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PROTOPLAST FUSION IN YEASTS

by

© RITA PAULINE VIDOLI, B.Sc.

A thesis submitted to the Faculty of
Graduate Studies and Research in partial fulfilment
of the requirements for the degree of
Master of Science

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ABSTRACT

Intraspecific fusion frequencies for Saccharomyces cerevisiae and Schwanniomyces alluvius were about 10^{-3} using 20% polyethylene glycol (PEG)-MW 4000 and about 10^{-7} using 40% PEG-MW 6000. Electric shock enhanced S. cerevisiae fusion by 260x with 40% PEG-MW 6000 only. No enhancement was found in Schw. alluvius fusions at either concentration of PEG. Encapsulation of fused protoplasts in Ca^{++} -alginate beads, compared to an agar overlay, showed 40x and 1000x increases for S. cerevisiae hybrid yields in regeneration and selection media, respectively. Preliminary experiments for Schw. alluvius gave regenerated colonies but no hybrids.

Intergeneric fusion between the two yeasts gave one hybrid at a frequency of about 5.2×10^{-8} . This failed to grow after four transfers. Irradiation pretreatments of the parental strains were used to facilitate a more specific, directional gene transfer. This gave a 10x increase in frequency. To obtain stable intergeneric hybrids, the use of such pretreatments merits serious consideration.

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LIST OF ABBREVIATIONS

Genetic Terms

<u>Schw. alluvius</u>	<u>Schwanniomyces alluvius</u>
<u>S. cerevisiae</u>	<u>Saccharomyces cerevisiae</u>
<u>SA-WT</u>	<u>Schw. alluvius</u> wild-type
<u>SA-LT2</u>	<u>Schw. alluvius</u> lysine and tryptophan double auxotroph
<u>SA-HT15</u>	<u>Schw. alluvius</u> histidine and threonine double auxotroph
<u>SS⁻</u>	mutation in soluble starch assimilation
<u>ts</u>	temperature-sensitive
<u>α</u>	alpha mating type
<u>a</u>	a mating type

Media

<u>YPD</u>	yeast extract-peptone-dextrose
<u>SD</u>	synthetic minimal media
<u>RA</u>	regeneration agar
<u>SS</u>	soluble starch media

Chemical Terms

UV	ultraviolet
EMS	ethylmethane sulfonate
MNNG	N-methyl-N'-nitro-nitroso-guanidine
DAPI	4',6-diamidino-2-phenyl-indol-2HCl
PEG	polyethylene glycol

Units

M ²	metre ²
cm	centimetre
cc	cubic centimetre
ml	millilitre
mM	millimolar
M	molar
ug	microgram
mg	milligram
g	gram
us	microseconds
min	minutes
hr	hours
rpm	rotations per minute
M Ω	megaohms
KV	kilovolts
Q	energy
c	capacitance
V	voltage
uf	microfarads
k μ ads	kilorads

Additional Term

MW	molecular weight
----	------------------

INTRODUCTION

Genetic exchange in nature has been known to play a vital role in providing the basis for genetic variability and evolution in biological systems. In more recent years, novel techniques have been developed in the laboratory which make it possible to accelerate the evolutionary process, or even to bypass it altogether. Such methods facilitate the transfer of genetic information from one microorganism to another. The best example of this is the widespread application of recombinant DNA techniques in both prokaryotes and eukaryotes. Here, a highly specific and unidirectional gene transfer makes it possible to introduce new genetic information into a host cell and to study foreign gene expression in a novel background.

In addition to this type of specific gene transfer, cell fusion provides a more general method for genetic exchange. The cell wall is removed to form a "protoplast" and, under given conditions, membrane-membrane interaction between protoplasts is facilitated. The end result is "fusion" - a complete pooling of cytoplasmic and nuclear material. The simplicity of the method (the ease with which protoplasts can be obtained, fused and the hybrid product recovered) lends the technique special merit. More importantly, it can be applied where polyploidy and/or lack of mating characteristics makes classical genetic crosses impossible, or where the complexity and lack of knowledge concerning the genetics of the organism chosen makes the

recombinant DNA approach somewhat difficult. Although protoplast fusion is usually a bidirectional means of gene transfer, it can be made into a more specific, directed exchange system if desired. The technique has been successfully attempted for a number of cell types, including plant and animal tissue culture systems, fungi and bacteria.

LITERATURE REVIEW

Protoplast Fusion

A typical protoplast fusion experiment can be divided into four major steps:

- 1) protoplast preparation
- 2) protoplast fusion
- 3) wall regeneration and reversion to the "normal" cell type
- 4) hybrid selection

The optimal conditions for protoplast preparation, fusion and regeneration must be empirically determined for each parental combination used. Efficient selection of the hybrid product depends upon the availability of stable, unequivocally characterized genetic markers for the parents involved.

Protoplasts are isolated through the use of lytic enzymes in the presence of an osmotic stabilizer. A number of enzymes are available (Hamlyn, et al., 1981; Stephen and Nasim, 1981). Often, a thiol pretreatment is employed to open up the molecular structure of the cell wall in order to make it more accessible to enzyme degradation (Sommer and Lewis, 1971). Cell density and cell growth phase can significantly affect the efficiency of protoplast formation (Peberdy, 1979). Generally, a density of $10^7 - 10^8$ cells/ml is used and protoplasts are formed from a logarithmic phase culture.

There exist a variety of chemical and biological agents which can induce the fusion process (Ahkong, et al., 1975; Nagata, 1978; Knutton and Pasternak, 1979). The use of polyethylene glycol (PEG) as a fusogen is currently the accepted choice (Peberdy, 1980). Its efficiency is enhanced by the addition of monovalent (Na^+ , K^+) or divalent (Ca^{++} , Mg^{++}) salts to the fusion mixture (Fernezy, et al., 1976). Ca^{++} (10 - 100 mM) is found to give the most significant increases. The mechanisms by which fusion occurs has been discussed (Ahkong, et al., 1975; Papahajopoulos, 1974; 1976; Maggio, et al., 1976; Ingolia and Koshland, 1978; Knutton, 1979; Knutton and Pasternak, 1979). First, protoplast aggregation takes place. This is probably due to charge interactions between negatively charged protoplasts, positively charged cations and PEG, a neutral molecule overall but with localized positively and negatively charged sites. Second, following removal of the fusogen, membrane-membrane interactions between closely apposed protoplasts take place. Membrane fusion results, but only at small localized sites denuded of intramembraneous protein and thus subject to lipid-lipid interaction. Third, localized fusion sites expand to form spherical fused protoplasts by a process of protoplast swelling.

Regeneration and reversion of the fused protoplast is essential. The hybrid product can not be recovered unless the original fused protoplast can regenerate the cell wall,

grow and finally divide to form a visible colony. The composition of the regeneration medium is of prime importance to the process of reversion. Some protoplasts can regenerate in liquid medium (Nečas, 1961). Others require a solid physical environment such as agar or gelatin for cell wall reformation (Nečas, 1961; Svoboda, 1966; Peberdy, 1979).

Selection of fusion products is usually based upon a nutritional complementation that occurs in hybrid protoplasts derived from two suitable parental lines (Sommer and Lewis, 1971). Where the goal of fusion is strain improvement, a predominance of one parental genome may be desired, with a very specific property of the second parental genome incorporated into it. This can be achieved by mutagenesis of the donor organism to very low survival levels (0.01%) (Hockney and Freeman, 1980; Hopwood and Wright, 1979; 1981). Such treatment disrupts the genome extensively. Therefore, the expression of any markers from the treated parental line in the hybrid product will most likely be a result of integration of a discrete DNA segment into the untreated genome. Such a procedure would be expected to yield a directional transfer of genetic information via protoplast fusion.

Historical Background

Protoplast fusion has a variety of applications over a wide range of organisms. It gained its popularity in plant tissue culture systems where entire plants could be regenerated from cultured protoplasts (Carlson, et al., 1972; Cocking, 1976; Constabel, 1978). It has been used to isolate hybrid varieties of plants (Gleba and Hoffman, 1980; Douglas, et al., 1981). The two major factors limiting success in this area are the lack of suitable mutants for selection and the lack of information regarding the regeneration of many plant cell types.

Protoplast fusion between microorganisms is a somewhat easier task in that a variety of mutants can be obtained from haploid strains and the number of factors contributing to cell wall regeneration and cell reversion are fewer than those for plant tissue culture systems. Bacteria and fungi hold other advantages over the more complex systems of plant and mammalian tissue culture in that the requirements for growth are minimal and the cell division time is relatively short. The two genera of bacteria which have received the most attention in protoplast fusion studies are Bacillus and Streptomyces (Gabor and Hotchkiss, 1979; Hopwood and Wright, 1981). They represent two of the most important groups of industrial microorganisms (Hopwood, 1981). Bacillus plays a vital role in commercial enzyme production and to a lesser extent, in antibiotic production. Streptomyces is valued for the production of glucose isomerase and for

the vast amount of naturally produced antibiotics.

Fungal protoplast fusion has become increasingly popular over the past few years. This is particularly true where polyploidy and lack of sexual mating systems prevents or impedes the genetic analysis of such microorganisms. Protoplast fusion has been successfully carried out in Aspergillus (Ferenczy, et al., 1975; Anné and Peberdy, 1976) and Penicillium (Anné and Peberdy, 1976). A number of yeasts have been fused both intra- and interspecifically to yield stable hybrid products. These include Kluyveromyces, Saccharomyces, Candida and Schizosaccharomyces (Ferenczy, 1981).

Yeasts are eukaryotes and so possess the relatively more complex processing machinery of this phylogenetic group while sharing some of the practical advantages of the prokaryotes. They are easy to culture and fast-growing; most laboratory strains are non-pathogenic (Petes, 1980). Yeasts draw particular attention with regards to protoplast fusion since many of the industrially significant strains are polyploid and/or lack mating characters (Stewart, 1981). Evaluation of the facts based on the dynamics of protoplast formation, microscopic morphology and the physical properties of protoplasts, strongly support the view that the majority of the protoplasts formed have completely lost the original cell wall (Necas, 1971). They would then fit the true definition of a protoplast--a "wall-less cell". Wall remnants could be present but they would have to be extremely elastic or very short so as to be discontinuous over the protoplast surface. With this last consideration in mind, the more

conservative scientist is reluctant to use the word "protoplast". The alternate choice is "spheroplast"--a cell in which the wall has almost been completely removed but short segments still remain. Hence, the conglomeration of "protoplast"/"spheroplast" denotations in the literature.

Intraspecific Fusion

Intact yeast cells of like mating types are incapable of sexual genetic exchange. Auxotrophic strains of Saccharomyces cerevisiae with identical and with opposite mating types yield recombinant cells upon PEG treatment of protoplasts (Christensen, 1979). Mating of hybrid diploids from fusions involving parents of opposite mating types leads to a tetraploid. Three types of diploid spores can be expected from the $4n$ regenerated cells. The first two groups are homozygous for each of the mating types. The third is heterozygous. Only the heterozygous spores can be induced to go through another reduction division. Tetrads are frequent in which all four diploid spores are heterozygous and further segregation of mating alleles in tetrad analysis gives the expected 8:8 ratio among haploid spores. Reduction division analysis plus the production of newly re-associated parental markers in hybrid recombinants lend credibility to the actual occurrence of fusion. Similar experiments employing like and unlike mating types of S. cerevisiae in which the donor

carries the mitochondrial erythromycin resistance marker have been carried out (Ferenczy and Maráz, 1977). The recipient is an ade⁻ (nuclear mutation), respiration-deficient (mitochondrial mutation) strain. Adenine prototrophs resistant to erythromycin are recovered. The hybrids have one nucleus per cell and hybrids from opposite mating type fusions can be sporulated. Up to 40% ade⁻ haploids can be isolated that are sufficient in respiration and erythromycin resistant. This is indisputable proof of successful nuclear and mitochondrial transfer.

Mechanisms concerning the transfer of the mitochondrial genome during protoplast fusion can only be speculated. At present, two possibilities seem to be favoured (Gunge and Sakaguchi, 1979):

- 1) the direct transfer of intact mitochondria during protoplast fusion or,
- 2) the transformation of mitochondrial DNA into protoplasts by some kind of membrane fusion between the mitochondria and the recipient protoplast membrane; the transformed DNA would then have to enter a mitochondria-like structure in the respiration deficient cell and eventually evolve into a functional organelle.

Intraspecific protoplast fusion has also been successfully carried out between like mating types of Schizosaccharomyces pombe (Sipiczki and Ferenczy, 1977; Lückemann, et al., 1979; Thuriaux et al., 1980). The genus Candida has also given intraspecific products (Fournier, et al., 1977; Sarachek, et al., 1981). Mitochondrial transfer via fusion has been demonstrated in the yeast Kluyveromyces lactis (Allmark, et al.,

1978; Morgan, et al., 1980).

