

Novel mobilizable prokaryotic two-hybrid system vectors for high-throughput protein interaction mapping in *Escherichia coli* by bacterial conjugation

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ABSTRACT

Since its initial description, the yeast two-hybrid (Y2H) system has been widely used for the detection and analysis of protein–protein interactions. Mating-based strategies have been developed permitting its application for automated proteomic interaction mapping projects using both exhaustive and high-throughput strategies. More recently, a number of prokaryotic two-hybrid (P2H) systems have been developed but, despite the many advantages such *Escherichia coli*-based systems have over the Y2H system, they have not yet been widely implemented for proteomic interaction mapping. This may be largely due to the fact that high-throughput strategies employing bacterial transformation are not as amenable to automation as Y2H mating-based strategies. Here, we describe the construction of novel conjugative P2H system vectors. These vectors carry a mobilization element of the IncP α group plasmid RP4 and can therefore be mobilized with high efficiency from an *E. coli* donor strain encoding all of the required transport functions *in trans*. We demonstrate how these vectors permit the exploitation of bacterial conjugation for technically simplified and automated proteomic interaction mapping strategies in *E. coli*, analogous to the mating-based strategies developed for the Y2H system.

INTRODUCTION

Protein–protein interactions play important functional roles in virtually every cellular process. They mediate many disease states and biological processes important for the pathogenesis

of bacterial and viral infections (1), and the specificity of these interactions makes them ideal targets for novel therapeutic agents (2–4). The identification of protein interactions can also be a critical step in the determination of unknown protein functions, and as many of the predicted genes in the ever growing list of completed genome sequences are of unknown function, the construction of protein interaction maps can aid the functional annotation of genome sequences and further our understanding of complex biological processes. The detection and analysis of such molecular interactions has thus become the latest challenge facing molecular biologists in the post-genomic era. High-throughput, easy to handle, and preferably automatable, technologies are required to facilitate the full exploitation of the enormous volume of genomic sequence data now available. As a result, a wide variety of biochemical and genetic-based assays have been developed for the detection of protein–protein interactions, and the yeast two-hybrid (Y2H) system (5) currently represents one of the most powerful genetic-based approaches for the detection of protein–protein interactions *in vivo*.

The Y2H system employs hybrid proteins, generally termed Bait and Prey hybrid proteins, for the detection of protein–protein interactions via reporter gene activation resulting in a readily detectable phenotype (see Figure 1A). The Y2H system is highly sensitive, often detecting interactions undetected by other techniques such as co-immunoprecipitation, and since its initial description, has spawned the development of a whole repertoire of similar genetic-based systems for the detection of a wide range of biomolecular interactions [reviewed in (6–9)]. The Y2H system itself has been widely applied not only to detect novel interacting partners for proteins of interest by performing library screens (10,11) but also to study interactions between defined protein pairs to identify minimal domains (12) and critical residues (13) required for interaction. The system has been adapted, through the use of yeast mating types (14), for high-throughput and automated protein interaction mapping strategies (see Figure 2A). It has

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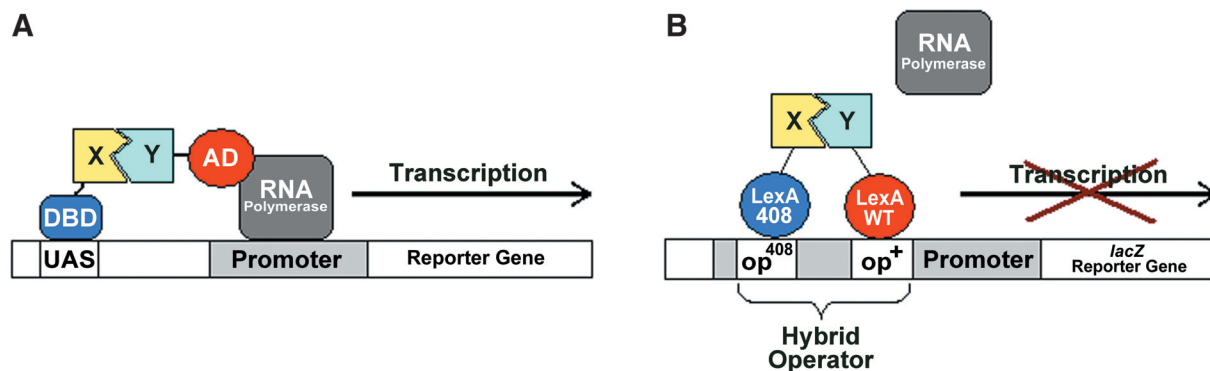


