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Construction of a *Schizosaccharomyces pombe* Gene Bank in a Yeast Bacterial Shuttle Vector and Its Use to Isolate Genes by Complementation

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Summary. A gene bank of partial *Sau*3A restriction fragments of *S. pombe* DNA has been constructed in the plasmid vector, pDB248′, which is capable of high frequency transformation of *S. pombe*. Procedures are described which enable plasmids to be recovered from *S. pombe* by their reintroduction into *E. coli*. These methods have been used to detect the *S. pombe* genes *lys* 1^+, *ade* 6^+ and *his* 2^+ in the gene bank by complementation of mutant gene functions, and to physically isolate the *lys* 1^+ gene.

Introduction

Plasmid DNA cloning vectors which can be propogated in both *Escherichia coli* and a eukaryotic organism are powerful tools for the isolation and investigation of eukaryotic genes. In principle, gene banks constructed in such vectors can be used to isolate any gene for which mutants are available; this is because the plasmid containing the appropriate gene can be selected on the basis of its complementation of the mutant function. Until now such vectors have only been available for the budding yeast, *Saccharomyces cerevisiae* (Hinnen et al. 1978; Beggs 1978), but recently we have described a vector, pDB248, which can be propogated in the fission yeast *Schizosaccharomyces pombe* as well as in *S. cerevisiae* and *E. coli* (Beach and Nurse 1981). The vector incorporates the bacterial plasmid pBR322, part of the *S. cerevisiae* 2 μM circle containing the origin of replication, and the *S. cerevisiae leu* 2^+ gene. The *leu* 2^+ gene complements the *S. pombe leu* 1.32 mutation, allowing *S. pombe* clones containing the vector to be isolated by selecting for leucine prototrophs after transformation of a *leu* 1.32 strain. In the present paper we describe the construction of an *S. pombe* gene bank in this vector, procedures for efficient *S. pombe* transformation, and recovery of the plasmids by reintroduction into *E. coli*. The gene bank has been used to complement mutations in the *lys* 1, *his* 2 and *ade* 6 genes, and the sequence complementing *lys* 1 has been recovered in *E. coli*.

Methods

Strains

The gene bank was constructed in the *E. coli* strain JA221, *rec*A1, *leu* B6, *trp* E5, *Hs*dR$^-$, *Hsd*M$^+$ *lac*Y C600 (obtained from J. Beggs) but BJ5183, r$^-$ *Rec*BC$^-$ *sbc*B *endo* I *Gal*$^-$ *meth*$^-$ *StrR thi biot hsd* (obtained from F. Lacroute), was used for recovery of plasmids from yeast. The *S. pombe* strains *leu* 1.32, *lys* 1.31h$^-$, *leu* 1.32, *his* 2$^-$h$^-$ and *leu* 1.32 *ade* 6.704 h$^-$ were used.

DNA Isolation

Plasmid DNA was prepared from *E. coli* by the cleared lysate procedure. 5 ml of cleared lysate was mixed with 5.15 gm caesium chloride and 0.73 ml 10 mg/ml ethidium bromide and centrifuged for not less than 40 h at 36,000 rpm. The plasmid band was recovered with a syringe, the ethidium extracted with isopropanol and desalted by centrifugation through a 5 ml Sephadex G-50 column. After incubation with protease K at 50 μg/ml for one hour, the protein was removed by phenol/chloroform extraction and the DNA precipitated from 300 mM NaCl with 2 volumes of ethanol. It was resuspended in 10 mM Tris pH 7.6, 1 mM EDTA (TE) at approximately 200 μg/ml. *S. pombe* DNA was isolated from 200 ml cultures grown to near saturation in yeast extract medium (5 g/l yeast extract, 30 g/l glucose, 75 mg/l adenine). The cells were harvested by centrifugation, washed in 20 mM sodium citrate/phosphate buffer pH 5.8 and resuspended in 10 ml 50 mM sodium citrate/phosphate pH 5.8, 1.2 M sorbitol, 50 mM β-mercaptoethanol, 40 mM EDTA, 2 mg/ml Novo SP234 (an extract of *Trichoderma harzianum*, Novo Enzymes). Once the cells had become osmotically sensitive they were lysed directly by addition of 20 ml 50 mM Tris pH 7.6, 50 mM EDTA, 2% SDS at 65° C. After approximately 2 min the lysate was extracted with an equal volume of phenol-chloroform. The aqueous upper layer was brought to 300 mM sodium acetate and the nucleic acids precipitated with a half volume of isopropanol. The precipitate was pelleted by centrifugation, resuspended in 6 ml T.E., and mixed with 6.4 gm caesium chloride and 0.6 ml 10 mg/ml ethidium bromide. After 40 h centrifugation one clear band formed in the centre of the gradient. This was removed with a syringe, extracted with isopropanol and diluted with 4 volumes of 300 mM NaCl. The DNA was precipitated with 2 volumes of ethanol and after washing with 70% ethanol redissolved at 200 μg/ml in T.E.