True intraspecific protoplast fusion involves two processes: heterokaryosis and karyogamy. Upon pooling nuclei and cytoplasms of double auxotrophic protoplast lines, prototrophic cells first recovered from fusions are heterokaryotic (Sarachek, et al., 1981). Mixing of cytoplasms does occur. However, the parental nuclei remain independent of each other. Such colonies are slow-growing and highly unstable, often reverting to either one of the parental types. Instability occurs because the nuclei undergo asynchronous division. Consequently, buds are frequently uninucleate. In many intraspecific fusions, the initial heterokaryons undergo further changes--"karyogamy". Here, nuclear fusion does occur giving rapidly growing prototrophic colonies that are uninucleate. Such lines remain stable as total diploids or undergo partial loss of chromosomes from one parental genome to attain a stable aneuploid state. Upon haploidization with p-fluorophenylalanine, these products yield recombinant segregants as a result of mitotic crossing-over and recombination.

Intraspecific protoplast fusions in the heterothallic yeasts Saccharomyces cerevisiae (Maráz, et al., 1978), Saccharomycopsis lipolytica (Stahl, 1978) and Schizosaccharomyces pombe (Sipiczki and Ferenczy, 1977) result in immediate karyogamy and establishment of stable diploid nuclei. In contrast, protoplast fusion within the genus Candida, an asexual yeast, involves the initial formation of slow-

growing heterokaryons followed by occasional karyogamy leading to the outgrowth of rapidly dividing uninucleate cells (Sarachek, et al., 1981; Fournier, et al., 1977). Genetic evidence of chromosome losses associated with karyogamy, as well as DNA analyses, are indicative of aneuploidy in Candida fusion products. The genus Candida is an arbitrary, phylogenetically heterogeneous taxon including all imperfect yeasts forming hyphae or pseudo-hyphae (van Uden and Buckley, 1970). Mating types have not been found in C. albicans or C. tropicalis.

The immediate formation of stable diploid nuclei when protoplasts of perfect yeasts fuse suggests that mating type loci may play a role in proper karyogamy (Sarachek, et al., 1981). The mating type characteristic permits the accommodation of two haploid genomes in nature. In fusion of like mating types, then, one is artificially mating two cell types that are incompatible in nature. However, the incompatibility may well exist at the physical/physiological level (wall proteins/receptors) rather than at the nuclear level. This may be so since, in perfect yeasts (those with distinct mating type properties), protoplast fusion involving opposite mating types results in stable complete diploid products which behave similarly to sexually formed diploids regarding sporulation and segregation.

Interspecific Fusion

Interspecific fusion products have been reported with the genus Saccharomyces (Hockney and Freeman, 1980; Spencer, et al., 1980). Mitochondrial transfer may also be feasible in such fusion events (Hockney and Freeman, 1980), although interspecific hybrids have proven to be less stable than those produced intraspecifically between like mating types (Weber and Spata, 1981).

Intergeneric Fusion

Attempts to obtain intergeneric fusion products using fungal protoplasts are outlined in the table below.

<u>Yeasts Fused</u>	<u>Selection</u>	<u>Remarks</u>	<u>References</u>
<u>Candida tropicalis</u>	prototrophy	10^{-5}	Provost, et al., 1978
x		genetic changes	
<u>Saccharomycopsis fibuligera</u>		followed by stability	
<u>Saccharomyces cerevisiae</u>	prototrophy,	10^{-6}	Svoboda, 1980
x	cell shape	highly unstable	
<u>Schizosaccharomyces pombe</u>			

<u>Yeasts fused</u>	<u>Selection</u>	<u>Remarks</u>	<u>References</u>
<u>Saccharomyces cerevisiae</u> x	prototrophy, highly growth on lactose	unstable	Stewart, et al., 1981
<u>Kluyveromyces lactis</u>			
<u>Saccharomyces cerevisiae</u> x	prototrophy, genetic p^+	changes followed by stability	Howes, 1981
<u>Kluyveromyces lactis</u>			
<u>Saccharomyces cerevisiae</u> x	prototrophy	2.5×10^{-7}	de van Broock, et al., 1981
<u>Saccharomycopsis lipolytica</u>			
<u>Saccharomyces cerevisiae</u> x	prototrophy, p^+	10^{-6} genetic changes followed by stability	Yamashita, et al., 1981
<u>Hansenula wingei</u>			
<u>Saccharomyces cerevisiae</u> x	prototrophy	6.5×10^{-6}	Wilson, et al., 1982
<u>Schwanniomyces alluvius</u>			

Numbers in Remarks column represent fusion frequencies.

p^+ --respiration sufficient

Most intergeneric studies have met with limited success.

Saccharomyces cerevisiae and Schizosaccharomyces pombe auxotrophic lines have been fused (Svoboda, 1980). Hybrids can be recognized microscopically since the two yeasts differ in cell morphology. Hybrids are recovered at a frequency of 10^{-6} but are slow-growing. They do not survive sub-culturing, making further genetic analysis impossible. Intergeneric products from Kluyveromyces lactis and Saccharomyces cerevisiae fusions (Howes, 1981; Stewart, et al., 1981), Schwanniomyces alluvius and S. cerevisiae fusions (Wilson, et al., 1982) and Candida tropicalis and Saccharomycopsis fibuligera fusions (Provost, et al., 1978) have all been obtained. In all cases, frequencies are low and fusion products are highly unstable.

Saccharomycopsis lipolytica and Saccharomyces cerevisiae fusion products have been obtained at a frequency of 10^{-6} (de van Broock, et al., 1981). The initial isolates undergo considerable spontaneous segregation upon sub-culturing but stable lines possessing characteristics from both parents are eventually established. This may be because the two yeasts involved here share closer phylogenetic links than the fusion pairs described above. Successful intergeneric fusion products have also been recovered by transferring mitochondria of Hansenula wingei into protoplasts of S. cerevisiae (Yamashita, et al., 1981). In the dividing cells of H. wingei, migration of the mitochondria into the buds or daughter cells precedes that of the nucleus. Protoplasts are formed and "mini-protoplasts" are isolated (anucleate buds with the mitochondria

only) in the supernatant after low speed centrifugation. The mini-protoplasts fuse with S. cerevisiae protoplasts at a frequency of 10^{-6} . The hybrid products undergo considerable spontaneous segregation but stable lines are obtained.

Studies involving intergeneric fusions, thus far, seem to demonstrate the difficulties in overcoming nature's inherent patterns. Fusion is possible among phylogenetically distinct yeasts, though fusion frequency is quite low. However, there are very real problems in maintaining the genetic integrity of the fused products. Chromosome loss is prevalent, often occurring as a nearly complete loss of one parental complement of genes. The result is a reversion to the other parental phenotype with perhaps one or two characteristics from the eliminated genome as a result of recombination. Most often, the hybrid colonies are very slow-growing and unresponsive to sub-culturing, making further analysis difficult. Such phenomena may be attributed to a number of occurrences within the hybrid. Parental differences in chromosome number, asynchronous entry into the cell division cycle and physiological incompatibility are, most likely, the major factors responsible for the high level of spontaneous segregation. Lack of homology between parental gene complements would also impede genetic recombination and a neutral tolerance for both genomes within the fused product (Hopwood and Wright, 1981). All of the genetic rearrangements so characteristic of

intergeneric fusates are representative of an attempt by the cell to attain a state of genetic equilibrium--that is, to have a genetic constitution that can readily "fit into" normal cell functions, namely, cell division.

Where the two parental pools hold more similarities (as with more closely related parents) or where one genetic pool is limited in its contribution (as with mini-protoplasts), genetic equilibrium/stability may eventually be reached. This probably occurs because the demands on the resulting hybrid are less. Chromosome homology is more extensive where phylogenetically "closer" parents are used. The number of genes introduced by the mini-protoplasts are considerably fewer than that of a regular nuclear fusion. In addition, nuclear fusion is not a requirement here since one parent is donating only the mitochondrial genes. Thus, by choosing two similar genetic pools or by restricting the contribution of one parental line, stable intergeneric fusates can be obtained subsequent to initial genetic rearrangements. Where phylogenetic distinctions are too great between the fused parental lines, nuclear fusion is never achieved in the resulting hybrid, making it highly unstable.

Objectives of This Study

It was the purpose of this study to investigate ways in which protoplast fusion frequency can be increased and/or genetic stability of the resulting hybrids enhanced. The

achievement of either would have obvious ramifications in both intra- and intergeneric fusions, leading to the isolation of more hybrid products in one case and the recovery of hybrid products that would lend themselves to extensive analysis, in the other. The yeasts we chose to work with were Saccharomyces cerevisiae and Schwanniomyces alluvius. S. cerevisiae intraspecific fusion systems were used to investigate new parameters for the desired effects. It is important, when introducing modifications to a given procedure, that one begins with a biological system in which the procedure is known to work, has been well-characterized and is reproducible. S. cerevisiae is the most extensively studied yeast (Petes, 1980). There are many reports in the literature dealing with intraspecific fusion for this organism (van Solingen and van der Plaat, 1977; Maráz, et al., 1978; Russell and Stewart, 1979; Sakaguchi, et al., 1980). Genetically marked strains are numerous, giving one easy access to a strong selection system.

Our second choice, Schwanniomyces alluvius, is a relatively "new" organism in that little is known about it. It is a budding yeast, believed to have four chromosomes (Ferreira and Phaff, 1959). Sexual mating is rare (Phaff, 1970). If diploidization does occur, one-spore and, occasionally, two-spore asci can be obtained (Phaff, 1970; Kurtzman, et al., 1972; Kreger-van Ru, 1977). The organism can assimilate a variety of substrates--glucose, maltose,

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cellobiose, inulin, soluble starch, xylose (Moranelli, et al., 1982). It can also ferment a number of substrates--glucose, maltose, inulin, soluble starch (Phaff, et al., 1960; Moranelli, et al., 1982). The yeast, therefore, has potential industrial uses. The lack of a defined sexual mating limits the employment of strain improvement programs and analyses by classical genetic techniques. Intraspecific fusion would not only provide a system for gene transfer in the organism but would also give one the opportunity to examine gene dosage effects, complementation, recombination, etc.

Recently, protoplast fusion in Schwanniomyces alluvius has been reported (Wilson, et al., 1982), but frequencies for hybrid recovery are low at 1.5×10^{-6} . Intraspecific fusion frequencies 10 - 100x higher have been achieved in other yeasts (Allmark, et al., 1978; Maráz, et al., 1978; Sipiczki and Ferenczy, 1979; Sarachek, et al., 1981). It was our aim to find fusion conditions for Schw. alluvius that would give a frequency of 10^{-3} to 10^{-5} for the recovery of hybrid products. Once this was established, the Schw. alluvius system was used to test any parameters that had proven effective in increasing fusion frequency in the S. cerevisiae system.

Two parameters have been examined in the intraspecific fusion systems: 1) electric shock and, 2) bead encapsulation. Under the action of a high electric field pulse, a pronounced stimulation of yeast protoplast fusion by polyethylene glycol

and Ca^{++} ions is found (Weber, et al., 1981a). The fusion rate is enhanced by a factor of 200 for different genetically marked strains of S. cerevisiae as compared with fusion without electric field treatment. An enhancement factor is found in intergeneric fusion systems as well (Weber, et al., 1981b). PEG creates the necessary intimate contact between protoplasts. The electric discharge for the protoplast mixture in PEG is thought to strengthen this contact and cause membrane labilization and breakdown (Weber, et al., 1981a). This "fluidization" of the membrane may permit the protoplasts to interact more readily with each other, resulting in a higher yield of hybrid products. The second parameter investigated, we termed "bead encapsulation". Here, we used Ca-alginate beads, rather than the routine agar overlay, to enclose protoplasts within a restricted volume. The entrapment of cells in calcium alginate has gained widespread appreciation in recent years due to the simplicity of the method and the properties of the gel material (Birnbaum, et al., 1981). It is non-toxic and quite inexpensive. The entrapment of living whole cells in the gel matrix does not seem to affect the biological integrity of the organism (Kierstan and Bucke, 1979; Takata, et al., 1977; Maleszka, et al., 1981; Veliky and Williams, 1982).

