are available for this purpose, but their utilization is limited because of their narrow spectrum of action and high cost. As the gene that we have cloned is expressed over a wide spectrum of yeasts, it can be applied extensively to the genetic manipulation of industrially important yeast strains.

CLONING GENES FOR METAL RESISTANCE

Resistance of cells to metals and production of protective peptides

Because yeast cells are usually insensitive to antibiotics such as chloramphenicol, ampicillin, tetracycline, etc, it is impossible to use antibiotic-resistance genes as selective markers. However, yeasts are sensitive to metal compounds, and become resistant when they are cultured in a medium containing such compounds. A gene for resistance to copper (*CUP1*) has been identified in yeasts (Weser *et al.*, 1977; Ruppe *et al.*, 1979) and its base sequence has been determined (Butt *et al.*, 1984; Karin *et al.*, 1984). The gene encodes a Cu-thionein peptide which is synthesized by *S. cerevisiae* when it is cultured with copper. Fogel and Welch (1982) showed that the *CUP1* locus is an important genetic determinant of copper resistance in yeasts and is present in multiple tandemly amplified copies in copper-resistant strains.

In general, metallothioneins (MT) are low-molecular-weight proteins that are unusual in their high cysteine content (about 30%) and their great affinity for various metals such as Zn^{2+}, Cd^{2+}, Hg^{2+}, Ag^{2+} and Cu^{2+} (Pulido, Kagi and Vallee, 1966). Since they were first detected in equine kidney (Margoshes and Vallee, 1957), various MTs have been identified in a variety of different species of organisms including yeast cells. They are thought to have an important role in the protection of cells against heavy metal toxicity and zinc homeostasis.

Cloning of yeast metal-resistance genes, their characterization and use for genetic manipulation

On the basis of the background and rationale outlined above we have attempted to clone the *S. cerevisiae* genes for resistance to cadmium and zinc. As before, this was accomplished by the selection of hybrid plasmids that would permit growth of transformant clones on high concentrations of these toxic chemicals. Plasmids pYZN1 and pYZN2 were selected on their ability to confer resistance to 10^{-2} M and 2×10^{-2} M $ZnCl_2$, respectively; similarly, plasmids pYCD1 and pYCD2 confer resistance to 10^{-4} M and 2×10^{-4} M $CdCl_2$, respectively.

The structures of these plasmids are shown in *Figure 10*. Although the inserted chromosomal fragments of each pair differ in size, in both cases there is sequence homology between them; thus, the sequence of the smaller insert is contained within the larger insert (*Figure 10*). However, there is no homology between the pairs, indicating that the pYZN and pYCD plasmids contain DNA derived from different chromosomal regions.

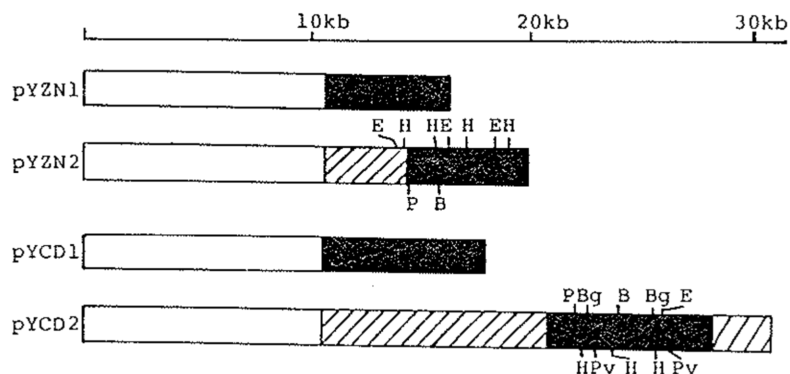

Figure 10. Physical maps of hybrid plasmids of pYZN and pYCD. □ vector plasmid YEp13; ■ homologous regions; ▨ unestablished regions; E: *Eco* RI; P: *Pst* I; B: *Bam* HI; H: *Hin* dIII; Bg: *Bgl* II; Pv: *Pvu* II. Restriction sites in pYZN1 and pYCD1 are the same as those in the homologous regions of pYZN2 and pYCD2, respectively. Although shown as linear, these plasmids are circular.