Figure 1. Schematic representation depicting the detection of protein–protein interactions in two-hybrid systems. (A) The Y2H system: X represents a given protein fused to a specific DNA binding domain (DBD). In library screens this protein is termed the ‘Bait’. Y represents a given protein, or a pool of proteins encoded by a DNA library fused to a transcriptional activation domain (AD). This fusion protein is often termed the ‘Prey’. If X and Y interact, the AD is brought to the vicinity of the DNA bound DBD and transcription is activated from the adjacent promoter resulting in a clearly detectable phenotype. (B) The LexA repressor-based P2H system: X represents a given Bait protein fused to a mutant LexA DBD (LexA408) and Y represents a given Prey protein, or library of Prey proteins, fused to a wild-type LexA DBD (LexAWT). Interactions between X and Y produce a heterodimeric LexA repressor capable of binding to a hybrid (op^{408}/op^+) operator site and repressing reporter gene expression.

been applied for the construction of protein interaction maps for functional groups of proteins (14–16), small proteomes (17,18) and more recently has been applied for the analysis of more complex proteomes (19–24).

In recent years, there has been a growing interest in the development of prokaryotic two-hybrid (P2H) systems as these present many advantages over Y2H systems and represent an experimental alternative to the yeast-based systems. A large number of P2H systems have been described previously (25–39) [reviewed in (40,41)], but despite their many advantages, P2H systems still have not been widely implemented for large-scale or proteomic scale protein interacting mapping in the same way as the Y2H system. Such applications of the P2H systems described to date, with the exception of the system developed by Joung and co-workers (31) that permits the exploitation of phage transduction, require the use of bacterial transformation to introduce plasmids into appropriate *Escherichia coli* reporter strains to test for interactions (42). While transformation-based strategies are possible, they are technically more involved and therefore less conducive to automation than the technically simplistic mating-based strategies developed for Y2H systems. It is probably for this very reason that the Y2H system has remained the preferred approach for large-scale protein interaction mapping.

Here, we describe the construction of novel Bait vectors for use with the P2H system developed by Dmitrova *et al.* (39) and based on the *E. coli* LexA repressor protein. The LexA protein comprises an N-terminal DNA-binding domain (DBD) and a C-terminal dimerization domain and only binds to operator sequences, comprising two inverted repeat half sites (op^+/op^+), as a dimer. It has been demonstrated that the natural dimerization domain of this repressor protein can be replaced with heterologous homodimerizing domains, and the resulting chimeric protein can still function as an efficient repressor (43,44). In order to permit the detection of heterologous protein–protein interactions, Dmitrova *et al.* (39) generated a mutant LexA DBD, the LexA408 DBD, with an altered binding specificity for a mutant operator sequence, op^{408} . One protein of an interacting pair, X, is fused C-terminally to this LexA408 DBD, while the other, Y, is fused C-terminally to a wild-type LexA DBD (LexAWT DBD). The hybrid

proteins are then co-expressed, from separate plasmids, in a specially constructed *E. coli* reporter strain, SU202, carrying a chromosomally integrated reporter gene. This reporter gene comprises a *lacZ* gene placed under the transcriptional control of a modified *sulA* promoter bearing hybrid op^{408}/op^+ LexA operator sequences. Only a heterologous interaction between X and Y generates a heterodimeric protein, with one LexAWT DBD and one LexA408 DBD, capable of binding the hybrid op^{408}/op^+ operator to repress the expression of the *lacZ* reporter gene (see Figure 1B).