The alginate is polymerized in a Ca^{++} solution as beads, but the polymerization is allowed to proceed only long enough to form a shell on the surface of the bead. The inner matrix is left as a viscous, unpolymerized gel-protoplast

suspension. Such an environment would, hopefully, permit three things, otherwise neglected in an agar overlay:

- 1) Temperature shock is avoided since the alginate beads are formed and dissolved at room temperature. The agar overlay requires temperatures of 45 - 50°C when mixed with the protoplast suspension.
- 2) The protoplasts are confined to a smaller volume in the beads compared to spreading on an agar overlay plate. This would perhaps bring protoplasts into closer physical proximity leading to increases in hybrid recovery.
- 3) The inner matrix of the bead is still a viscous, fluid or gel-like material so that protoplasts are "free-floating" during incubation. In an agar overlay, the agar solidifies almost instantly, fixing protoplasts in a certain position on the plate. The bead environment, then, may permit a prolonged period for protoplast interaction which could again lead to significant increases in hybrid recovery.

Once efficient intraspecific fusion systems were established for S. cerevisiae and Schw. alluvius, intergeneric fusion of the two yeasts was attempted. Since conventional protoplast fusion techniques have demonstrated limited success, a modified approach was employed here. The use of ultraviolet (UV) irradiation to increase fusion frequency has been reported for the bacterium Streptomyces (Hopwood and Wright, 1979; 1981). The same technique has been employed for interspecific fusion systems of brewery yeast (Hockney and Freeman, 1980). Here, one of the parental protoplast preparations is given a high dose of UV prior to fusion with unirradiated protoplasts of the other parental line. Applications of this technique may include cases where counter-selectable markers are available in one strain but not in the other with which it is to be recombined. It may also be of use

where limited regions of the genome of one strain are to be introduced into a second strain without disturbing the rest of its genotype (Hopwood and Wright, 1981).

Our approach for the intergeneric fusion system was to heavily irradiate Schw. alluvius protoplasts with gamma rays (0.1 - 0.01% survival) while exposing S. cerevisiae protoplasts to a low dose of UV (80 - 90% survival). Gamma rays are known to induce single and double strand breaks in yeast DNA (Resnick and Martin, 1976). A low dose of UV will induce repair enzymes. The idea is to break the Schw. alluvius genome into a series of small DNA fragments. The extent of damage must be such that fragment size is large enough to accommodate at least one entire gene yet small enough to facilitate uptake via insertion into the S. cerevisiae genome. UV irradiation induces thymidine dimers in the DNA which are subsequently removed by repair enzymes leaving nicks in the DNA (Unrau, et al., 1971). Thus, the S. cerevisiae genome may be receptive to recombinational insertion of the newly introduced Schw. alluvius DNA fragments.

The purpose of such an approach is to create a crude transformation system where Schw. alluvius is the "donor" and S. cerevisiae the "recipient". Selection is relatively more specific for the transfer of a small DNA segment and the transfer is unidirectional. In this way, the general pooling of two genomes via routine protoplast fusion could perhaps be changed into a more specific gene transfer system.

The demands on the recipient cell may be less because it is not required to accommodate an entirely new and complete complement of genetic material. It seems reasonable to assume that in order to obtain stable products from intergeneric fusions, approaches similar to the one outlined above will have to be considered seriously in future experiments involving protoplast fusion.

MATERIALS AND METHODS

A. Yeast Strains

The haploid Schwanniomyces alluvius wild-type strain was obtained from the American Type Culture Collection (ATCC #26074) and used to isolate various mutants for protoplast fusion. All Saccharomyces cerevisiae strains, except AH22, were kindly provided by Dr. A.P. James of the National Research Council, Ottawa. AH22 was kindly provided by Dr. G.R. Fink of Cornell University, New York. The designation and genotype of the various strains used in this study are presented in the Results section (Table 1).

Wild-type strains were maintained on YPD media (Yeast Extract-Peptone-Dextrose) and auxotrophic mutants were maintained on SD media (Yeast Nitrogen Base w/o Amino Acids-Dextrose) with the appropriate supplements (20 ug/ml). Cultures used routinely were stored on plates as individual colonies at 4°C and transferred every 2-4 weeks. All other cultures were stored as slants and transferred every 4 months.

B. Media and Solutions

All media and solutions were prepared in distilled water and sterilized by autoclaving at 121°C for 20 min, unless otherwise indicated. Filter sterilization was conducted with Nalgene disposable filter units.

Media

1. YEAST EXTRACT-PEPTONE-DEXTROSE MEDIUM (YPD)

Yeast extract	1.0	g
Peptone	2.0	g
Dextrose	2.0	g
Agar	2.0	g

in 100 ml water. Autoclave.

For YPD in KCl, add 4.47 g KCl for 0.6 M KCl prior to autoclaving.

2. SYNTHETIC MINIMAL MEDIUM (SD)

Yeast nitrogen base		
w/o amino acids (Difco)	0.67	g
Dextrose	2.0	g
Agar	2.0	g

in 100 ml water. Autoclave.

For SD in KCl, add 4.47 g KCl for 0.6 M KCl prior to autoclaving.

For supplemented media, add required stocks (2 mg/ml, filter sterilized) subsequent to autoclaving, at 20 ug/ml final concentration.

3. REGENERATION AGAR (RA)

Yeast nitrogen base	
w/o amino acids	0.67 g
Dextrose	2.0 g
KCl	4.47 g
Agar	3.0 g

in 100 ml water. Weigh flask prior to autoclaving. Bring to original weight with sterile water after autoclaving. Maintain media at 50°C until ready for use to avoid gelatinization. Add required supplements at 20 ug/ml final concentration.

4. SOLUBLE STARCH MEDIUM (SS)

Yeast nitrogen base	
w/o amino acids	0.5 g
Soluble starch (Sigma)	0.5 g
Agarose (Sigma)	0.5 g

in 100 ml water. Dissolve with gentle heating and autoclave. Add required supplements at 20 ug/ml final concentration.

5. PRESPORULATION MEDIUM

Yeast extract	0.8 g
Peptone	0.3 g
Dextrose	10.0 g
Agar	2.0 g

in 100 ml water. Autoclave. Add required supplements at 40 ug/ml final concentration.

6. SPORULATION MEDIUM

Yeast extract	0.5 g
Dextrose	5.0 g
Agar	2.0 g

in 100 ml water. Autoclave. Add required supplements at 40 ug/ml final concentration.

SolutionsI Mutagenesis

1. 0.05 M potassium phosphate buffer pH 7.1

KH_2PO_4	1.7 g
K_2HPO_4	2.2 g

in 450 ml water. Adjust pH to 7.1 with 2 N NaOH.
Bring volume to 500 ml and autoclave.

2. 0.1 M citrate buffer pH 5.5

Citrate	2.1 g
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in 90 ml water. Adjust pH to 5.5 with 2 N NaOH.
Bring volume to 100 ml and autoclave.

3. Ethylmethane sulfonate (EMS) (Terochem Lab)

4. N-methyl-N'-nitro-nitrosoguanidine (MNNG) stock

MNNG	10.0 mg
(Aldrich Chemical Co.)	

in 10 ml sterile citrate buffer pH 5.5. Dissolve by alternately passing quickly through a flame and gently agitating. No further sterilization is required.

5. 6% sodium thiosulfate

Sodium thiosulfate	6.0 g
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in 100 ml water. Autoclave. Store at 4°C.

II Protoplast Fusion

1. 1.2 M sorbitol

Sorbitol 109.3 g
in 500 ml water. Filter sterilize.

2. zymolyase stock

Zymolyase 60,000 10.0 mg
(Miles Lab)

in 5.0 ml sterile sorbitol. No further sterilization
is required. Store at 4°C.

3. 1.2 M sorbitol/10 mM CaCl_2 pH 8.0

Sorbitol 21.84 g
 CaCl_2 0.15 g

in 90 ml water. Adjust pH to 8.0 with 1 N NaOH.
Bring volume to 100 ml and autoclave.

4. 100 mM CaCl_2

CaCl_2 1.5 g

in 100 ml water. Autoclave. Store at 21°C.

5. 20% (w/v) PEG-MW 4000/10 mM CaCl_2 /10 mM Tris-HCl pH 8.0

Trizma-HCl 0.12 g

in 60 ml water. Adjust pH to 8.0 with 2 N NaOH.
Add 20.0 g polyethylene glycol (PEG)-MW 4000 (Baker).
Bring volume to 90 ml and autoclave. As used, add
1.0 ml sterile 100 mM CaCl_2 stock per 9.0 ml PEG solution.

6. 40% (w/v) PEG-MW 6000/10 mM CaCl_2 pH 6.2 (unadjusted)

PEG-MW 6000 (Baker) 40.0 g

in 50 ml water. Bring volume to 90 ml and autoclave.
As used, add 1.0 ml 100 mM CaCl_2 stock per 9.0 ml PEG solution.

7. 0.6 M KCl

KCl 23.35 g

in 500 ml water. Filter sterilize.

III Bead Encapsulation

1. 50 mM CaCl_2 /0.6 M KCl

CaCl_2 0.74 g

KCl 4.47 g

in 100 ml water. Autoclave.

2. sodium alginate

Sodium alginate (BRL) 1.0 g

OR

Sodium alginate (Sigma) 0.75 g

in 45 ml water pre-heated to 55-60°C and stirring gently. Do not let boil as excessive heating could change the structure of the polymer. Vigorous stirring causes bubbles and so should be avoided. Bring volume to 50 ml. Autoclave 10 min with the stir bar above the liquid to avoid bubbling over. Cool slowly to 21°C with gentle stirring.

3. 0.1 M citrate buffer pH 6.0

Citrate 2.1 g

in 90 ml water. Adjust pH to 6.0 with 2 N NaOH.
Bring volume to 100 ml and autoclave.

IV Nuclear Staining

1. acridine orange stock

Acridine orange 10.0 mg
(Fisher)

in 100 ml 50% aqueous ethanol for 10 ug/ml final
concentration. Prepare as needed and store at 21°C
in the dark until ready for use.

2. 4',6-diamidino-2-phenylindol-2HCl (DAPI) stock

DAPI 10.0 mg
(Serva, Feinbiochemical
Heidelberg)

in 10.0 ml water for 1 mg/ml final concentration.
Store at 21°C.

C. Procedures

I Mutagenesis

The haploid Schwanniomyces alluvius wild-type strain (ATCC #26074) was used for the isolation of all mutant strains. Dose-effect curves were obtained for EMS, MNNG and ultraviolet (UV) irradiation as follows:

EMS: A 48 hr Schw. alluvius wild-type (SA-WT) culture was grown in 20 ml YPD at 30°C. Stationary phase cells were washed twice with 0.05 M phosphate buffer pH 7.1 and resuspended in 20 ml buffer. 2.0 ml washed cells, 7.85 ml buffer and 0.15 ml EMS was added to a flask for 1.5% EMS (v/v) final concentration and 2×10^7 cells/ml final cell density. The flask was incubated at 30°C in a water bath, shaking at 100 rpm. Samples were withdrawn every 15 min over a 90 min period. The first dilution was made in cold 6% sodium thiosulfate; further dilutions were made in water. Samples were plated in triplicate on YPD to determine percentage survival. Plates were scored for visible colonies after 7 days at 30°C.

MNNG: A 24 hr SA-WT culture was grown in 20 ml YPD at 30°C. It was diluted to 5×10^6 cells/ml in 20 ml YPD and regrown 4 hr to 2×10^7 cells/ml. Cells were washed twice with

0.1 M citrate buffer pH 5.5 and resuspended in the same volume. To 9.5 ml cells was added 0.5 ml MNNG stock (1 mg/ml) for a final concentration of 50 ug/ml. The flask was incubated at 30°C in a water bath, shaking at 100 rpm. Samples were withdrawn every 30 min over a 120 min period. Dilutions and platings were carried out as described for EMS.

UV: A 48 hr SA-WT culture was grown in 20 ml YPD at 30°C. Cells were washed twice with water and resuspended in 20 ml. 2 ml of the cell suspension was added to 8 ml water for 2×10^7 cells/ml. The cell suspension was placed in a sterile glass petri dish and UV-irradiated with a dose ranging from 20-100 joules/M². Samples were diluted in water and plated in triplicate on YPD to determine percentage survival. Plates were scored for visible colonies after 7 days at 30°C.

Single auxotrophic mutants of SA-WT were isolated using EMS mutagenesis as described for the dose-effect curve. A 1.5% EMS (v/v) concentration was used with a treatment time of 90 min. Dilutions were made so as to give 100-150 colonies per plate, 10 plates for the untreated sample and 90 plates for the treated cell suspension. Plates were incubated at 30°C for 7 days. Colonies from the treated sample were replica-plated onto

SD and incubated at 30°C for 4 days. Replica plates were compared to the YPD masters and colonies failing to grow on SD were picked. These were characterized by transferring, with a toothpick, onto SD plus various supplement pools as described in Cold Spring Harbor Laboratory Manual Methods in Yeast Genetics (Sherman et al., 1979). Identified auxotrophs were tested for reversion frequency on SD.