In confirmation of this we noted that the pYCD plasmids could confer resistance only to cadmium, whereas the pYZN plasmids could confer resistance only to zinc, suggesting that the plasmids encoded enzymes with different recognition specificities. Interestingly, however, we found that the pYZN plasmids could confer resistance to cadmium also, in the presence of zinc ions. Thus it seems that the metal resistance gene carried on the pYZN plasmids is inducible by zinc ions.

We also observed that pYCD conferred resistance to a higher concentration of $CdCl_2$ (2×10^{-4} M) than pYCD1 (10^{-4} M). It is possible that the gene sequence carried on pYCD1 lacks some important expression signals that are present on pYCD2. Both pYZN1 and pYZN2 were able to confer resistance to the same concentration of $ZnCl_2$ (2×10^{-2} M).

We have been able to use the zinc and cadmium resistance phenotypes of the pYZN and pYCD plasmids for the selection of transformants of several industrial strains. These have included a bakers' yeast (*S. cerevisiae* IFO 2375), a sake yeast (*S. cerevisiae* IFO 3247) and a beer yeast (*S. carlsbergensis* or *S. uvarum* IFO 0641). All of these strains are prototrophic, thus precluding the use of conventional amino-acid auxotrophic complementation markers for the selection of transformants. Again, as with the MG resistance marker, these metal-resistance markers will be particularly useful for genetic manipulation of these types of yeast strains.

Conclusions

In this review I have summarized our work on the manipulation of yeast strains by recombination technology. I have tried to show how this technology can be successfully applied to improve cells for use in a bioreactor for the production of large amounts of metabolites such as ATP, ethanol and glutathione (Kimura, 1985, 1986). In order to do this it has been necessary to develop two important aspects. First, we needed a reliable and efficient method for transformation of yeast cells, which allowed easy identification of the clones of interest: the KU method was developed for this purpose. Secondly, it was important to develop genetic markers which could be used for plasmid transformation of industrial wild-type yeast strains: the plasmids carrying determinants for the resistance to MG, zinc and cadmium can be used for this purpose. These markers, which contain only yeast DNA, were isolated without the need for sensitive or auxotrophic mutants by selecting for a higher resistance level brought about by increased gene dosage. In principle, the method should be applicable to the isolation of many genes involved in determining resistance mechanisms. By using toxic analogues as selective agents it may even be possible to clone genes encoding the enzymes of amino-acid and nucleic-acid base biosynthetic pathways.

Genetic engineering techniques become most effective when combined with a broad knowledge of microbiology and other related techniques such as cell immobilization, chemical engineering, fermentation and processing. The combination of recombinant DNA technology with other techniques will therefore become essential for the production of effective microbial bioreactors and biocatalysis systems. We propose the term 'syntechno-system' to describe this combined-technology approach to biotechnological problems.

References

ASADA, M., SHIRAI, Y., NAKANISHI, K., MATSUNO, R., KIMURA, A. AND KAMIKUBO, T. (1981). ATP regeneration with enzymes of the alcohol fermentation pathway and kinases of yeast and its computer simulation. *Journal of Fermentation Technology* **59**, 239–245.

BUTT, T.R., STERNBERG, E.J., GORMAN, J.A., CLARK, P., HAMER, D., ROUNBERB, L. AND CROOKE, S.T. (1984). Copper metallothionein of yeast, structure of the gene, and regulation of expression. *Proceedings of the National Academy of Sciences of the United States of America* **81**, 3332–3336.

DUDANI, A.K., SRIVASTAVA, L.D. AND PRASAD, R. (1984). Glyoxalase-I activity and cell cycle regulation in yeast. *Biochemical and Biophysical Research Communications* **119**, 962–967.

EGYUD, L.G. AND SZENT-GYORGYI, A. (1965). Cell division, SH, ketaoaldehydes, and cancer. *Proceedings of the National Academy of Sciences of the United States of America* **55**, 388–393.

FOGEL, S. AND WELCH, J.W. (1982). Tandem gene amplification mediates copper resistance in yeast. *Proceedings of the National Academy of Sciences of the United States of America* **79**, 5342–5346.