The new Bait vectors that we have constructed carry a region of the conjugative plasmid RP4, called a *mob* site (mobilization element). These vectors can therefore be transferred by highly efficient bacterial conjugation from a donor *E. coli* strain called S17-1, bearing a chromosomally integrated RP4 plasmid encoding all of the required plasmid mobilization functions (45), into a recipient reporter *E. coli* strain already harbouring the Prey vector for the system. The resulting transconjugant *E. coli* reporter strain carries both the Prey and Bait vectors for the system and can be assayed to detect protein–protein interactions. This bacterial mating therefore achieves the same result as the mating of yeast cells (see Figure 2B). Using the model interaction of the eukaryotic Jun and Fos leucine zippers, we demonstrate how selected Bait clones can be mated with pools of Prey clones for technically simplified screening of libraries to identify interacting partners with an efficiency unattainable using bacterial transformation procedures or yeast mating. We also demonstrate how each clone in a Bait library could be mated with each individual clone in an arrayed Prey library in a matrix format using a technically simplistic and automatable protocol (see Figure 3). Exhaustive screening in this fashion would reveal all possible interactions between proteins in the Prey and Bait libraries. The strategies we describe here are analogous to those currently implemented for high-throughput proteomic interaction mapping using Y2H mating-based approaches (15,19,21,22,46–48) and should therefore permit the application of this LexA P2H system, or any of the previously described P2H systems modified to incorporate such a mobilizable vector, for similar proteomic interaction mapping projects.

