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STERILE HOST YEASTS (SHY): A EUKARYOTIC SYSTEM OF BIOLOGICAL CONTAINMENT FOR RECOMBINANT DNA EXPERIMENTS

(*Saccharomyces cerevisiae*; HV2; pBR322 plasmid vectors; 2-μ plasmid; nonmating mutants)

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SUMMARY

A system of biological containment for recombinant DNA experiments in *Saccharomyces cerevisiae* (Brewer's/Baker's yeast) is described. The principle of containment is sterility: the haploid host strains all contain a mating-type-non-specific sterile mutation. The hosts also contain four auxotrophic mutations suitable for selection for the various kinds of vectors used. All vectors are derivatives of pBR322 which can be selected and maintained in both yeast and *Escherichia coli*. The system has recently been certified at the HV2 level by the National Institutes of Health.

INTRODUCTION

For reasons of prudence and of politics, precautions are taken when carrying out some classes of "recombinant DNA" experiments (i.e., experiments involving joining in vitro of DNA from unlike species). These precautions are aimed at "containment" of the living products of these experiments on the theory that novel combinations of DNA might produce living organisms capable of doing some harm if dispersed into the environment. In most countries, rules are in effect governing the precautions required for different kinds of experiments, and governing bodies charged with interpreting the rules have been formed. In the United States, the rules are made by the

National Institutes of Health and its Guidelines (1978) are administered by its Office of Recombinant DNA Activities (ORDA). The NIH Guidelines divide "containment" into two categories: physical and biological. Biological containment means the use of hosts and vectors in recombinant DNA experiments which render the living products of these experiments substantially less able to survive and multiply outside the laboratory environment. Until now, all certified biological containment systems have been based upon the bacterium *E. coli* K-12 and its bacteriophages.

In this paper we describe briefly a eukaryotic system of biological containment which we have devised based on the yeast *Saccharomyces cerevisiae*. We describe only the system itself and the theory behind its containment. Full details of the extensive experimental basis for its recent certification as an HV2 system by NIH (DHEW, NIH, 1979; see also Szybalski, 1979) are available from ORDA.

The theoretical basis of the containment system begins with the idea that laboratory strains of *Saccharomyces cerevisiae* (Brewer's/Baker's yeast) are already intrinsically "contained". No member of the genus *Saccharomyces* colonizes man or animals and none causes any disease. This species is not a normal inhabitant of the sewers or the air, which are considered the principal non-human "escape routes" out of the laboratory for microbes carrying recombinant DNA. Furthermore, the laboratory strains do not compete well with their "wild" ancestors (e.g., strains used in industrial fermentation for wine, beer, and bread). Thus the principal route of "escape" for a segment of DNA "cloned" in a *Saccharomyces* host is hypothesized to be the possibility of a mating between the in vitro recombinant and a hardier "wild" yeast strain. For this reason the primary basis for the biological containment built into the SHY strains is sterility: i.e., the inability to mate with other yeasts.

DESCRIPTION OF THE SHY HOSTS

Four SHY derivatives have been tested, certified by NIH, and are equivalent in their properties. Their genotypes are:

SHY1: α *ste*-VC9 *ura3*-52 *trp1*-289 *leu2*-3 *leu2*-112 *his3*-Δ1 *ade1*-101
SHY2: α *ste*-VC9 *ura3*-52 *trp1*-289 *leu2*-3 *leu2*-112 *his3*-Δ1 *can1*-100
SHY3: a *ste*-VC9 *ura3*-52 *trp1*-289 *leu2*-3 *leu2*-112 *his3*-Δ1 *ade1*-101 *can1*-100
SHY4: a *ste*-VC9 *ura3*-52 *trp1*-289 *leu2*-3 *leu2*-112 *his3*-Δ1

The *ste*-VC9 mutation is a mating-type-non-specific sterility-causing mutation isolated by MacKay and Manney (1974a,b) and classified class 6 (i.e. unable to mate regardless of mating type; makes no detectable α factor, a mating hormone made by α cells). Strains carrying the VC9 mutation mate, under optimal conditions, about a million-fold less frequently than the wild-type parental strain from which the VC9 mutation was isolated.

The *ura3*-52 mutation is stable (less than 10^{-9} revertants in a culture) and

causes uracil to be required for growth. This mutation allows the selection of vectors carrying the *URA3* gene.

The *trp1*-289 mutation reverts at low frequency (about 10^{-7} revertants in a culture) and causes tryptophan to be required for growth. This mutation allows the selection of vectors carrying the *TRP1* gene.

The *leu2*-3 and *leu2*-112 mutations are each frameshift mutations; the double mutants revert very infrequently (less than 10^{-8} revertants in a culture) and cause leucine to be required for growth. This allows the selection of vectors carrying the *LEU2* gene.