Gene Bank Construction

The vector pDB248′ was used for construction of an *S. pombe* gene bank. This molecule, constructed at the same time as pDB248 (see figure in Beach and Nurse 1981), con-

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tains a tandem duplication of the 1.5 kb *Eco*RI fragment which carries part of the *leu* 2^+ gene. This inadvertant duplication which occured during construction of pDB248′, is removed at high frequency by intramolecular recombination in *S. pombe* (see results) and does not affect the properties of the vector.

5 μg of pDB248′ was digested to completion with *Bam*HI (Bethesda Research Labs) and ligated with 5 μg of wild type *S. pombe* DNA partially digested with *Sau* 3A (B.R.L.). The ligated DNA was used to transform $CaCl_2$ treated *E. coli* (JA221) to ampicillin resistance. 30,000 transformants were generated and of these approximately 10,000 were found to be tetracycline sensitive. The tet^s colonies were pooled and grown in a 1 litre culture from which plasmid was prepared. The average size of *S. pombe* inserts was estimated from 25 clones and found to be 4 kilobases. The bank thus contains 40,000 kilobases of *S. pombe* DNA, which is twice the genome size of this yeast.

S. pombe Transformation

The procedure for transformation of *S. pombe* was based on that described previously (Bean and Nurse, 1981). Cells were grown at 25° C to approximately 1×10^7 cells/ml in 200 mls minimal medium (Mitchison 1970, as modified by Nurse 1975), supplemented with 50 mg/l amino acids as required. The medium contained 0.5% rather than the normal level of 2.0% glucose as this greatly enhances the ease of formation of spherical protoplasts. The cells were harvested by centrifugation, washed with 1.2 M sorbitol, 20 mM citrate-phosphate pH 5.6, 40 mM EDTA, 150 mM β-mercaptoethanol and after recentrifugation resuspended at approximately 3×10^8 cells/ml 1.2 M sorbitol, 50 mM citrate-phosphate pH 5.6, 30 mM β-mercaptoethanol, 5 mg/ml Novo SP234. The cells were incubated at 30° C for 20–60 min until at least 50% of the cells had become spherical. They were washed 3 times in not less than a total of 100 ml 1.2 M sorbitol 10 mM Tris pH 7.6, taking great care to gently resuspend the pelleted cells between each wash. The protoplasts were finally resuspended at 5×10^8 cells/ml in 1.2 M sorbitol, 10 mM Tris pH 7.6, 10 mM $CaCl_2$ to which was added salmon sperm DNA (Sigma, London) at 10 μg/ml. The cells to be transformed were dispensed in 100 μl aliquots to which plasmid DNA was added in less than 10 μl, bringing the final DNA concentration to 10–20 μg/ml. After incubation for 15 min at 22° C, 1 ml of 10 mM Tris pH 7.6, 10 mM $CaCl_2$ 20% (w/v) polyethylene glycol 4,000 (B.D.H.) was added to each tube and left for 15 min. The cells were finally pelleted and resuspended in 200 μl 10 mM Tris pH 7.6, 10 mM $CaCl_2$, 1.2 M sorbitol, 0.5 mg/ml yeast extract, 5 μg/ml leucine for 20–60 min before plating directly onto the surface on 2. agar minimal selective plates containing 1.2 M sorbitol. Transformant colonies grew up in 3–5 days at 29° C.