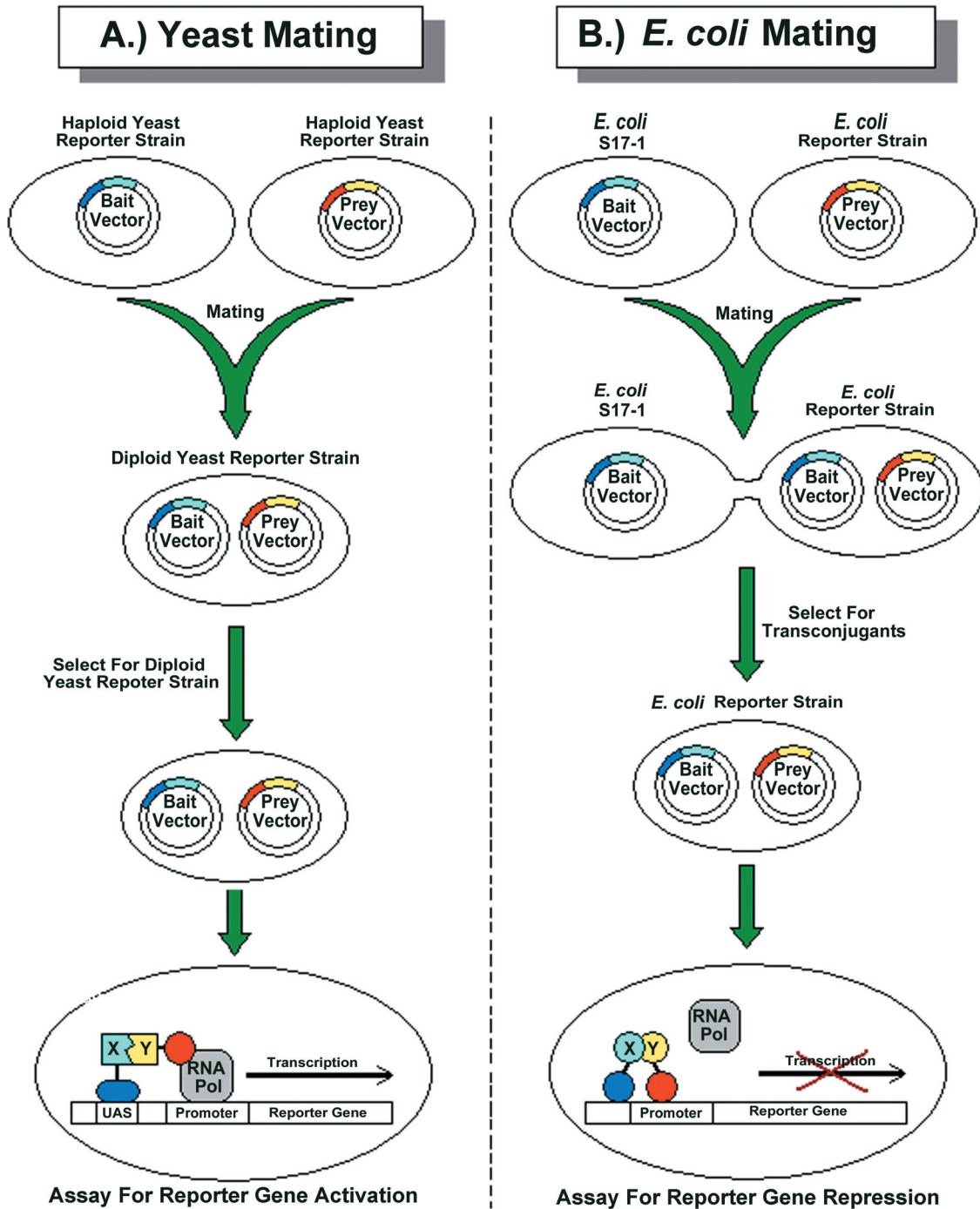


Figure 2. Mating-based protein–protein interaction mapping. (A) Application of yeast mating for protein–protein interaction mapping: The Bait vector carrying a cloned gene of interest is transformed into a haploid yeast reporter strain of one mating type (MAT a). This is then mated either with individual Prey clones, or with pools of Prey clones, maintained in a haploid yeast reporter strain of opposite mating type (MAT α). Diploid yeast reporter strains carrying both a Bait and a Prey vector are selected and assayed for reporter gene activation to detect protein–protein interactions. (B) Application of bacterial mating for protein–protein interaction mapping using the LexA repressor-based P2H system. This is analogous to yeast-mating strategies. A mobilizable Bait vector carrying a cloned gene of interest is transformed into *E.coli* strain S17-1. This is then mated either with individual Prey clones, or with pools of Prey clones, maintained in the *E.coli* reporter strain for the P2H system. Transconjugant *E.coli* reporter strain cells carrying both a Bait and a Prey vector are selected for and then assayed for reporter gene repression to detect protein–protein interactions.

MATERIALS AND METHODS

The plasmids and strains utilized in this work are listed in Table 1 and a more detailed description of some of these is given below.

Bacterial strains and plasmids

Plasmids pMS604 and pDP804 are designated in this work as Prey and Bait vectors, respectively (see Figure 4). Plasmid pMS604 (44) expresses a chimeric protein consisting of a