FUKUDA, Y., YAMAGUCHI, S., SHIMOSAKA, M., MURATA, K. AND KIMURA, A. (1983). Cloning of the glucokinase gene in *Escherichia coli* B. *Journal of Bacteriology* **156**, 922–925.

FUKUDA, Y., YAMAGUCHI, S., HACHIMOTO, H., SHIMOSAKA, M. AND KIMURA, A. (1984). Cloning of glucose phosphorylating genes in *S. cerevisiae* by the KU-method and application to ATP production. *Agricultural and Biological Chemistry* **48**, 2877–2881.

HASHIMOTO, H., MORIKAWA, H., YAMADA, Y. AND KIMURA, A. (1985). A novel method for transformation of intact yeast cells by electroinjection of plasmid DNA. *Applied Microbiology and Biotechnology* **21**, 336–339.

HINNEN, A., HICKS, J.B. AND FINK, G.R. (1978). Transformation of yeast. *Proceedings of the National Academy of Sciences of the United States of America* **75**, 1929–1933.

ITO, H., MURATA, K. AND KIMURA, A. (1984). Transformation of intact yeast cells treated with alkali cations or thiol compounds. *Agricultural and Biological Chemistry* **48**, 341–347.

ITO, H., FUKUDA, Y., MURATA, K. AND KIMURA, A. (1983). Transformation of intact yeast cells treated with alkali cations. *Journal of Bacteriology* **153**, 163–168.

KARIN, M., NAJARIAN, R., HASLINGER, A., VALENZUELA, P., WELCH, J. AND FOGEL, S. (1984). Primary structure and transcription of an amplified genetic locus: The CUP1 locus of yeast. *Proceedings of the National Academy of Sciences of the United States of America* **81**, 337–341.

KIMURA, A. (1982). Application of plasmid to production of energy (ATP) and glutathione. In *Proceedings of the IVth International Symposium on Genetics of Industrial Microorganisms, Kodansha, Kyoto*, pp. 277–282.

KIMURA, A. (1985). Novel techniques for breeding of microorganisms and application of the microorganisms to production of glutathione. In *Industrial Aspects of Biochemistry and Genetics. NATO ASI Series, Series A: Life Sciences, Volume 87* (N.G. Aleaddinoglu, A.L. Demain and G. Lancini, Eds), pp. 35–47. Plenum Press, New York and London.

KIMURA, A. (1986). Application of recDNA Techniques to production of ATP and glutathione by 'syn-techno system'. In *Advances in Biochemical Engineering* (A. Fiechter, Ed.), pp. 29–51. Springer-Verlag, Heidelberg.

KIMURA, A. AND OKUDA, M. (1976). Disappearance of phosphorylation activity of nucleotides from mitochondria-rich cells of a yeast, *Hansenula jadinii*; a modified Pasteur effect. *Agricultural and Biological Chemistry* **40**, 1373–1380.

KIMURA, A., ARIMA, K. AND MURATA, K. (1981). Biofunctional change in yeast cell surface on treatment with Triton X-100. *Agricultural and Biological Chemistry* **45**, 2627–2629.

KIMURA, A., OKUDA, M. AND FUKUDA, H. (1979). Separation and comparison of the hexokinases of aerobic and anaerobic cells of the yeast *Hansenula jadinii*. *Journal of Applied Biochemistry* **1**, 127–138.

KIMURA, A., HIROSE, K., KARIYA, Y. AND NAGAI, S. (1976). Phosphorylation of mononucleotides and formation of cytidine 5′-diphosphate-choline and sugar nucleotides by respiration-deficient mutants of yeasts. *Journal of Bacteriology* **125**, 744–746.

KIMURA, A., TATSUTOMI, Y., FUKUDA, H. AND MORIOKA, H. (1980). Effect of acriflavine on the hexokinase isozyme pattern of a yeast, *Hansenula jadinii*. *Biochimica et biophysica acta* **629**, 217–224.