Recovery of Plasmids in E. coli

Transformant yeast colonies were smeared as 1 cm squares onto selective plates and incubated for 1–2 days. The cells were scraped off the plates into 0.5 ml 20 mM Tris pH 7.6, 20 mM EDTA, 1% Triton X-100 to which was added an equal volume of 0.45–0.5 mm glass beads. The cells were vortexed vigorously for 30 s and immediately extracted with an equal volume of phenol/chloroform. The aqueous supernatant was precipitated with a half volume of isopropanol from 300 mM sodium acetate, resuspended in 100 μl T.E. containing 5 μg RNase and digested for 1 h at 37° C. The sample was extracted with phenol/chloroform and precipitated with 2 volumes of ethanol from 300 mM NaCl. The DNA was resuspended in 20 μl T.E. and 1–10 μl samples used to transform *E. coli*, strain BJ5183. Up to 100 ampicillin resistant transformants were routinely obtained.

Results and Discussion

Optimisation of Transformation Procedure

The transformation procedure we have described previously (Beach and Nurse 1981) can yield $0.5–1 \times 10^4$ transformants/μg vector DNA and 1 transformant per 10^4 viable regenerated protoplasts. However, rather variable results were obtained and we have investigated the procedure to see if changes could be made to optimize it.

An important factor influencing the efficiency of transformation is the method of protoplast preparation. It was observed that the original protoplasting procedure sometimes gave spherical true protoplasts and sometimes rod shaped spheroplasts which were osmotically sensitive but were still bounded by remnants of the cell wall. The true protoplasts were transformed far more efficiently. If cells are grown in minimal medium with the glucose concentration reduced from 2% w/v to 0.5% w/v then true protoplasts were formed consistantly.

Addition of salmon sperm carrier DNA improved efficiently especially if the vector DNA was present at low concentration. The optimal concentration of carrier was 15 μg/ml; further increase above this level gave no further improvement. Incubating the DNA with the protoplasts for 15 min before addition of PEG 4000 was optimal but reducing the time to 1 min still yielded 60% of this optimal value. Increasing the polyethylene glycol (PEG) concentration from 20% to 40% and inclusion of 1.2 M sorbitol in the PEG solution both dramatically reduced transformation.

The concentration of protoplasts when incubated with DNA was found to be important. For the most efficient transformation rates per μg vector DNA a protoplast concentration $3–5 \times 10^8$/ml was best. This routinely gave $2–3 \times 10^4$ transformants/μg pDB248′. Increasing protoplast concentration resulted in a dramatic fall off in efficiency down to 2–3,000 transformants/μg at 2×10^9/ml. At $3–5 \times 10^8$/ml 1 transformant was formed per 2–3,000 viable regenerated protoplasts. Reducing the protoplast concentration to 2×10^7 ml increased the protoplast rate to 1 transformant per 500 viable regenerated protoplasts but was more wasteful of plasmid DNA where the rate was reduced to 5,000 transformants/μg pDB248′.

As a result of these experiments the following changes of the original procedure were made: (1) The cells were grown in minimal medium with 0.5% glucose: (2) Protoplasts were incubated with the DNA at $3–5 \times 10^8$/ml: (3) Salmon sperm carrier DNA was added to give a final DNA concentration of 15 μg/ml. The full procedure is described in detail in the Methods.

Stability of pDB248′

The plasmid pDB248′ is unstable in *S. pombe*. When it is introduced into a *leu* 1.32 strain and the subsequent trans-

formants are cultured on minimal medium about 20% of the cells have lost the plasmid. This can be shown by plating from the minimal culture onto yeast extract and replica plating the resultant colonies onto minimal medium to determine which are *leu* 1^-. Despite its instability it is difficult to lose the plasmid all together. After relaxing selection for 15 generations by culturing on yeast extract 50% of the cells are still *leu* 1^+. The instability of pDB248′ is a useful property when isolating genes by complementation. If the gene to be isolated is on the plasmid then all cells which become *leu* 1^- should also lose the gene with a subsequent loss of the ability to complement.