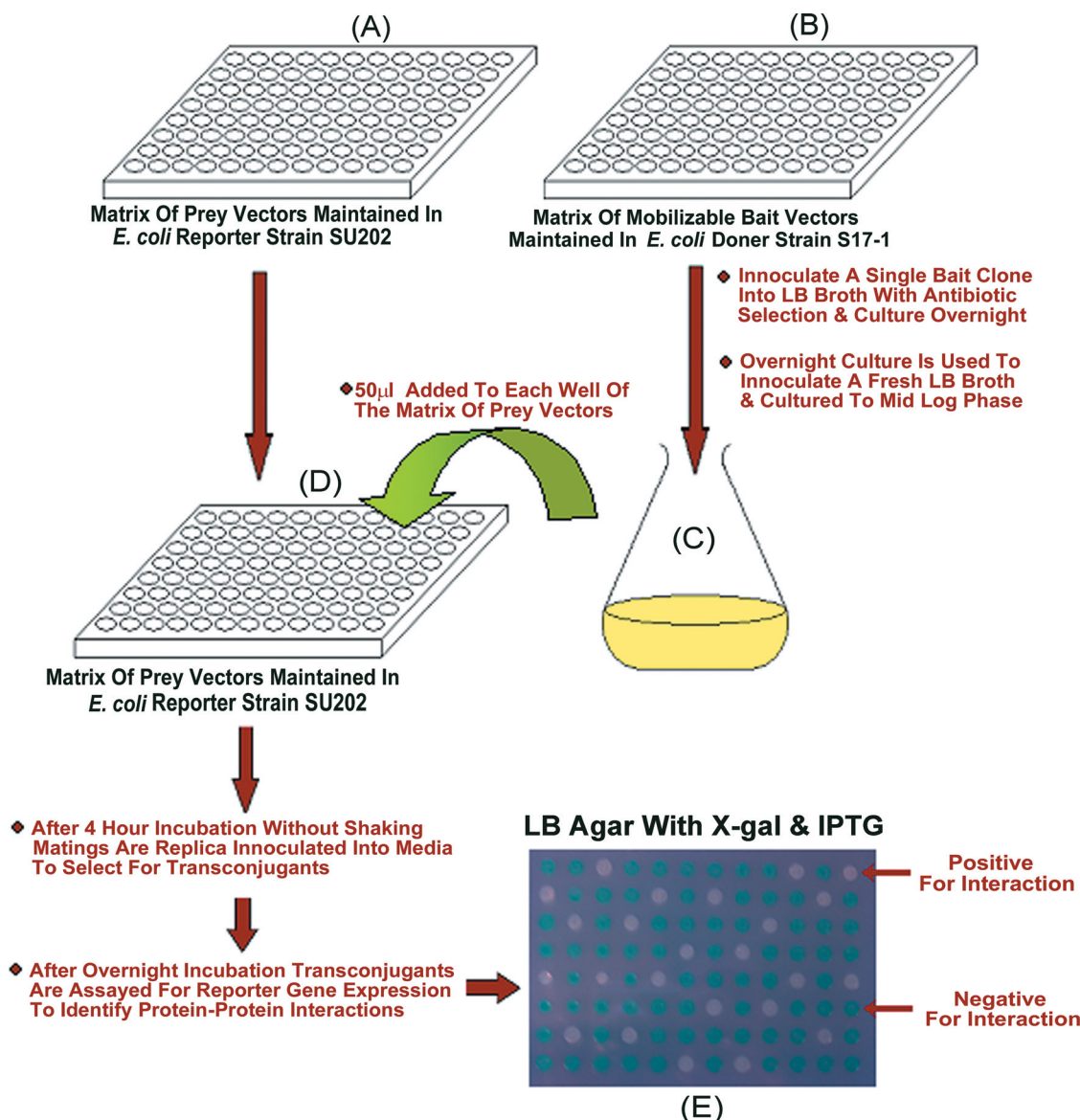


Figure 3. Exhaustive protein interaction mapping in *E. coli* using a P2H system and bacterial conjugation. In order to carry out exhaustive protein interaction mapping, matrices of Prey and Bait clones would be constructed by cloning PCR-amplified open reading frames (ORFs) into the Prey and Bait vectors for the P2H system. The cloning of amplified ORFs would initially be carried out using standard *E. coli* strains and once constructed Prey vectors would be transformed into the P2H systems reporter strain SU202 to form a matrix of recipient Prey clones (A), and Bait vectors would be transformed into an *E. coli* strain, such as S17-1, to form a matrix of donor Bait (B) clones, respectively. Each donor Bait clone would then mated with each Prey clone by simply culturing the Bait clone in bulk in LB broth (C) and adding an aliquot to each well of a freshly grown plate of recipient Prey clones (D). Following a 4 h incubation, matings would be replica inoculated into fresh plates to select for transconjugants, incubated overnight and finally a sample of each transconjugant would be spotted onto the surface of selective media to screen for protein-protein interactions. Above transconjugants exhibiting the jun and fos interaction, and thus harbouring plasmids pPC810 and pMS604, were detected by spotting onto LB IPTG X-gal indicator plates (E) and were identified as white culture spots and after 24 h incubation at 37°C. Transconjugants not exhibiting the jun and fos interaction, and thus harbouring plasmids pPC810 and pPC605, appeared as blue culture spots.

wild-type LexA DBD fused to the Fos leucine zipper (LexAWT-Fos). Plasmid pDP804 (39) expresses a chimeric protein consisting of a mutated LexA DBD, called LexA408, fused to the Jun leucine zipper (LexA408-Jun). The hybrid proteins on both of these plasmids are expressed from UV5lac promoters and thus expression can be induced using isopropyl- β -D-thiogalactopyranoside (IPTG).

Plasmid pJQ200-sk (49) carries an RP4 *mob* site and can thus be mobilized with high efficiency from an *E. coli*

strain, such as S17-1 described below. Plasmid pJQ200-NS (see Figure 4) was derived from plasmid pJQ200-sk. The NotI-SacII-SacI restriction sites of the multiple cloning site (MCS) of pJQ200-sk were eliminated by restricting the vector sequentially with NotI and then with SacI, filling in the resulting cohesive ends with Klenow and then re-ligating the blunt ends. This was carried out to permit the incorporation of these restriction sites as unique sites within a newly designed MCS. The pJQ200-sk plasmid and