A number of double mutants were isolated from an SA-HIS2 single auxotroph. MNNG mutagenesis was conducted as outlined for the dose-effect curve. A 50 ug/ml MNNG concentration was used with a treatment time of 120 min. Platings were done on YPD as for EMS. A variety of mutants were isolated by failure to grow on replica plates containing:

- 1) SD + his. Double auxotrophs were characterized using the CSH supplement pool scheme (Sherman et al., 1979).
- 2) YPD pre-warmed to 36°C. Platings were done by removing only 3 plates at a time from the incubator and returning them immediately after plating to avoid any significant temperature drop. Incubation was continued at 36°C for 7 days. The histidine, temperature-sensitive mutants were designated his⁻ts⁻.
- 3) SS + his. Histidine, soluble starch mutants were designated his⁻SS⁻.

An SA-LYS1 single auxotroph was used to recover double auxotrophs for lysine and tryptophan. MNNG mutagenesis was carried out, 50 ug/ml for 120 min. Cells were plated on SD + lys + trp and treated cells were replica-plated onto SD + lys. Those failing to grow on the replica plates were picked as lys⁻trp⁻ double auxotrophs.

II Protoplast Fusion

a) Time Course Studies to Determine Optimal Conditions for Protoplast Formation and Regeneration

A 24 hr culture was grown in 20 ml YPD at 30°C. It was diluted in 100 ml YPD to 10^7 cells/ml and regrown 3 hr at 30°C. Logarithmic phase cells were washed twice with water, once with 1.2 M sorbitol and resuspended in 10 ml sorbitol. Schwanniomyces alluvius cultures were sonicated at this point to disrupt cell aggregates. Each culture was plated to determine the initial number of colony forming units. Protoplasts were formed in 10 ml final volume with the appropriate zymolyase concentration. The cell-enzyme mixture was incubated at 30°C with gentle shaking (75 rpm). Samples were taken at regular intervals. Percentage protoplasts was determined by diluting in water, vortexing and plating directly, to obtain the number of intact cells, on SD and the necessary supplements. Plates were incubated at 30°C for 4 days.

b) Intraspecific Protoplast Fusions

Saccharomyces cerevisiae: For S. cerevisiae intraspecific fusions, XY207-5C α ade⁻leu⁻his⁻ and C540-5B α ade⁻trp⁻arg⁻ were used. 24 hr cultures were grown in 20 ml YPD at 30°C. These were diluted to 10^7 cells/ml in 100 ml YPD and regrown 3 hr at 30°C. Cells were washed twice with water, once with

1.2 M sorbitol and resuspended in 10 ml sorbitol. Each culture was plated to obtain the initial number of colony forming units. Protoplasts were formed with 40 ug/ml zymolyase for 10 min at 30°C, shaking at 75 rpm. Protoplasts were carefully washed three times in sorbitol, centrifuged 5 min at 2000 rpm and resuspended gently by hand. Vortexing was avoided. Washed protoplasts were resuspended in 0.25 ml 1.2 M sorbitol/10 mM CaCl₂ pH 8.0. Controls were plated for each culture in an agar overlay of RA + ade. 0.2 ml of each culture was mixed in a 15 ml Corex centrifuge tube and left 15 min at 21°C. 4.0 ml 20% PEG-MW 4000/10 mM CaCl₂/10 mM Tris-HCl pH 8.0 was slowly layered on top of the mixture, 1.0 ml at a time followed by thorough yet gentle hand-mixing. The protoplast-PEG suspension was left 20 min at 21°C, then centrifuged 5 min at 2000 rpm. The pellet was drained, resuspended in 0.15 ml YPD in KCl and incubated 20 min at 30°C. The suspension was then mixed in 10 ml 0.6 M KCl. Appropriate dilutions were made in water and vortexed for percentage intact cells and in KCl and hand-mixed for percentage regeneration and fusion frequencies. Regenerated colonies were recovered in an agar overlay of RA + ade/trp/arg/his/leu. Fusion products were recovered in an agar overlay of RA + ade. Intact cells were recovered by plating directly on SD + ade/trp/arg/his/leu. All plates were incubated at 30°C for 4 days.

Schwanniomyces alluvius: For Schw. alluvius intraspecific fusions, SA-HT15 his⁻thr⁻ and SA-LT2 lys⁻trp⁻ were used.

Fusion was conducted as described above except:

- 1) Washed cell suspensions were sonicated prior to protoplast formation to disrupt cell aggregates.
- 2) For protoplast formation, SA-HT15 was treated with 20 ug/ml zymolyase 30 min and SA-LT2 was treated with 40 ug/ml zymolyase 15 min.
- 3) Regenerated colonies were recovered in an agar overlay of RA + his/thr/lys/trp. For fusion products and controls, samples were plated in an agar overlay of RA. Intact cells were recovered by plating directly on SD + his/thr/lys/trp.

c) Enhancement of Protoplast Fusion Frequency

Electric Shock: S. cerevisiae and Schw. alluvius intraspecific protoplast fusions were carried out with electric shock treatments. Fusion was conducted as described in Procedures, section IIb), with the following modifications:

- 1) Washed protoplasts were mixed in 0.1:0.1 ml aliquots. A separate mixture was prepared for each voltage dose used.
- 2) 2.0 ml of 40% PEG-MW 6000/10 mM Tris-HCl pH 6.2 (unadjusted) was added to each mixture.
- 3) Electric shock was applied for treated samples using the apparatus depicted in Fig A-D.
- 4) Following shock treatment, samples were incubated with PEG at 30°C for 30 min.
- 5) The samples were then centrifuged 5 min at 2000 rpm and resuspended in 0.15 ml 0.6 M KCl. This was mixed in 5.0 ml KCl and the appropriate dilutions were made for plating.

FIG A-D: APPARATUS USED FOR ELECTRIC SHOCK
TREATMENT DURING PROTOPLAST FUSION

The circuit shown in Fig A is used to generate a high voltage, short duration pulse between the electrodes of a sample cell.

FIG A

The spark gap is adjustable to control the peak value of this pulse. The pulse shape is shown below at 6.2 kilovolts (KV).

Input is provided by a DEL H.V. supply with a variable output from 0 to 10 kilovolts. This is fed into the circuit in Fig A and the charging of the capacitors begins. As the supply voltage is increased to approximately 7 KV the gap discharges as the threshold is exceeded. The pulse is applied to the solution in the cell and the power supply turned off to avoid a repeated pulse after the next charging cycle.

The current limit was set up to provide just enough current to operate the charging circuit as the operator turns up the supply. Also a safety interlock was provided to remove power to the high voltage supply whenever the sample chamber is open.

The cell and spark gap chamber is presented in Fig B (top view) and Fig C (front view).

CELL AND SPARK GAP

FIG B: TOP VIEW

FIG C: FRONT VIEW

The cell itself is made from teflon with an inner size of 1 cm by 1 cm by 1.5 cm deep, to accommodate 1 cc of solution (Fig D). Two pieces of platinum foil were used as electrodes going to the full depth of the cell on either side. This is mounted on two banana plugs to facilitate easy removal.

FIG D

Bead Encapsulation: Cells were prepared and protoplasts formed as outlined in Procedures, section IIb). Duplicate tubes were set up for fusion, containing 0.1 ml of each washed protoplast suspension. The mixture was left 15 min at 21°C. 2.0 ml 20% PEG-MW 4000/10 mM CaCl_2 /10 mM Tris-HCl pH 8.0 was slowly added, mixed and left 20 min at 21°C. The PEG suspensions were centrifuged and drained. One pellet was brought through the routine agar overlay method described in Procedures, section IIb), except that the initial dilution was made in 5.0 ml KCl rather than 10 ml.

The other pellet was resuspended in 0.15 ml KCl. This was mixed with 2 ml sodium alginate at 21°C. 70-80 Ca-alginate beads were formed by extruding the alginate-cell suspension through a 0.5 ml capacity Eppendorf pipette tip into a gently stirring 50 mM CaCl_2 /0.6 M KCl solution. The beads were removed from the CaCl_2 solution within 5-8 min after the first bead was formed and divided equally between two 50 ml Erlenmeyer flasks. Both flasks contained 5.0 ml SD broth/0.6 M KCl/10 mM CaCl_2 . The "regeneration flask" was supplemented with all the nutritional requirements for regeneration. The "selection flask" contained no supplements for Schw. alluvius fusions and adenine only for S. cerevisiae fusions. Beads were incubated at 30°C for 4 days, without shaking. These were washed twice with 5.0 ml KCl by gently swirling in the flask and decanting the liquid. 3.0 ml 0.1 M citrate buffer pH 6.0 was added to each flask and the beads were dissolved in 10 min at 30°C, shaking at 75 rpm.

The dissolved mixtures were then diluted in water and plated directly on SD and all the nutritional requirements for regeneration. Fusion products were recovered on the appropriate selection media. Plates were incubated at 30°C for 5-7 days.

Intergeneric Protoplast Fusions: For intergeneric fusions, Saccharomyces cerevisiae AH22 a his⁻leu⁻ and Schwanniomyces alluvius LT2 lys⁻trp⁻ were used. 24 hr cultures were grown in 20 ml YPD at 30°C. These were diluted to 10^7 cells/ml in 100 ml YPD and regrown 3 hr at 30°C. Cells were washed once in water. S. cerevisiae AH22 was resuspended in 100 ml water and plated on SD + his + leu to obtain an initial cell count. The remaining culture was UV-irradiated with a dose of 10 joules/M². Percentage survival was determined by plating on SD + his + leu. Schw. alluvius LT2 was resuspended in 20 ml water, sonicated and gamma-irradiated with a dose of 360 krads. An initial cell count prior to irradiation and percentage survival following irradiation were determined by plating on SD + lys + trp. The irradiated cultures were centrifuged and washed once in 1.2 M sorbitol. These were resuspended in 10 ml sorbitol. Protoplasts were formed at 30°C, with 80 ug/ml zymolyase for 25 min for AH22 and 40 ug/ml zymolyase for 15 min for SA-LT2. Protoplasts were washed, mixed and treated with PEG as described for intraspecific fusions in Procedures, section IIb). Regenerated colonies were recovered in an agar overlay

of RA + his/leu/lys/trp. For fusion products and controls, samples were plated in an agar overlay of RA + his. Intact cells were recovered by plating directly on SD + his/leu/lys/trp. Plates were incubated at 30°C for 2 weeks.

III Sporulation

Fusion products from Schw. alluvius intraspecific fusion experiments were sporulated using a modification of the method described by Wilson, et al., 1982. Fusion products were streaked on presporulation media and parent strains were streaked on presporulation media plus the required amino acids (40 ug/ml). These plates were incubated at 30°C for 2 days. Cells were then transferred to sporulation media plus the required amino acids (40 ug/ml) and incubated at 30°C. Samples were checked for spores at three day intervals. Cells were resuspended in water and mixed with an equal volume of 50% Ficoll for 25% Ficoll final concentration. Mixtures were observed on a Reichert phase microscope using a Fluor Oel, Ph100/1.3 objective lens. Photographs were taken with Kodak Plus-X pan film and developed with HC110 developer.

IV Nuclear Staining

Parental strains and fusion products from Schw. alluvius intraspecific experiments were stained to examine the nuclei. Cells were grown in 2.0 ml YPD at 30°C, shaking at 150 rpm, to $1-4 \times 10^7$ cells/ml. Cells were sonicated and 0.2 ml aliquots were added to an equal volume of 50% aqueous ethanol. These were mixed and left at 21°C for 15 min. 0.4 ml acridine orange (1 ug/ml) in 50% ethanol was added, mixed and left at 21°C for 10 min. The mixtures were centrifuged 1 min at 2000 rpm and the supernatants decanted. The pellets were resuspended in the remaining liquid. An equal volume of DAPI (0.1 ug/ml) in water was added and mixed. Cells were observed on a Reichert Zetopan microscope with an Epiilluminator using a Fluor Oel 100/1.3 objective lens. Photographs were taken with Kodak Tri-X pan film and developed with Microdol developer.

RESULTS

A number of different strains of Saccharomyces cerevisiae and Schwanniomyces alluvius were used in the present study. The S. cerevisiae strains were obtained from other investigators whereas Schw. alluvius strains were isolated in the present study. The details of the genotypes and the sources of strains are presented in Table 1.