Complementation of Auxotrophic Mutants

S. pombe DNA was partially digested with *sau* 3A and the resultant quasi-random fragments ligated into the *Bam* H1 site located in the *tet*^{r} gene of pDB248′. Approximately 10,000 recombinant plasmids generated in *E. coli* were pooled to form an *S. pombe* gene bank (see Methods). The gene bank was transformed into a *leu* 1.32 *lys* 1.131 h^- *S. pombe* strain and about 20,000 *leu* 1^+ clones were selected by plating onto sorbitol minimal medium plus lysine. These were replica plated onto minimal medium and 8 clones were found to be *lys* 1^+. The 8 clones were streaked to single colonies on yeast extract plates and were replica plated to minimal medium plus leucine and minimal medium plus lysine. Both *leu* 1^+ and *lys* 1^+ were lost from about 20% of the colonies and usually together. However colonies which were *leu* 1^+ *lys* 1^- were occasionally observed. These result from cotransformation of plasmids and are discussed later.

Similar experiments were carried out with the *leu* 1.32 *ade* 6.704 and *leu* 1.32 *his* 2^- strains and clones which were *leu* 1^+ *ade* 6^+ or *leu* 1^+ *his* 2^+ were isolated. In both cases stability tests showed that loss of *leu* 1^+ was nearly always accompanied by loss of *ade* 6^+ or *his* 2^+.

Recovery of Plasmids from S. pombe

A small scale DNA preparation (see Methods) was made from an *S. pombe* transformant unstable for *leu*$^+$ and *lys*$^+$. This was used to generate approximately 100 ampicillin resistant transformants in the *E. coli* strain BJ5193. This *rec*BC strain can be transformed at least 100 fold more efficiently by yeast crude DNA preparations than can more conventional *rec*A strains. This is despite the fact that BJ5193 is transformed at only 5–10 fold higher frequency than JA221 or HB101 by caesium chloride purified plasmid preparation. However BJ5183 gives a poor yield of plasmid and those of interest were usually transfered to JA221 for further investigation.

Ten independent Amp^r bacterial clones generated by transformation with *S. pombe* DNA were grown up in 5 ml cultures and small scale plasmid preparations obtained. These were retransformed into the *leu 1.32*, *lys* 1.131h^- *S. pombe* strain to give leucine prototrophs which were tested for complementation of *lys* 1^- by replica plating to lysing free plates. Five of the plasmids gave *leu*$^+$ transformants essentially all of which were *lys*$^+$. These plasmids all generated the same restriction fragments. The other five showed no lysine complementation and were found by restriction digests to be a different plasmids. These probably arose by co-transformantion of plasmids from the original gene bank. The presence of more than one type of plasmid in the yeast transformant accounts for the occurance of *leu*$^+$ *lys*$^-$ colonies segregating from a colony which is *leu*$^+$ *lys*$^+$.