Table 1. Bacterial strains and plasmids used in this study

<i>E. coli</i>	Relevant genotype			Reference
XL1-Blue	<i>recA1, hsdR17</i> (r _k ⁻ , m _k ⁺), <i>supE44, lac</i> , [F', <i>proAB</i> ⁺ <i>lacI</i> ^q , <i>lacZ</i> ΔM15::Tn10(Tet ^R)]			Stratagene
SU202	<i>lexA71::Tn5</i> (Def) <i>sulA211 ΔlacU169</i> , F' <i>[lacF⁺lacZ</i> ΔM15::Tn9]			(39)
S17-1	<i>thi pro res</i> ⁻ <i>mod</i> ⁺ Sm ^R Tp ^R <i>recA1</i> RP-4-2[Tc::Mu; Km::Tn7]			(45)
Plasmids	Origin	Resistance	Features	Reference
pMS604	ColE1	Tet ^r	<i>lexAWT-fos</i> construct	(44)
pPC605	ColE1	Tet ^r	<i>lexAWT-MCS</i> construct	This study
pDP804	p15a	Amp ^r	<i>lexA408-jun</i> construct	(39)
pJQ200ks	p15a	Gm ^r	<i>mob sacB</i>	(49)
pJQ200-NS	p15a	Gm ^r	<i>mob sacB</i>	This study
pPC810	p15a	Gm ^r	<i>mob sacB lexA408-jun</i> construct	This study
pPC811	p15a	Gm ^r	<i>mob sacB lexA408-MCS</i> construct	This study

pJQ200-NS also carry a *sacB* gene that is lethal to *E. coli* cells grown in the presence of 5% sucrose. *E. coli* strains cured of any plasmid carrying this gene can be easily selected by streaking colonies onto Luria–Bertani (LB) agar lacking antibiotic selection for the plasmid and containing 5% sucrose.

The *E. coli* strain S17-1 (45) was used as a donor strain for bacterial matings. This strain harbours a chromosomally integrated IncPα group plasmid RP4 encoding all of the transfer functions necessary for plasmid mobilization. These products can function *in trans* mediating the mobilization of any plasmid carrying a recognizable RP4 mobilizable element called a *mob* site.

The *E. coli* strain SU202 (39) was the reporter strain used for the detection of heterologous protein–protein interactions. The strain is both kanamycin resistant (due to the chromosomally inserted Tn5) and chloramphenicol resistant (due to the Tn9 inserted on the F' factor), but kanamycin was preferentially used for transconjugant selection. This is because the F factor does not carry a mutation in the *traD* gene and thus was found to be transferable during bacterial matings complicating transconjugant selection. The strain also carries a chromosomally integrated reporter gene consisting of a *lacZ* gene placed under the transcriptional control of a modified *sulA* promoter bearing a hybrid op⁴⁰⁸/op⁺ LexA operator sequence.

E. coli strains were routinely cultured on LB medium (50). Transformation was by the method of Inoue *et al.* (51) and *E. coli* XL1-Blue was used for routine transformation and plasmid preparation. Antibiotics were used at the following concentrations: 20 μg kanamycin ml⁻¹, 10 μg tetracycline ml⁻¹, 10 μg gentamicin ml⁻¹ and 50 μg streptomycin ml⁻¹. IPTG was used at a concentration of 1 mM for the induction of hybrid protein expression, and β-galactosidase activity was assayed by plating onto either LB agar containing IPTG and the chromogenic substrate X-gal at a concentration of 50 μg ml⁻¹ or onto MacConkey agar containing 10% lactose and IPTG (available from Oxoid).

Molecular biology techniques were performed using standard protocols (50). Plasmid DNA was isolated from cultures by the alkaline lysis method (52). PCRs were carried out using the temperature gradient block in a Thermo Hybaid PCR Express thermocycler. Restricted DNA fragments for sub-cloning were cut from ethidium bromide-stained gels as required and purified using the Perfectprep gel clean up kit as directed by the manufacturers (Eppendorf).

Construction of the mobilizable Bait vector pPC810 expressing a LexA408-Jun hybrid protein

The region of plasmid pDP804 carrying the entire *lexA408-jun* expression construct, including the *P_{lac} UV5* promoter region, was sub-cloned onto the mobilizable plasmid pJQ200-NS. Plasmid pDP804 was linearized by digestion with EcoRI, treated with Klenow to fill in the cohesive ends generated and subsequently digested with BglIII. The 524 bp blunt-BglIII *lexA408-jun* fragment was then gel purified. Plasmid pJQ200-NS was linearized by digestion with XhoI, the cohesive ends were filled by Klenow treatment and the vector was subsequently digested with BamHI. The blunt-BamHI pJQ200-NS plasmid was then ligated with the gel-purified *lexA408-jun* blunt-BglIII fragment and the resulting vector was named pPC810 (see Figure 4A).