TABLE 1: YEAST STRAINS USED IN THIS STUDY

<u>Strain Designation</u>	<u>Mating Type</u>	<u>Genotype</u>	<u>Source</u>
<u>Saccharomyces cerevisiae</u>			
<u>XY207-5C</u>	<u>α</u>	<u>ade</u> ₂ <u>leu</u> ₁ <u>his</u> ₅	Dr. A.P. James
<u>C540-5B</u>	<u>α</u>	<u>ade</u> ₁ <u>trp</u> ₂ <u>arg</u> ₆	Dr. A.P. James
<u>AH22</u>	<u>a</u>	<u>his</u> ₄₋₅₁₉ <u>leu</u> _{2-3,2-112}	Dr. G.R. Fink
<u>Schwanniomyces alluvius</u>			
<u>SA-WT</u>		wild-type	ATCC # <u>26074</u>

I Mutagenesis

Since mutants of Schw. alluvius (SA-WT) were not available, it was necessary to isolate strains with suitable markers for subsequent protoplast fusion experiments. Little work has been

done thus far regarding mutation induction in this yeast. Therefore, a number of mutagenic agents were tested to compare relative mutation frequencies. Two chemical mutagens, EMS and MNNG, as well as ultraviolet (UV) irradiation were tested. Dose-effect curves for each of the three mutagenic treatments are shown in Fig 1-3. The survival curves show that Schw. alluvius is quite similar to S. cerevisiae in its sensitivity to killing by the two chemical mutagens and UV. For instance, following a UV dose of 60 joules/M² approximately 30% survival is obtained in the two yeasts.

A treatment time giving 5 - 15% survival levels was used for large scale mutagenesis experiments (Table 2). EMS was used to isolate single auxotrophs of SA-WT. UV and MNNG were subsequently employed to recover a variety of double auxotrophic strains. Among the amino acid requirements histidine, lysine and threonine mutations appeared most frequently. Temperature-sensitive strains as well as those failing to grow on soluble starch were also picked. The present data show that UV gives a mutation frequency 5 - 10x lower than either of the chemical mutagens at comparable survival levels.

In some of these early experiments, the technique for enrichment using nystatin was also tested using different concentrations of the drug. Equal concentrations of wild-type and pink adenine mutant lines were incubated overnight in various minimal media containing 20 - 200 ug/ml nystatin. Cells were placed on YPD to check if nystatin was indeed killing only growing cells (wild-type) and thereby enriching

FIG 1 AND 2: DOSE-EFFECT CURVES FOR Schwanniomyces alluvius
WILD-TYPE (SA-WT) FOLLOWING TREATMENTS WITH
EMS AND MNNG

FIG 3: DOSE-EFFECT CURVE FOR SA-WT
EXPOSED TO ULTRAVIOLET IRRADIATION

for non-growing mutant lines. No enrichment was observed. Both the mutant and the wild-type were sensitive to 20 ug/ml nystatin.

TABLE 2: ISOLATION OF VARIOUS MUTANTS FROM Schwanniomyces alluvius WILD-TYPE AND SINGLE AUXOTROPHS

<u>Mutagenic Agent</u>	<u>Dose Used</u>	<u>% Survival</u>	<u>Mutation Frequency</u>	<u>Mutant Characterization</u>
*EMS	1.5% 90 min	10%	1.5×10^{-3}	<u>his</u> ⁻ (3) <u>lys</u> ⁻ (1)
UV	80 joules/M ²	15%	2.6×10^{-4}	<u>his</u> ⁻ <u>lys</u> ⁻ (1)
*MNNG	50 ug/ml 120 min	5%	2.3×10^{-3}	<u>his</u> ⁻ <u>lys</u> ⁻ (15) <u>his</u> ⁻ <u>thr</u> ⁻ (12) <u>his</u> ⁻ <u>trp</u> ⁻ (10) <u>his</u> ⁻ <u>asp</u> ⁻ (3) <u>his</u> ⁻ <u>met</u> ⁻ (2) <u>his</u> ⁻ <u>arg</u> ⁻ (2) <u>his</u> ⁻ <u>ile</u> ⁻ (1) <u>his</u> ⁻ <u>ts</u> (33) <u>his</u> ⁻ <u>ss</u> (14) <u>lys</u> ⁻ <u>trp</u> ⁻ (4)

*Cells were mutagenized in 0.05 M phosphate pH 7.1.
Parentheses indicate the number of mutants isolated.
ts temperature-sensitive mutants selected at 36°C.

II Protoplast Fusion

a) Optimal Conditions for Protoplast Formation and Regeneration

Mutant strains to be used in fusion experiments were examined for protoplast formation and regeneration frequencies. In all cases, zymolyase 60,000 was the enzyme chosen for protoplast isolation. The initial choice was glusalase but it was found that no consistency existed between different commercial preparations. This made it necessary to determine optimal conditions for protoplast formation and regeneration for each bottle of glusalase opened. Zymolyase, though considerably more expensive, was highly reproducible and effective at very low concentrations. Regeneration media including various osmotic stabilizers and agar or agarose concentrations were also tested. The best results were obtained with SD + 3% agar + 0.6 M KCl.

The data for Saccharomyces cerevisiae strains are presented in Table 3. The same treatment conditions proved optimal for C540-5B and XY207-5C, namely, 40 ug/ml enzyme for 10 min. Both strains demonstrated similar trends in protoplast formation and regeneration. Within 10 min incubation with the enzyme, 98 - 100% protoplasts were obtained. Prolonged exposure gave a decrease in regeneration frequency to one third that attained at the optimum over an additional 10 min period. AH22 behaved quite differently. The appropriate conditions for protoplast formation and regeneration required 80 ug/ml zymolyase for 15 - 25 min. This was twice the enzyme concentration optimal for C540-5B

and XY207-5C and a 1.5 - 2.5x longer incubation period. 99% protoplasts were obtained within 15 min incubation. Regeneration was maintained at a constant 30 - 35% up to 10 min after protoplast formation appeared to be complete. Since prolonged incubation of AH22 in the enzyme did not affect the regeneration ability of the protoplasts, the longer incubation time was chosen for protoplast formation in later fusion experiments.

TABLE 3: PROTOPLAST FORMATION AND REGENERATION FOR Saccharomyces cerevisiae STRAINS USED IN INTRASPECIFIC AND INTERGENERIC FUSION

<u>Strain Used</u>	<u>Zymolyase</u> (ug/ml)	<u>Time</u> (min)	<u>%</u> <u>Protoplasts</u>	<u>%</u> <u>Regeneration</u>
<u>XY207-5C</u>	40	0	----	----
		5	83.6	15.2
		*10	99.9	8.5
		15	99.9	6.5
		20	99.9	3.1
<u>C540-5B</u>	40	0	----	----
		5	72.1	12.8
		*10	97.5	6.0
		15	99.0	4.2
		20	99.7	2.0
<u>AH22</u>	80	0	----	----
		5	89.2	28.7
		10	96.9	27.3
		15	99.1	32.9
		20	99.7	32.7
		*25	99.7	34.9

*Treatment time used for protoplast formation during fusion experiments.

Logarithmic phase cells were used in all experiments.

Data presented are the averages of three separate experiments.

Protoplast formation and regeneration frequencies for Schwanniomyces alluvius strains were also determined for use in later fusion experiments. Each strain had its own optimal conditions for protoplast isolation (Table 4). SA-LT2 required 40 ug/ml zymolyase for 15 min. SA-HT15 required 20 ug/ml zymolyase for 30 min. The general trends apparent for S. cerevisiae strains XY207-5C and C540-5B also applied to the Schw. alluvius lines. Regeneration ability dropped significantly as the incubation period was extended past the optimum.

TABLE 4: PROTOPLAST FORMATION AND REGENERATION FOR Schwanniomyces alluvius STRAINS USED IN INTRASPECIFIC AND INTERGENERIC FUSION

<u>Strain Used</u>	<u>Zymolyase</u> (ug/ml)	<u>Time</u> (min)	<u>%</u> <u>Protoplasts</u>	<u>%</u> <u>Regeneration</u>
<u>SA-LT2</u>	40	0	----	----
		10	85.0	13.0
		*15	98.0	8.1
		20	99.0	5.0
		25	99.3	3.4
		30	99.7	1.3
<u>SA-HT15</u>	20	0	----	----
		15	71.3	32.6
		20	90.9	27.4
		25	96.3	14.2
		*30	98.8	8.9

*Treatment time used for protoplast formation during fusion experiments.

Logarithmic phase cells were used in all experiments.

Data presented are the averages of three separate experiments.

b) Intraspecific Protoplast Fusions

Having established the proper conditions for protoplast isolation and regeneration of the appropriate mutant lines, it was now possible to set up a number of fusion experiments. First, routine procedures were used to obtain intraspecific fusion frequencies for S. cerevisiae and Schw. alluvius. Two concentrations of polyethylene glycol (PEG) were examined (Table 5): 20% PEG-MW 4000 and 40% PEG-MW 6000. Control experiments, in which cells were brought through the regular fusion procedure using 20% PEG-MW 4000, gave a frequency of less than 10^{-8} for both yeasts. Protoplasts fused under similar conditions resulted in a hybrid recovery of 10^{-3} for S. cerevisiae and Schw. alluvius. Regeneration of the final plating mixture was 5 - 10% for S. cerevisiae and 0.2 - 0.5% for Schw. alluvius. These products, upon sub-culturing, continued to grow on selection media. The use of 40% PEG-MW 6000 resulted in as much as a 100 - 500x decrease in fusion frequency for both yeasts. Regeneration frequency dropped to one half that obtained with 20% PEG-MW 4000. This marked difference between 20% and 40% PEG clearly demonstrates the significance of experimental conditions in determining the fusion frequency and points to the possibility of obtaining relatively higher hybrid yields by varying the protocols.

TABLE 5: INTRASPECIFIC FUSION FREQUENCIES FOR
Saccharomyces cerevisiae and Schwanniomyces
alluvius USING TWO DIFFERENT PEG CONCENTRATIONS

		<u>Fusion Frequency</u>	
<u>Parental Strains</u>	<u>Experiment</u>	<u>20% PEG</u> <u>MW 4000</u>	<u>40% PEG</u> <u>MW 6000</u>
<u>Saccharomyces</u>			
<u>cerevisiae</u>			
<u>XY207-5C</u>	Cells		
(<u>α</u> <u>ade</u> ⁻ <u>his</u> ⁻ <u>leu</u> ⁻)	Fused	$< 7.4 \times 10^{-7}$	-----
x			
<u>C540-5B</u>	Protoplasts		
(<u>α</u> <u>ade</u> ⁻ <u>trp</u> ⁻ <u>arg</u> ⁻)	Fused	1.0×10^{-3}	2.0×10^{-7}
<u>Schwanniomyces</u>			
<u>alluvius</u>			
<u>SA-LT2</u>	Cells		
(<u>lys</u> ⁻ <u>trp</u> ⁻)	Fused	$< 1.2 \times 10^{-8}$	-----
x			
<u>SA-HT15</u>	Protoplasts		
(<u>his</u> ⁻ <u>thr</u> ⁻)	Fused	9.0×10^{-4}	$< 8.3 \times 10^{-6}$

Data presented are the averages of three separate experiments.

Sporulation: To confirm that the colonies picked from
Schwanniomyces alluvius intraspecific fusions were indeed
 hybrids, fusates and parental strains were both checked for
 sporulation (Fig 7-9). The parent SA-LT2 failed to sporulate.
SA-HT15 did sporulate, though infrequently. However, upon

FIG 4 - 6: NUCLEAR STAINING OF Schwanniomyces alluvius
PARENTS AND HYBRIDS FROM INTRASPECIFIC FUSIONS
USING DAPI AND ACRIDINE ORANGE

FIG 4: Nuclear staining of logarithmic phase cells from
SA-HT15. A. Interphase nuclei. B, C. Nuclei migrating
into bud from mother cell. D. Nuclear migration almost
complete as bud begins to separate from mother cell.

FIG 5: Nuclear staining of logarithmic phase cells from
SA-LT2. A. Nucleus migrating into bud from mother
cell. B. Nucleus just beginning to migrate into
bud. C. Interphase nuclei.

FIG 6. Nuclear staining of logarithmic phase cells from Schw. alluvius hybrids. A. Interphase nuclei and a single cell where nuclear migration is just beginning. B. Interphase nuclei. C, D, E. Successive stages of migration. F. Migration of nucleus is nearly complete.

FIG 7 - 9: Schwannomyces alluvius PARENTS AND
HYBRIDS FROM INTRASPECIFIC FUSIONS
AFTER INCUBATION ON SPORULATION MEDIA

FIG 7. SA-HT15 after six days on sporulation media.

FIG 8. SA-LT2 after six days on sporulation media.
A, B. Vegetative cells grown in YEPD.
C, D. Cells from culture on sporulation media
appear sickly or dead.

FIG 9: Schw. alluvius intraspecific fusion hybrid following six days on sporulation media. ' A. Single ascus with dead bud attached. Equatorial ridge is evident; surface appears mottled. B, C, D. Other cell forms found in a sporulated culture.

testing for auxotrophic requirements it still failed to grow on minimal media and retained the nutritional markers originally selected. Sixteen fusion products were tested and all sporulated within six days on the appropriate media. Under these conditions, the time that it took cultures to sporulate was considerably shorter compared to that reported earlier (Wilson, et al., 1982). Since very little genetics has been done with Schw. alluvius, the ability to achieve maximal sporulation within short incubation times would prove quite useful for future studies.