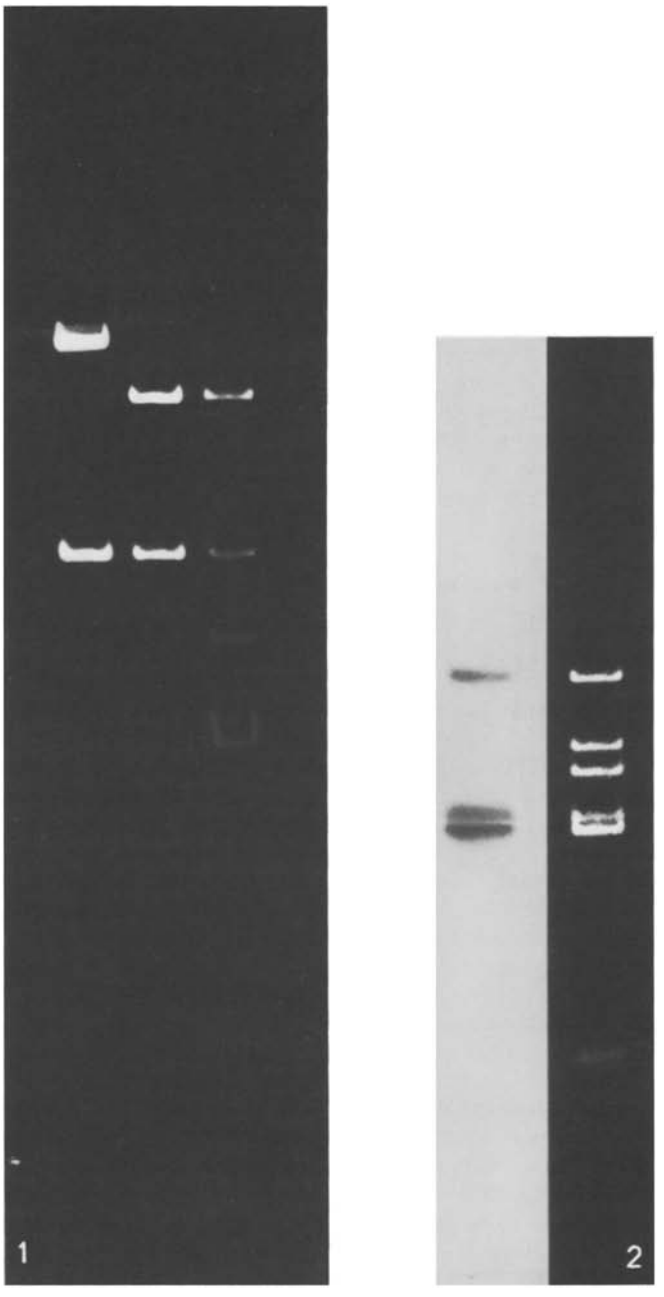

Fig. 1. Sal I/Hind III double digests of pDB248′ pDB248 and pLys 1. The upper band of the pDB248′ digest (*left hand track*) is larger than that seen for pDB248 (middle track). This is because of the presence of an extra 1.5 kb *Eco*R1 fragment. This band has been removed in vivo to generate the smaller upper band seen in pLys 1 (*right hand track*). The other bands in pLys 1 consist of chromosomal insert DNA

Fig. 2. Autoradiograph of Southern transfer of *Eco*R1 digested *S. pombe* DNA hybridised with [P^{32}] pLys 1. *Left hand track* shows 3 hybridising bands in the autoradiograph. *Right hand track* shows an Ethidium stained bands of pLys 1 digested with *Eco*R1. Three of the bands are in common

The plasmid which complemented both *leu* 1 and *lys* 1 mutations, p*Lys* 1, was analysed further. Restriction digest with Hind III and *Sal* I revealed that the 7.7 kb parental fragment of pDB248′ had reduced to 6.0 kb. (Fig. 1). This results from intramolecular recombination in the tandemly repeated region of pDB248′ and deletion of the repeat. In order to confirm that pLys 1 contains sequences derived from *S. pombe* the plasmid was nicktranslated to high specific activity and used as a probe in a Southern transfer of *Eco*RI digested DNA. Figure 2 shows that there is hybridization between pLys 1 and the *S. pombe* chromosome.

The *lys* 1 gene was initially isolated because it is closely linked to the centromere of chromosome I (Kohli et al. 1977). There was thus the possibility that pLys 1 would contain the centromere. Centromeres of *S. cerevisiae* have been shown to stabilize plasmids in that organism (Clarke and Carbon 1980) though not in *S. pombe*. However, the *S. pombe* transformants of pLys 1 were as mitotically unstable as those of pDB248′. Thus the plasmid probably does not contain the centromere sequence but could readily be used to "chromosome walk" to the centromere.

In this paper we have described methods which in principle can be used to isolate any gene in *S. pombe* for which mutants are available as long as the mutant function can be complemented by the wild type gene on a plasmid. These procedures have been used to isolate not only *lys* 1 but also the *mat* P mating-type gene (Beach, Nurse and Egel 1982). DNA sequences can now be readily introduced into and reisolated from *S. pombe*. The vector used in this study can also be propogated in *S. cerevisiae* (Beach and Nurse 1981). This provides the unique opportunity in eukaryotic cells to isolate genes from *S. pombe* and *S. cerevisiae* and test for their function in either yeast. This possibility will be particularly powerful for identifying genes with homologous functions from the two yeasts.

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