The *his3*-Δ1 mutation is a chemically constructed deletion in the *his3* gene (Scherer and Davis, in preparation), and reversion is, as expected, undetectable (less than 10^{-9} revertants in a culture). This mutation causes histidine to be required for growth, and allows selection of vectors carrying the *HIS3* gene.

The *ade1*-101 mutation is a simple point mutation causing a requirement for adenine, and is in some of the strains because of the method of their construction. Similarly, the *can1*-100 mutation is sometimes present because of the method of construction, and causes resistance to the arginine analogue canavanine.

The various mutations were combined in a complicated series of crosses and tetrad analysis carried out by standard methods. The *ste*-VC9 mutation does allow mating with opposite mating type at a very low frequency, resulting in diploids which sporulate normally, giving rise to two fertile and two sterile spores. Mating of steriles was accomplished by using complementary auxotrophic markers to select the very rare diploids.

TABLE I

PROPERTIES OF SHY STRAINS

	Mating[a]	Doubling time[b] (min)	Transformability[c]
SHY1	$3 \cdot 10^{-6}$	144	$2 \cdot 10^{-5}$
SHY2	$8 \cdot 10^{-6}$	108	$1 \cdot 10^{-6}$
SHY3	$4 \cdot 10^{-6}$	156	$4 \cdot 10^{-5}$
SHY4	$4 \cdot 10^{-6}$	150	$3 \cdot 10^{-6}$
ste$^+$	(1)	123	10^{-6} to 10^{-4}
Budweiser	—	84	—

[a] Mating tested as described below and calculated relative to a *ste*$^+$ control. As shown below, the test detects more than 90% of the *ste*$^+$ cells as maters.
[b] In YEP-glucose (standard complete medium).
[c] To either Ura$^+$ or Leu$^+$ using plasmids YEp24 and YEp20, respectively. 3 μg of plasmid were applied exactly as described by Hinnen et al. (1978). Values are transformants per viable regenerated cell.

In the final crosses, the *ste*-VC9 mutation was essentially repeatedly backcrossed to strains closely related to S288C. All four SHY strains are segregants from a single cross selected for their sterility and ability to be transformed by DNA carrying either the *URA3* or *LEU2* genes. A summary of their properties is given in Table I.

DESCRIPTION OF PLASMID VECTORS TO BE USED IN THE PROPOSED HV2 YEAST SYSTEM

All of the plasmids described here have in common several characteristics. They all contain a bacterial replicon which makes possible the amplification of the DNA in *E. coli.* For the purposes of an HV2 system, all the DNA of prokaryotic origin is derived from a certified EK2 plasmid vector pBR322 (Bolivar et al., 1977).

Another common characteristic is the presence of markers which enable selection in *E. coli* for the presence of the plasmid. Again, some of these markers are drug-resistance genes derived from pBR322. Others, however, are yeast DNA fragments which are expressed in *E. coli* K-12: these include the *URA3*, *LEU2*, *HIS3*, and *TRP1* genes, all of which will suppress mutations in *E. coli* mutations which inactivate the analogous bacterial functions. Although only χ1776, which has none of the relevant auxotrophic mutations (Curtiss et al., 1978) is certified for EK2 level experiments, the vectors themselves can be carried and amplified using prototrophic selection in EK1 hosts carrying the relevant auxotrophic mutations.

Most of the vectors also carry one or more genes of yeast origin which can be used to select the presence of the vector in yeast. The *URA3*, *LEU2*, *TRP1*, and *HIS3* genes again are usable in this way. The SHY strains are arranged to make any or all of these genes selectable. The exceptional vectors (YEp2, YEp4, and YIp25, see below) are intended for uses in which selection for function encoded by the cloned DNA is intended, or may be provided in the future with one of the four selectable markers, a modification which we would hope will be regarded as a "trivial modification" under the NIH Guidelines.

Finally, all the vectors carry fragments of yeast DNA which will, in some way, provide for the maintenance of the plasmid in yeast. Three ways in which this is possible are all represented among the vectors, and the nature of each is indicated by the nomenclature. One form of maintenance is integration into the yeast genome. Such vectors (YIp, for *Y*east *I*ntegrating *P*lasmid) transform yeast at very low efficiency, but result in stable additions of the entire plasmid to the yeast genome (Hinnen et al., 1978). Another form of maintenance is inclusion of all or part of the yeast 2-μ plasmid, which transforms at high frequency and results in episomal maintenance, usually very unstable, of the entire vector plasmid (Beggs, 1978). Such vectors are called YEp, for *Y*east *E*pisomal *P*lasmid. The third form of maintenance is inclusion of a yeast sequence allowing autonomous replication,