Asci were ovoid with a point at one end. All asci observed contained a single spore. The surface of the ascus appeared mottled and a ridge was sometimes apparent to one side of the spore.

Nuclear Staining: Nuclear staining was also conducted using the fluorescent stain DAPI and the counter-stain acridine orange. All strains, sixteen fusates and both parents, appeared to be uninucleate (Fig 4-6). Stages of cell division could be seen as the nucleus migrated from the mother cell to the bud and eventually divided into two distinct nuclei.

Fusates also grew faster than the parental strains from which they were derived (data not presented).

c) Enhancement of Protoplast Fusion Frequency

Electric Shock: Electric shock was applied to the protoplast-PEG mixture and the remainder of the procedure was carried out as for previous experiments. Table 6 summarizes the effect of electric shock on an S. cerevisiae intraspecific fusion system using 20% PEG-MW 4000 and 40% PEG-MW 6000. No enhancement effect was noted with 20% PEG-MW 4000 and electric shock. Up to 260x increases were achieved with 40% PEG-MW 6000 followed by electric shock treatment (1 joule). A lower dose of 0.25 joules gave only a 6.5x increase while higher doses of 2 and 3 joules gave 130 and 180x increases, respectively. However, even with a 260x enhancement effect, protoplast fusion frequency approached 10^{-4} . This was no higher than that already achieved with 20% PEG-MW 4000 alone. So, it seems reasonable to conclude that an effect similar to the one reported earlier for electric shock (Weber, et al., 1981a) can be obtained by using a lower PEG concentration.

TABLE 6: INTRASPECIFIC FUSION FREQUENCIES FOR Saccharomyces cerevisiae USING TWO DIFFERENT PEG CONCENTRATIONS WITH AND WITHOUT ELECTRIC SHOCK

⁺ Energy (joules)	<u>Fusion Frequency</u>		<u>*Enhancement</u>	
	<u>20% PEG</u>	<u>40% PEG</u>	<u>20% PEG</u>	<u>40% PEG</u>
	<u>MW 4000</u>	<u>MW 6000</u>	<u>MW 4000</u>	<u>MW 6000</u>
0	9.0×10^{-4}	3.1×10^{-7}	---	---
0.25	9.8×10^{-4}	2.0×10^{-6}	1.1	6.5
1.0	6.3×10^{-4}	8.1×10^{-5}	0.7	260
^a 2.0	-----	4.2×10^{-5}	---	130
^b 3.0	1.7×10^{-4}	5.7×10^{-5}	0.2	180

*Enhancement = $\frac{\text{Fusion frequency of shocked protoplasts}}{\text{Fusion frequency of control protoplasts (0 joules)}}$

^aTwo 6.4 KV shocks were discharged across the spark gap.

^bThree 6.4 KV shocks were discharged across the spark gap.

⁺See appendix for derivation of energy from voltage applied.

Data presented are the averages of three separate experiments.

A Schw. alluvius intraspecific fusion system was also tested for similar responses. Electric shock treatment had virtually no enhancement effect with either PEG concentration. Only a 2x increase in fusion frequency was found over a dose range of 0 - 3 joules. The efficiency of such treatments thus clearly seems to depend upon the yeast used and is even likely to be strain dependent.

Bead Encapsulation: The second parameter investigated for an enhancement effect on intraspecific fusion frequency was "Bead Encapsulation". The bead procedure resulted in a

substantial improvement of hybrid yield in an S. cerevisiae fusion (Table 7). Beads incubated in regeneration media (supplemented with all parental requirements) gave 40x more hybrid colonies compared to the conventional agar overlay method. Beads incubated in selection media (adenine supplement only) gave a 1000x increase in the recovery of fusion products. Time-course experiments where beads were dissolved at 24 hr intervals over a 5 day incubation period demonstrated that cells did divide within the bead matrix. Division did not occur until 48 hours after incubation. Most likely, this is indicative of the time required for protoplasts to regenerate and revert to the normal cell type.

TABLE 7: COMPARISON OF Saccharomyces cerevisiae INTRASPECIFIC FUSION FREQUENCIES USING THE AGAR OVERLAY AND ALGINATE BEAD ENCAPSULATION METHODS

<u>Methods Used</u>	<u>Incubation Media</u>	<u>Fusion Frequency</u>	<u>*Enhancement</u>
Agar Overlay	----	4.4×10^{-4}	----
Alginate Bead	Regeneration	1.7×10^{-2}	38.6
Encapsulation	Selection	4.4×10^{-1}	1000

$$*Enhancement = \frac{\text{Fusion frequency obtained with beads}}{\text{Fusion frequency obtained with agar overlay}}$$

Data presented are the averages of three separate experiments.

Preliminary experiments with bead entrapped fusion mixtures of Schw. alluvius were also carried out. Viable

colonies were recovered upon plating the dissolved bead mixture on fully supplemented SD media. However, no fusion products were obtained on selection media (SD).

Intergeneric Protoplast Fusions: The third approach used to increase fusion frequency and possibly achieve some degree of genetic stability was tested in an intergeneric fusion between Saccharomyces cerevisiae and Schwanniomyces alluvius. In some of the earlier experiments fusions were attempted without any prior treatment. As a result of these different experiments only one fusate was obtained which was tested further by repeated sub-culturing (Table 8). However, this turned out to be unstable, failing to grow on selection media after four transfers.

Additional experiments were conducted to determine the effect of irradiation pretreatment of protoplasts followed by fusion (Table 8). SA-LT2 was exposed to a heavy dose of gamma irradiation to break the DNA into small fragments. AH22 was given a light dose of UV irradiation to induce repair and recombination enzymes to facilitate integration of newly introduced Schw. alluvius DNA fragments into the S. cerevisiae genome. Pretreatment did produce a 10x increase in fusion frequency compared to that of the control, from 5.2×10^{-8} to 5.1×10^{-7} . However, even the frequency obtained with the modified procedure was quite low. Furthermore, after a two week incubation period, hybrids were recovered as

microcolonies only. This made isolation and sub-culturing of fusion products very difficult.

TABLE 8: INTERGENERIC FUSION FREQUENCIES FOR Schwanniomyces alluvius LT2 (lys⁻trp⁻) AND Saccharomyces cerevisiae AH22 (a his⁻leu⁻)

<u>Experiment</u>	<u>Fusion Frequency</u>	<u>*Enhancement</u>
Conventional protoplast fusion.	5.2×10^{-8}	----
Heavy gamma irradiation of SA-LT2 protoplasts and light UV irradiation of AH22 protoplasts followed by fusion.	5.1×10^{-7}	10

$$\text{*Enhancement} = \frac{\text{Fusion frequency obtained with treatment}}{\text{Fusion frequency obtained with control}}$$

Data presented are the averages from three separate experiments.

DISCUSSION/CONCLUSIONS

Protoplast fusion as a technique for gene transfer in yeast has become increasingly popular. It is of particular value where the microorganism studied lacks a defined mating type or its ploidy and genetic characteristics are so complex that conventional methodology is not suitable. The applications of protoplast fusion in fungal genetics are not only of academic interest but also of practical importance, particularly in the field of industrial strain improvement. As discussed, great success has been achieved with intraspecific and to a lesser extent, with interspecific fusions. However, intergeneric studies thus far have been discouraging. Fusion can be achieved but the frequency is low and the products are highly unstable.

When working with a protoplast fusion system, there are a number of factors to consider. The first should be the development of a strong selection scheme for the recovery of hybrid products. This is usually based upon a nutritional complementation derived from suitably marked auxotrophic parents (Sommer and Lewis, 1971). Selection of hybrid products on minimal media is convenient. This selection is particularly strong where parents have multiple markers, making the likelihood of spontaneous reversion very small (10^{-12} for double auxotrophs). Mitochondrial mutations for antibiotic resistance (Spencer and Spencer, 1980) and respiration deficiencies (Packer, et al., 1973) can be readily induced in most industrial yeast strains. Where temperature-sensitive and growth mutants are desired,

selection conditions must be absolute. There should be no background growth for either parent to ensure that colonies picked from fusions are truly hybrid products. The isolation of his⁻SS⁻ mutants of Schw. alluvius (Table 2) required the testing of a number of soluble starch media before a "clean" selection system was obtained. Higher concentrations of yeast nitrogen base (0.67%) and the use of 2% agar rather than 0.5% agarose resulted in the formation of microcolonies by XY207-5C, a strain known to be soluble starch negative (van der Walt, 1970).

There are a number of significant factors involved in the optimization of the fusion itself. Protoplast formation and regeneration are indeed strain specific as noted in Table 3 and 4. The choice of lytic enzyme is critical. One looks for reproducibility and effectiveness. With the vast number of commercial preparations (Hamlyn, et al., 1981; Stephen and Nasim, 1981), the choice is virtually unlimited. Most marketed products are usually a composite of cellulases, proteases, xylanases, sulfatases and glucanohydrolases. This makes it somewhat easier in that:

- 1) the addition of a number of isolated components is no longer necessary and,
- 2) thiol pretreatment can often be neglected.

However, the action of commercial preparations can be quite severe. Time must be taken to ascertain the enzyme concentration and incubation period necessary to obtain protoplasts that will fuse and regenerate. If enzyme treatment is too short, wall fragments may impede the fusion

process. If enzyme treatment is too long, the protoplasts may fuse but not regenerate. Protoplasts demonstrate lower levels of DNA synthesis compared to normal cells. However, RNA and protein synthesis in protoplasts are comparable to that of normal cells (Doi and Doi, 1979). Such functions are very important for the reformation of the cell wall (Kopecká and Farkas, 1979). Excessive stripping by the enzyme may destroy protoplast integrity.

For intraspecific Schw. alluvius fusions, treatment times of 10 min for SA-LT2 and 25 min for SA-HT15 were initially selected to form protoplasts (Table 4). The idea was to achieve a higher regeneration frequency. However, no fusates were recovered in three separate experiments. When the enzyme treatment was extended another 5 min for each parent, a fusion frequency of about 10^{-3} was reproducible (Table 5). Regeneration of the fusion mixture, however, was considerably lower than that expected from the results in Table 4. Under the "protoplast conditions" used, individual strains regenerated with a frequency of about 10%. Subsequent to fusion, this frequency dropped by a factor of 20 - 50, to 0.2 - 0.5%. No such effect was observed with S. cerevisiae, where regeneration of individual strains was comparable to that of the final fusion mixture. The poor regeneration for Schw. alluvius fusion mixtures may be a result of extensive aggregation and/or increased sensitivity to the manipulations carried out in the procedure. Regeneration of the final fusion mixture could be enhanced by shorter enzyme incubations but no fusates were recovered in these

experiments, as previously discussed. Thus, the significance of protoplast formation and regeneration conditions in making the fusion successful should not be underestimated.

Table 5 clearly illustrates the effect of PEG concentration on fusion frequency. There is a 100 - 500x decrease in hybrid recovery with 40% PEG-MW 6000 compared to 20% PEG-MW 4000. Schwanniomyces alluvius fusion was first reported using 40% PEG (Wilson, et al., 1982). Fusates were recovered at a frequency of 1.5×10^{-6} . Using 20% PEG, intraspecific hybrids were obtained at a frequency of 10^{-3} , some 1000x higher (Table 5). Regeneration in both experiments was 0.2%. The fusates recovered in this study grew repeatedly in minimal media, grew faster than either parent, sporulated readily (Fig 9) and were uninucleate (Fig 6).

The great drop in fusion frequency for 40% PEG may be attributed to two major factors (Peberdy, 1979). First, PEG is toxic at excessive levels. Second, PEG concentrations higher than 30% make the media hypertonic. This causes protoplasts to shrink and as a result, leads to less surface contact. Mechanisms of fusion centre on membrane-membrane interaction (Ahkong, et al., 1975; Papahajopoulos, 1974; 1976; Maggio, et al., 1976; Ingolia and Koshland, 1978; Knutton, 1979; Knutton and Pasternak, 1979). Therefore, a decrease in surface contact between protoplasts would certainly account for a decrease in fusion frequency.

Other factors, such as pH and Ca^{++} concentration do play a major role in achieving optimal protoplast fusion conditions. However, these criteria are standardized. A 10 mM CaCl_2 concentration is used most widely and a pH range from 6 to 9 seems conducive to the fusion process (Anne and Peberdy, 1976; van Solingen and van der Platt, 1977; Peberdy, 1979).