Construction of the mobilizable Bait vector pPC811 facilitating genetic fusions with LexA408

A region of pDP804 extending from its unique SacII site to the end of the LexA408 DBD coding sequence was amplified using the following primers: LexA-F1, 5'-CCGCGGCCCTCTC-ACTTCCTTGTTAAGTATCTTCC-3' and LexA-R1, 5'-AG-ATCTAAGCTTCTAGACTCGAGACTAGTGAGCTCGGA-TCCTGGTTCACCGGCAGCCACACGACCTACC-3'. The reverse primer was designed to incorporate a MCS, terminating in a BglIII site, C-terminally to the *lexA408* sequence (underlined bases correspond to the added MCS sequence). The resulting 717 bp PCR product was cloned into the pCR2.1 vector of a TA cloning kit (Invitrogen) and verified by sequencing. The fragment was sub-cloned as a SacII–BglIII fragment back into the original pDP804 plasmid replacing the original *lexA408-jun* construct with a *lexA408-MCS* construct and the resulting plasmid was named pPC807 (see Figure 4B). The *lexA408-MCS* construct was then sub-cloned from plasmid pPC807 onto pJQ200-NS as follows. Plasmid pPC807 was linearized by digestion with EcoRI, treated with Klenow to fill in the cohesive ends generated and subsequently digested with XbaI. The resulting 426 bp blunt-XbaI *lexA408-MCS* fragment was then gel purified. Plasmid pJQ200-NS was linearized by digestion with XhoI, the cohesive ends were filled by Klenow treatment and the vector was subsequently digested with XbaI. The blunt-XbaI pJQ200-NS plasmid was then ligated to the gel-purified *lexA408-MCS* blunt-XbaI