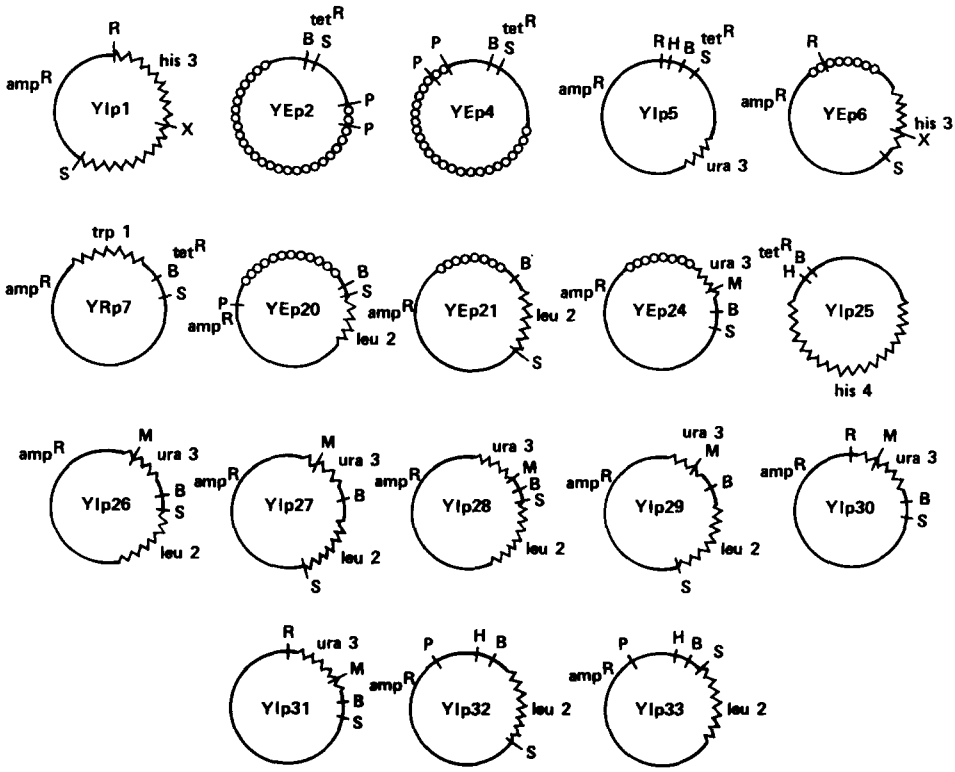

Fig.1. Vectors currently certified for use with the SHY strains for HV2 level experiments. Each circle represents the DNA composition of the vector. Each is only roughly to scale; refer to Table II for absolute size. ——, represents pBR322 DNA; ∧∧∧∧ , represents yeast chromosomal DNA; oooooo represents 2-μ plasmid DNA. Some restriction endonuclease sites are indicated as follows: R = *Eco*RI; B = *Bam*HI; S = *Sal*I; M = *Sma*I; P = *Pst*I; H = *Hin*dIII; X = *Xho*I; V = *Pvu*I.

presumably because the yeast DNA carries a normal chromosomal replicon (Struhl et al., 1979). These give high frequency of transformation and result in unstable transformants, and are called YRp, for *Y*east *R*eplicating *P*lasmid.

The properties of these plasmids, all containing well-defined fragments from pBR322 (the only source of prokaryotic sequences), the yeast 2-μ plasmid, and/or the yeast chromosome are summarized in Table II and Fig. 1.

With respect to biological containment, all these vectors are equivalent. The basis for the biological containment of yeast HV2 systems is failure of cloned segments to be transferred to robust "wild" strains, and these cannot be transferred from the SHY strains because they are unable to mate. With respect to *E. coli*, these vectors are all based on pBR322 and thus are EK2.