Fusion frequency (see appendix) is determined as follows:

$$\frac{\# \text{ colonies with fused phenotype (A} \cdot \text{B)}}{\# \text{ colonies with fused phenotype (A} \cdot \text{B)} + \# \text{ colonies with unfused phenotype (A} + \text{B)}}$$

where A is one parent
 B is the other parent
 A·B is the hybrid.

There are inherent difficulties with the practical applications of this formula. Since PEG induces protoplast aggregation, a given regenerated colony of either the fused or unfused phenotype may most likely be initiated from a protoplast "clump". The visible colony may even be a conglomeration of a number of colonies that have arisen from different regenerated cells within a single aggregate.

The numerical values regarding fusion frequency may, therefore, have questionable value. However, this stands as the only way to express hybrid yield thus far. Though not entirely justified, there is no way at this time of circumventing the problem. Aggregation is a requirement for fusion and so, can not be eliminated until regeneration

and reversion to the normal cell type has occurred. Disruption of aggregates at this point would ensure an accurate fusion frequency by the above formula. However, the length of time that it takes a protoplast to regenerate and form a viable cell is not presently known. Disruption prior to this point would result in loss of the potential fusion products due to the fragile nature of the protoplasts themselves.

Enhancement of fusion frequency and/or improvements in genetic stability of the hybrid products recovered are two obvious goals for protoplast fusion. This applies particularly to intergeneric studies where fusion products thus far have appeared at very low frequencies (10^{-6} or less) and have inevitably segregated into one of the original parental phenotypes. The protoplast fusion technique may be strengthened by:

- 1) attempting to improve the physiological/physical aspects involved in fusion to further stimulate protoplast-protoplast interaction and so increase hybrid yield and,
- 2) altering the genetic constitution of the organisms involved to facilitate genome-genome tolerance and so improve on stability of the fused product.

Electric shock treatment did enhance fusion frequency in *S. cerevisiae* intraspecific system, but only at excessive levels of PEG (40%) (Table 6). It is thought to affect the physiological state of the membrane, making it more fluid and labile, thus permitting protoplasts to interact more readily (Weber and Spata, 1981a). Fusion frequencies comparable to that obtained with the electric shock enhancement effect were achieved by simply using

a lower PEG concentration (20%). This made the electric shock approach impractical for the system examined in this study. In Schw. alluvius, no enhancement was found with electric shock treatment at either PEG concentration. These effects are obviously unique to each system. Electric shock may be helpful where untreated fusions give low frequencies for hybrid recovery.

Table 7 clearly shows an increase in the number of hybrid products recovered in an S. cerevisiae intraspecific fusion using "Bead Encapsulation" rather than the routine agar overlay. Here, the modification is still at the level of protoplast-protoplast interaction. However, it concerns the physical microenvironment of the protoplasts as opposed to the physiological status of the protoplast membrane. 40x and 1000x increases were found in regeneration and selection conditions, respectively. Due to the viscous nature of the inner bead matrix, protoplasts are "free-floating" within a restricted volume. Thus, the opportunity for interaction is stimulated by the increase in protoplast density and by protoplast mobility. Polymerization throughout the entire bead was achieved simply by extending the time of freshly made beads in CaCl_2 solution. Solid beads produced viable cells on fully supplemented media but no fusates were recovered. This strongly suggests that the viscosity of the bead matrix is of primary importance in obtaining fusates. A suitable alginate concentration and degree of polymerization must be empirically determined

for each system used. This may account for the failure to obtain hybrids for Schw. alluvius fusions via "Bead Encapsulation".

A 25x increase in fusion frequency was obtained with selection conditions (where only fusates can grow) over that with regeneration conditions (where both fusates and parents can grow). Once protoplasts had fused and/or reverted back to their normal cell form, they did divide within the bead (data not presented). Cell division was evident no sooner than 48 hr after bead encapsulation. Selection conditions favour the recovery of hybrid products. Since the number of unfused parental cells should remain the same, hybrid fusion frequency calculated from dissolved bead suspensions would be higher. However, for beads incubated in regeneration media, both unfused parentals and hybrid products can divide. Hybrids may be faster-growing than unfused cells but this is insignificant compared to the imbalance of growth rates in selection conditions. Since the number of unfused cells recovered from dissolved bead suspensions increases, by the formula presented earlier, fusion frequency decreases. The enhancement effect for beads in regeneration media is a more accurate assessment of the "Bead Encapsulation" procedure compared to the agar overlay. Beads incubated under selection conditions may be useful when one is interested in recovering a large number of hybrid products from interspecific and intergeneric fusions. Many of the

colonies recovered will be the division products of a single mother cell. Where genetic equilibrium is a step for fusion recovery, these products may very well be representative of the intermediates along the way to the final stable product.

The third approach used to enhance fusion frequency and genetic stability was tested in an intergeneric system between S. cerevisiae AH22 and Schw. alluvius LT2.

Modification occurs then at the genetic level. Heavy gamma irradiation (0.1% survival) of SA-LT2 was used to disrupt the genome extensively so as to produce fragments of DNA. Simultaneously, AH22 was exposed to light UV irradiation (40% survival) to induce recombination and repair enzymes in an effort to stimulate recombinational events. Fusion was carried out as usual. The purpose was to make the gene transfer a specific and unidirectional event. Since SA-LT2 was pretreated to very low survival levels, the genes from this strain could only be expressed as a result of integration into the S. cerevisiae genome. UV induction of S. cerevisiae would, hopefully, make the recipient cell much more receptive to recombinational events (Fig 10).

Clearly intergeneric fusion via conventional methodology will not be successful. Genetic incompatibilities between phylogenetically distant organisms are just too great to achieve complete genome tolerance through protoplast fusion. The technique, therefore, must be modified such that the demands on the cell to accommodate new information are somewhat

FIG 10: A MODIFICATION OF THE EXPERIMENTAL PROCEDURE FOR PROTOPLAST FUSION TO FACILITATE A MORE SPECIFIC GENE TRANSFER AND GENETIC STABILITY OF INTERGENERIC PRODUCTS

alleviated. Our approach does this by first reducing the amount of genetic material to be transferred. Second, it directs the transfer; third, it facilitates integration. Preliminary studies did give a 10x increase in fusion frequencies where irradiation pretreatment was included (Table 8) and thus the approach seems promising. Judging from current literature (Symposium: Abstracts of IVth International Symposium on Genetics of Industrial Microorganisms, June, 1982), it is evident that there is a great deal of interest in further exploiting the technique of protoplast fusion. Numerous novel approaches such as the use of liposomes are currently being used. With the advent of other breakthroughs in molecular biology, the cell fusion techniques can also be expected to see a great deal of refinement.

REFERENCES

1. Ahkong, Q., Fisher, D., Tampion, W. and Lucy, J. 1975. Mechanisms of cell fusion. NATURE 253: 194 - 195.
2. Allmark, B., Morgan, A. and Whittaker, P. 1978. The use of protoplast fusion in demonstrating chromosomal and mitochondrial inheritance of respiratory-deficiency in Kluyveromyces lactis, a petite-negative yeast. MOLEC GEN GENET 159: 297 - 299.
3. Anne, J. and Peberdy, J. 1976. Induced fusion of fungal protoplasts following treatment with polyethylene glycol. J GEN MICROBIOL 92: 413 - 417.
4. Birnbaum, S., Pendleton, R., Larson, P. and Mosbach, K. 1981. Covalent stabilization of alginate gel for the entrapment of living whole cells. BIOTECH LETT 3: 393 - 400.
5. de van Broock, M., Sierra, M. and de Figueroa, L. 1981. Intergeneric fusion of yeast protoplasts. In: Stewart, G. and Russell, I. (ed). Current Developments in Yeast Research (Vth International Yeast Symposium, London, Ont.). Willowdale: Pergamon Press, pp. 171 - 176.
6. Carlson, P., Smith, H. and Dearing, H. 1972. Parasexual interspecific plant hybridization. PNAS (USA) 69: 2292 - 2294.
7. Christensen, B. 1979. Somatic hybridization in Saccharomyces cerevisiae: Analysis of products of protoplast fusion. CARLSBERG RES COMMUN 44: 225 - 233.
8. Cocking, E. 1976. Fusion and somatic hybridization of higher plant protoplasts. In: Peberdy, J. (ed). Microbial and Plant Protoplasts. London: Academic Press, pp. 189 - 200.
9. Constabel, F. 1978. Development of protoplast fusion products, heterokaryocytes and hybrid cells. In: Thorpe, T. (ed). Frontiers of Plant Tissue Culture. Calgary: University of Calgary, pp. 141 - 149.
10. Doi, A. and Doi, K. 1979. Mitotic behavior of yeast spheroplasts in liquid culture. CELL STRUCTURE AND FUNCTION 4: 261 - 266.

11. Douglas, G., Keller, W. and Setterfield, G. 1981. Somatic hybridization between Nicotiana-rustica and Nicotiana-tabacum. 2. Protoplast fusion and selection and regeneration of hybrid plants. CAN J BOTANY 59: 220 - 227.
12. Ferenczy, L. 1981. Microbial protoplast fusion. In: Symposium 31. The Society for General Microbiology. Cambridge: University Press, pp. 1 - 34.
13. Ferenczy, L., Kevei, F. and Szegedi, M. 1975. Increased fusion frequency of Aspergillus nidulans protoplasts. EXPERIENTIA 31: 50 - 52.
14. Ferenczy, L., Kevei, F., Szegedi, M., Frankó, A. and Rojik, I. 1976. Factors affecting high-frequency fungal protoplast fusion. EXPERIENTIA 32: 1156 - 1158.
15. Ferenczy, L. and Maráz, A. 1977. Transfer of mitochondria by protoplast fusion in Saccharomyces cerevisiae. NATURE 268: 524 - 525.
16. Ferreira, J. and Phaff, H. 1959. Life cycle and nuclear behavior of a species of the yeast genus Schwanniomyces. J BACTERIOL 78: 352 - 361.
17. Fournier, P., Provost, A., Bourguignon, C. and Heslot, H. 1977. Recombination after protoplast fusion in the yeast Candida tropicalis. ARCH MICROBIOL 115: 143 - 149.
18. Gabor, M. and Hotchkiss, R. 1979. Parameters governing bacterial regeneration and genetic recombination after fusion of Bacillus subtilis protoplasts. J BACTERIOL 137: 1346 - 1353.
19. Gleba, Y. and Hoffman, F. 1980. "Arabidobrassica": A novel plant obtained by protoplast fusion. PLANTA 149: 112 - 117.
20. Gunge, N. and Sakaguchi, K. 1979. Fusion of mitochondria with protoplasts in Saccharomyces cerevisiae. MOLEC GEN GENET 170: 243 - 247.
21. Hamlyn, P., Bradshaw, E., Mellon, F., Santiago, M., Wilson, J. and Peberdy, J. 1981. Efficient protoplast isolation from fungi using commercial enzymes. ENZYME AND MICROBIAL TECHNOLOGY 3: 321 - 325.

22. Hockney, R. and Freeman, R. 1980. Construction of polysaccharide-degrading brewing yeasts by protoplast fusion. In: Ferenczy, L. and Farkas, G. (ed). Advances in Protoplast Research. Oxford: Permagon Press, pp. 139 - 144.
23. Hopwood, D. 1981. Genetic studies with bacterial protoplasts. ANN REV MICROBIOL 35: 237 - 272.
24. Hopwood, D. and Wright, H. 1979. Factors affecting recombinant frequency in protoplast fusions of Streptomyces coelicolor. J GEN MICROBIOL 111: 137 - 143.
25. Hopwood, D. and Wright, H. 1981. Protoplast fusion in Streptomyces: Fusions involving ultraviolet-irradiated protoplasts. J GEN MICROBIOL 126: 21 - 27.
26. Howes, C. 1981. Changes in the genome of Kluyveromyces lactis after fusion with Saccharomyces cerevisiae. PROCEEDINGS OF AUSTRALIAN BIOCHEMICAL SOCIETY 14: 89.
27. Ingolia, T. and Koshland Jr., D. 1978. The role of calcium in fusion of artificial vesicles. J BIOLOGICAL CHEMISTRY 253: 3821 - 3829.
28. Kandoian, A., DeRosa, L. and Colin, R. (ed). 1972. Reference Data for Radio Engineers. 5th Edition. USA: Howard W. Sams and Co.
29. Kierstan, M. and Bucke, C. 1977. The immobilization of microbial cells, subcellular organelles and enzymes in calcium alginate gels. BIOTECH AND BIOENG 19: 387 - 397.
30. Knutton, S. 1979. Studies on membrane fusion. III Fusion of erythrocytes with polyethylene glycol. J CELL SCIENCE 36: 61 - 72.
31. Knutton, S. and Pasternak, C. 1979. The mechanism of cell-cell fusion. TIBS Oct: 220 - 223.
32. Kopecká, M. and Farkaš, V. 1979. RNA synthesis and the formation of the cell wall. Effect of lomofungin on regenerating protoplasts of Saccharomyces cerevisiae. J GEN MICROBIOL 110: 453 - 463.
33. Kreger-van Rij, N. 1977. Electron microscopy of sporulation in Schwanniomyces alluvius. ANTONIE VAN LEEUWENHOEK 43: 55 - 64.