KONDO, N., IMAI, K., ISOBE, M., GOTO, T., MURASUGI, A., WADA-NAKAGAWA, C. AND HAYASHI, Y. (1984). Cadystin A and B, major unit peptides comprising cadmium binding peptides induced in a fission yeast—separation, revision of structures and synthesis. *Tetrahedron Letters* **25**, 3869–3872.

MARGOSHES, M. AND VALLEE, B.L. (1957). A cadmium protein from equine kidney cortex. *Journal of the American Chemical Society* **79**, 4813–4814.

MURASUGI, A., WADA, C. AND HAYASHI, Y. (1981a). Calcium-binding peptide induced in fission yeast, *Schizosaccharomyces pombe*. *Journal of Biochemistry* **90**, 1561–1564.

MURASUGI, A., WADA, C. AND HAYASHI, Y. (1981b). Purification and unique properties in UV and CD spectra of Cd-binding peptide 1 from *Schizosaccharomyces pombe*. *Biochemical and Biophysical Research Communications* **103**, 1021–1028.

MURATA, K., FUKUDA, Y., SHIMOSAKA, M., WATANABE, K., SAIKUSA, T. AND KIMURA, A. (1985a). Phenotypic character of the methylglyoxal resistance gene in *Saccharomyces cerevisiae*: expression in *Escherichia coli* and application to breeding wild-type yeast strains. *Applied and Environmental Microbiology* **50**, 1200–1207.

MURATA, K., FUKUDA, Y., SHIMOSAKA, M., WATANABE, K., SAIKUSA, T. AND KIMURA, A. (1985b). Metabolism of 2-oxoaldehyde in yeasts; Purification and characterization of NADPH-dependent methylglyoxal-reducing enzyme from *Saccharomyces cerevisiae*. *European Journal of Biochemistry* **151**, 631–636.

MURATA, K., INOUE, T., SAIKUSA, T., WATANABE, K., FUKUDA, Y., SHIMOSAKA, M. AND KIMURA, A. (1986). Metabolism of α-ketoaldehydes in yeasts: inducible formation of methylglyoxal reductase and its relation to growth arrest of *Saccharomyces cerevisiae*. *Journal of Fermentation Technology* **64**, 1–4.

NEUMANN, E., SCHAEFER-RIDDER, M., WANG, Y. AND HOFSCHNEIDER, P.H. (1982). Genetic transfer into mouse lyoma cells by electroporation in high electric fields. *EMBO Journal* **1**, 841–845.

PULIDO, P., KAGI, J.H.R. AND VALLEE, B.L. (1966). Isolation and some properties of human metallothionein. *Biochemistry* **5**, 1768–1777.

RUPPE, H., CAMMACK, R., HARTMANN, H-J. AND WESER, U. (1979). Oxidation–reduction reactions of copper–thiolate centres in Cu–thionein. *Biochimica et biophysica acta* **578**, 462–475.

RUSSELL, D.H. AND MCVICKER, T.A. (1972). Polyamines in the developing rat in supportive tissue. *Biochimica et biophysica acta* **259**, 247–258.

SHIVAROVA, N., FORSTER, W., JACOB, H-E. AND GRIGOROBA, R. (1983). Microbiological implications of electric field effects. VII. Stimulation of plasmid transformation of *Bacillus cereus* protoplasts by electric field pulses. *Zeitschrift für allgemeine Mikrobiologie* **23**, 595–599.

SZENT-GYORGYI, A. (1965). Cell division and cancer. *Science* **149**, 34–37.

UMEMURA, I., FUKUDA, H. AND KIMURA, A. (1982). Controlling mechanism of ATP regeneration by glycolytic pathway in a yeast: *Hansenula jadinii*. *European Journal of Applied Microbiology and Biotechnology* **15**, 133–137.

WESER, U., HARTMANN, H-J., FRETZDORFF, A. AND STROBEL, G.-J. (1977). Homologous copper (1)-(thiolate) chromophores in yeast copper thionein. *Biochimica et biophysica acta* **493**, 465–477.

YAMAGUCHI, S., FUKUDA, Y., SHIMOSAKA, M. AND KIMURA, A. (1984). Phosphorylation of AMP to ATP by dried *Escherichia coli* B cells. *Journal of Fermentation Technology* **62**, 29–33.