TABLE II

CHARACTERISTICS OF HV2 YEAST VECTORS

Vector	Size (kb)	Phenotypes	Cloning sites	Source
YIp1	9.8	Amp His3	*Eco*RI *Sal*I *Xho*I	Stanford Univ. (R.W.D.)
YEp2	10.4	Tet	*Pst*I *Bam*HI *Sal*I	Stanford Univ. (R.W.D.)
YEp4	10.4	Tet	*Pst*I *Bam*HI *Sal*I	Stanford Univ. (R.W.D.)
YIp5	5.5	Amp Tet Ura3	*Eco*RI *Bam*HI *Sal*I *Hin*dIII *Sma*I[a]	Stanford Univ. (R.W.D.)
YEp6	7.9	Amp His3	*Eco*RI *Xho*I *Sal*I	Stanford Univ. (R.W.D.)
YRp7	5.7	Amp Tet Trp1	*Bam*HI *Sal*I	Stanford Univ. (R.W.D.)
YEp20	10.4	Amp Leu2	*Bam*HI *Sal*I *Pst*I	Cold Spring Harbor Lab.
YEp21	8.8	Amp Leu2	*Bam*HI *Sal*I	Cold Spring Harbor Lab.
YEp24	7.6	Amp Ura3	*Bam*HI *Sma*I[a] *Sal*I	Univ. of Strasbourg
YIp25	11.9	Tet His4	*Bam*HI *Hin*dIII	Cornell Univ.
YIp26	7.8	Amp Leu2 Ura3	*Bam*HI *Sal*I *Sma*I[a]	M.I.T.
YIp27	7.8	Amp Leu2 Ura3	*Bam*HI *Sal*I *Sma*I[a]	M.I.T.
YIp28	7.8	Amp Leu2 Ura 3	*Bam*HI *Sal*I *Sma*I[a]	M.I.T.
YIp29	7.8	Amp Leu2 Ura 3	*Bam*HI *Sal*I *Sma*I[a]	M.I.T.
YIp30	5.5	Amp Ura3	*Eco*RI *Bam*HI *Sal*I *Sma*I[a]	M.I.T.
YIp31	5.5	Amp [Tet][b] Ura3	*Eco*RI *Bam*HI *Sal*I *Sma*I[a]	M.I.T.
YIp32	6.7	Amp Leu2	*Bam*HI *Sal*I *Pst*I *Hin*dIII	M.I.T.
YIp33	6.7	Amp Leu2	*Bam*HI *Sal*I *Pst*I *Hin*dIII	M.I.T.

[a] *Sma*I site is near the end of *URA3* DNA, but has not yet been used for cloning.
[b] Brackets indicate low level resistance.

CONCLUSION

With the certification by NIH of the SHY strains a convenient principle of biological containment (sterile mutants of *Saccharomyces cerevisiae*) has been established. In the future many new vectors consisting of certified EK2 plasmids or phages (especially the latter) should be certifiable and thereby increase the usefulness of the system.

Preliminary description of this system was announced in the Recombinant DNA Techn. Bull. (Vol. 2, No.2, July, 1979, pp. 49—54), but that communication contains several errors (especially in Fig. 1) which are corrected in the present paper.

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APPENDIX

GENEALOGY OF SHY STRAINS

Origin of the markers:

ste-VC9 from VC9 = *ade6 his6 leu1 met1 trp5 gal2 can1* (MacKay and Manney, 1974a). *leu2*-3 *leu2*-112 from AH22 = a *leu2*-3 *leu2*-112 *his4 can1* (Hinnen et al., 1978). *ura3*-52 from DBY630 = α *ura3*-52. (collection of F. Lacroute)
his3-Δ1 *trp1*-289 from Scherer and Davis (1979), who through a series of crosses constructed DBY703 = α *his3*-Δ1 *trp1*-289 *ura3*-52.

cross 1: VC9* × a *ura3*-56 (a deletion); select prototrophs (10^{-7}):
Yields DBY748* = α *ste*-VC9 *met1 can1*

cross 2: AH22 × DBY630
Yields DBY704 = a *his4 ura3*-52 *leu2*-3 *leu2*-112
and DBY736 = α *his4 ura3*-52 *leu2*-3 *leu2*-112

cross 3: DBY703 × DBY704
Yields DBY747 = a *his3*-Δ1 *leu2*-3 *leu2*-112 *ura3*-52 *trp1*-289

cross 4: DBY748* × DBY747; select ten prototrophs, dissect all which sporulate:
Yields DBY749* = a *ste*-VC9 *his3*-Δ1 *trp1*-289 *ura3*-52 *can1*

cross 5: DBY736 × a *ade1*-101
Yields DBY745 = α *leu2*-3 *leu2*-112 *ade1*-101 *ura3*-52

cross 6: DBY749* × DBY745; select ten Ade$^+$ His$^+$, dissect those which sporulate:
Yields SHY1 = α *ste*-VC9 *ura3*-52 *trp1*-289 *leu2*-3 *leu2*-112 *his3*-Δ1 *ade1*-101
SHY2 = α *ste-VC9 ura3*-52 *trp1*-289 *leu2*-3 *leu2*-112 *his3*-Δ1 *can1*-100
SHY3 = a *ste-VC9 ura3*-52 *trp1*-289 *leu2*-3 *leu2*-112 *his3*-Δ1 *ade1*-101 *can1*-100
SHY4 = a *ste*-VC9 *ura3*-52 *trp1*-289 *leu2*-3 *leu2*-112 *his3*-Δ1

In this cross, all heterozygous markers segregated 2:2 including *ste*-VC9 and mating type.

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*All sterile strains (strains unable to mate) are marked with an asterisk.