34. Kurtzman, C., Smiley, M. and Baker, F. 1972. Scanning electron microscopy of ascospores of Schwanniomyces. J BACTERIOL 112: 1380 - 1382.
35. Lückemann, G., Sipiczki, M. and Wolf, K. 1979. Transmission, segregation and recombination of mitochondrial genomes in zygote clones and protoplast fusion clones of yeast. MOLEC GEN GENET 177: 185 - 187.
36. Maggio, B., Ahkong, Q. and Lucy, J. 1976. Poly(Ethylene glycol), surface potential and cell fusion. BIOCHEM J 158: 647 - 650.
37. Maleszka, R., Veliky, I. and Schneider, H. 1981. Enhanced rate of ethanol production from D-xylose using recycled or immobilized cells of Pachysolen tannophilus. BIOTECH LETT 3: 415 - 420.
38. Maráz, A., Kiss, M. and Ferenczy, L. 1978. Protoplast fusion in Saccharomyces cerevisiae strains of identical and opposite mating types. FEMS MICROBIOL LETT 3: 319 - 322.
39. Moraneli, F., Yaguchi, C., Champagne, C., Veliky, I., Nasim, A. and Walker, T. 1982. Schwanniomyces: Yeasts of potential industrial importance. In: Proceedings of Fourth Bioenergy R & D Seminar, Winnipeg, Manitoba (in press).
40. Morgan, A., Hall, J., Brunner, A. and Whittaker, P. 1980. Protoplast fusion in the study of mitochondrial genetics in the petite-negative yeast, Kluyveromyces lactis. In: Ferenczy, L. and Farkas, G. (ed). Advances in Protoplast Research. Oxford: Pergamon Press, pp. 93 - 98.
41. Nagata, T. 1978. A novel cell-fusion method of protoplasts by polyvinyl alcohol. NATURWISSENSCHAFTEN 65: 263 - 264.
42. Nečas, O. 1961. Physical conditions as important factors for regeneration of naked yeast protoplasts. NATURE 192: 580 - 581.
43. Nečas, O. 1971. Cell wall synthesis in yeast protoplasts. BACTERIOLOGICAL REVIEWS 35: 149 - 170.
44. Packer, L., Williams, M. and Criddle, R. 1973. Freeze-fracture studies on mitochondria from wild-type and respiration-deficient yeasts. BIOCHIM BIOPHYS ACTA 292: 92 - 104.

45. Papahadjopoulos, D., Poste, G., Schaeffer, B. and Vail, W. 1974. Membrane fusion and molecular segregation in phospholipid vesicles. BIOCHIM BIOPHYS ACTA 352: 10 - 28.
46. Papahadjopoulos, D., Vail, W., Pangborn, W. and Poste, G. 1976. Studies on membrane fusion. III Induction of fusion in pure phospholipid membranes by calcium ions and other divalent metals. BIOCHIM BIOPHYS ACTA 448: 265 - 283.
47. Peberdy, J. 1979. Fungal protoplasts: Isolation, reversion and fusion. ANN REV MICROBIOL 33: 21 - 39.
48. Peberdy, J. 1980. Protoplast fusion--a tool for genetic manipulation and breeding in industrial microorganisms. ENZYME AND MICROBIAL TECHNOLOGY 2: 23 - 28.
49. Petes, T. 1980. Molecular genetics of yeast. ANN REV BIOCHEM 49: 845 - 876.
50. Phaff, H. 1970. Schwanniomyces Klöcker. In: Lodder, J. (ed). The Yeasts. 2nd Edition. Amsterdam: North-Holland Publishing Co., pp. 756 - 766.
51. Phaff, H., Miller, M. and Bridge Cooke, W. 1960. A new species of Schwanniomyces: Schwanniomyces alluvius. ANTONIE VAN LEEUWENHOEK 26: 182 - 188.
52. Provost, A., Bourguignon, C., Fournier, P., Ribet, A. and Heslot, H. 1978. Intergeneric hybridization in yeasts through protoplast fusion. FEMS MICROBIOL LETT 3: 309 - 312.
53. Resnick, M. and Martin, P. 1976. The repair of double-strand breaks in the nuclear DNA of Saccharomyces cerevisiae and its genetic control. MOLEC GEN GENET 143: 119 - 129.
54. Russell, I. and Stewart, G. 1979. Spheroplast fusion of brewer's yeast strains. J INST BREW 85: 95 - 98.
55. Sakaguchi, K., Ochi, K., Gunge, N. and Uchida, K. 1980. In: Sakaguchi, K. and Okanishi, M. (ed). Molecular Breeding and Genetics of Applied Microorganisms. New York: Academic Press, pp. 85 - 105.
56. Sarachek, A., Rhoads, D. and Schwarzhoff, R. 1981. Hybridization of Candida albicans through fusion of protoplasts. ARCH MICROBIOL 129: 1 - 8.

57. Sherman, F., Fink, G. and Lawrence, C. 1979. Methods in Yeast Genetics. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.
58. Sipiczki, M. and Ferenczy, L. 1977. Protoplast fusion of Schizosaccharomyces pombe auxotrophic mutants of identical mating-type. MOLEC GEN GENET 151: 77 - 81.
59. van Solingen, P. and van der Plaat, J. 1977. Fusion of yeast spheroplasts. J BACTERIOL 130: 946 - 947.
60. Sommer, A. and Lewis, M. 1971. Effect of dithiothreitol on yeast: Sphaeroplast formation and invertase-release. J GEN MICROBIOL 68: 327 - 335.
61. Spencer, J. and Spencer, D. 1980. The use of mitochondrial mutants in the isolation of hybrids involving industrial yeast strains. MOLEC GEN GENET 177: 355 - 358.
62. Spencer, J., Land, P. and Spencer, D. 1980. The use of mitochondrial mutants in the isolation of hybrids obtained by fusion of protoplasts of brewing yeasts. In: Ferenczy, L. and Farkas, G. (ed). Advances in Protoplast Research. Oxford: Pergamon Press, pp. 145 - 150.
63. Stahl, U. 1978. Zygote formation and recombination between like mating types in the yeast Saccharomycopsis lipolytica by protoplast fusion. MOLEC GEN GENET 160: 111 - 113.
64. Stephen, E. and Nasim, A. 1981. Production of protoplasts in different yeasts by mutanase. CAN J MICROBIOL 27: 550 - 553.
65. Stewart, G. 1981. The genetic manipulation of industrial yeast strains. CAN J MICROBIOL 27: 973 - 990.
66. Stewart, G., Russell, I. and Panchal, C. 1981. The genetic manipulation of industrial yeast strains. In: Stewart, G. and Russell, I. (ed). Current Developments in Yeast Research (Vth International Yeast Symposium, London, Ont.). Willowdale: Pergamon Press, pp. 17 - 24.
67. Svoboda, A. 1966. Regeneration ability of protoplasts of different yeast species. DEUTSCHE AKADEMIE DER WISSENSCHAFTEN ZU BERLIN. KLASSE FUER MEDIZIN ABHANDBENGEN 6: 31 - 35.

68. Svoboda, A. 1980. Intergeneric fusion of yeast protoplasts: Saccharomyces cerevisiae and Schizosaccharomyces pombe. In: Ferenczy, L. and Farkaš, G. (ed). Advances in Protoplast Research. Oxford: Pergamon Press, pp. 119 - 124.
 69. Symposium: Abstracts of IVth International Symposium on Genetics of Industrial Microorganisms. June, 1982. Kyoto, Japan.
 70. Takata, I., Tosa, T. and Chibata, I. 1977. Screening of matrix suitable for immobilization of microbial cells. J SOLID-PHASE BIOCHEM 2: 225 - 236.
 71. Thuriaux, P., Sipiczki, M. and Fantes, P. 1980. Genetical analysis of a sterile mutant by protoplast fusion in the fission yeast Schizosaccharomyces pombe. J GEN MICROBIOL 116: 525 - 528.
 72. van Uden, N. and Buckley, H. 1970. Candida Berkhout. In: Lodder, J. (ed). The Yeasts. 2nd Edition. Amsterdam: North-Holland Publishing Co., pp. 893 - 1087.
 73. Unrau, P., Wheatcroft, R. and Cox, B. 1971. The excision of pyrimidine dimers from DNA of ultraviolet-irradiated yeast. MOLEC GEN GENET 113: 359 - 362.
 74. Veliky, I. and Williams, R. 1981. The production of ethanol by Saccharomyces cerevisiae immobilized in polycation-stabilized calcium alginate gels. BIOTECH LETT 3: 275 - 280.
 75. van der Walt, J. 1970. Saccharomyces Meyen Emend Reess. In: Lodder, J. (ed). The Yeasts. 2nd Edition. Amsterdam: North-Holland Publishing Co., pp. 555 - 718.
 76. Weber, H., Förster, W., Berg, H. and Jacob, H. 1981a. Parasexual hybridization of yeasts by electric field stimulated fusion of protoplasts. CURRENT GENETICS 4: 165 - 166.
 77. Weber, H., Förster, W., Jacob, H. and Berg, H. 1981b. Enhancement of yeast protoplast fusion by electric field effects. In: Stewart, G. and Russell, I. (ed). Current Developments in Yeast Research (Vth International Yeast Symposium, London, Ont.). Willowdale: Pergamon Press, pp. 219 - 224.
-

78. Weber, H. and Spata, L. 1981. Characterization of yeast protoplast fusion products. In: Stewart, G. and Russell, I. (ed). Current Developments in Yeast Research (Vth International Yeast Symposium, London, Ont.). Willowdale: Pergamon Press, pp. 213 - 218.
79. Wilson, J., Khachatourians, G. and Ingledew, W. 1982. Protoplast fusion in the yeast, Schwanniomyces alluvius. MOL GEN GENET (in press).
80. Yamashita, K., Fukuda, M., Murata, K. and Kimura, A. 1981. Transfer of mitochondria of Hansenula wingei into protoplasts of Saccharomyces cerevisiae by mini-protoplast fusion. FEBS LETT 132: 305 - 307.

APPENDIX A

For electric shock treatment of protoplast-PEG suspensions, a single, double, or triple pulse of known voltage was applied across the spark gap chamber. To derive the amount of energy released (Table 6) during the shock procedure, the following equation was used:

$$Q = 1/2 c V^2 \times \# \text{ pulses (Kandoian, et al., 1972)}$$

where

Q is energy in joules

c is capacitance in farads

V is voltage in volts

pulses is the number of times the voltage was applied

Then, for a single pulse of 6400 V

$$\begin{aligned} Q &= 1/2 (0.05 \times 10^{-6}) (6400)^2 (1) \text{ joule} \\ &= (0.025 \times 10^{-6}) (4.0 \times 10^7) \text{ joule} \\ &= (0.1) (10) \text{ joules} \\ &= 1 \text{ joule} \end{aligned}$$

and, for a double pulse of 6400 V

$$\begin{aligned} Q &= 1/2 (0.05 \times 10^{-6}) (6400)^2 (2) \text{ joules} \\ &= (1) (2) \text{ joules} \\ &= 2 \text{ joules} \end{aligned}$$

APPENDIX B

Fusion frequency is calculated as:

$$\frac{\# \text{ colonies with fused phenotype (A} \cdot \text{B)}}{\# \text{ colonies with fused phenotype (A} \cdot \text{B)} + \# \text{ colonies with unfused phenotype (A} + \text{B)}}$$

where A is one parent
 B is the other parent
 A·B is the hybrid.

By varying the concentration ratio of the two parent strains, one might be able to enhance fusion frequency. For instance, if the fusion of A with B is desired, one might limit the self-fusions of one parent, say A. This could be achieved by increasing the concentration of B far above that of A in the protoplast fusion mixture. The likelihood of two A protoplasts meeting and interacting will then be small due to the relative scarcity of the A cell type. B·B self-fusions will still occur. However, all A protoplasts that fuse will most probably fuse with B because B is present in such high numbers. Similarly, one can limit B and mix it with an excess of A to ensure that all B protoplasts fuse with A rather than with themselves. By "swamping" the system with one parent, fusions that lead to a hybrid product are encouraged while the self-fusions of the other parent are reduced at the same time. Looking at the equation, then, the numerator will increase (# hybrids) and the denominator will decrease (# colonies with unfused phenotype). Therefore overall fusion frequency will increase.

END

1 2 10 9 18 4

FIN