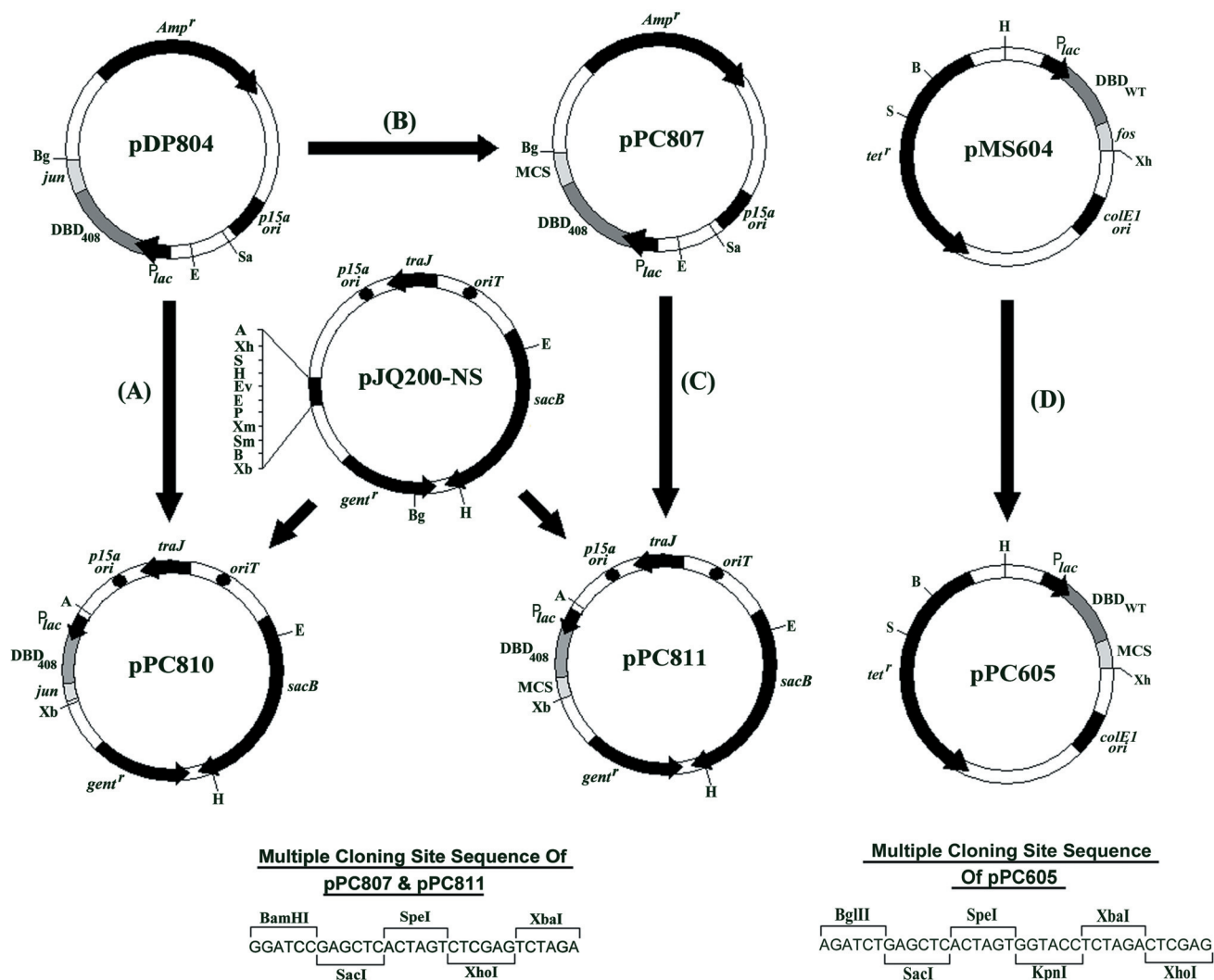


Figure 4. Schematic representation depicting the construction of novel mobilizable Bait vectors for use with a LexA repressor-based P2H system (see Materials and Methods for full construction details). Plasmids pDP804, pPC807, pPC810 and pPC811 all carry a LexA408 DBD coding sequence (DBD₄₀₈) while plasmids pMS604 and pPC605 carry a LexAWT DBD coding sequence (DBD_{WT}). Restriction sites are given as follows: BglIII (Bg), EcoRI (E), SacII (Sa), ApaI (A), XhoI (Xh), Sall (S), HindIII (H), EcoRV (Ev), PstI (P), XmaI (Xm), SmaI (Sm), BamHI (B), XbaI (Xb).

fragment and the resulting vector was named pPC811 (see Figure 4C).

Construction of a Prey vector pPC605 facilitating genetic fusions with LexAWT

A region of pMS604 extending from the unique HindIII site to the end of the LexAWT DBD coding sequence was amplified using the following primers; LexAW-F1: 5'-CTGTG-ATAAACTACCGCATTAAGCTTATCGAT-3' and LexAW-R1: 5'-CTCGAGTCTAGAGGTACCACTAGTGCAGCTCAGATCTGGTTCACCGGCAGCCACACGACCTACC-3'. The reverse primer was designed to incorporate a MCS, terminating in an XhoI site, C-terminally to the *lexAWT* sequence (underlined bases correspond to the added MCS sequence). The resulting 465 bp PCR product was cloned into the pCR2.1 vector of a TA cloning kit (Invitrogen) and sequenced. The fragment was then sub-cloned as a HindIII-XhoI fragment back into the original pMS604 plasmid replacing the original *lexAWT-fos*

construct with a *lexAWT-MCS* construct and the resulting plasmid was named pPC605 (see Figure 4D).

Performing *E. coli* surface matings for the detection of protein-protein interactions

Bacterial conjugation was carried out according to the following protocol. Donor S17-1 strains, carrying either pPC810 or pPC811, and recipient SU202 strains, carrying either pMS604 or pPC605, were inoculated into LB broth with appropriate antibiotic selection and incubated overnight at 37°C. Overnight donor and recipient cultures were used to inoculate fresh LB broth cultures without antibiotic selection and grown to mid-log phase (OD₆₀₀ 0.3–0.5). Donor and recipient cultures were then mixed in a 4:1 ratio (based on culture OD₆₀₀) in a 1.5 ml microfuge tube (usually 800 µl of donor culture with 200 µl of recipient culture) and the cells were pelleted by centrifugation. The pellet was resuspended in 40 µl of LB and spotted onto 0.45 µm filter paper placed on an LB agar

plate. After a 4 h incubation at 37°C, the filter paper was immersed in 2 ml of LB broth, agitated, the resulting cell suspension diluted and plated on selective media. Total recipient and total transconjugant counts were determined by plating on LB agar supplemented with kanamycin, tetracycline and LB agar supplemented with kanamycin, tetracycline and gentamycin, respectively. The transfer frequency (Tf) was then calculated as the number of transconjugants per recipient according to the equation:

$$Tf = \frac{\text{Total number of transconjugants}}{\text{Total number of recipients}}$$

For the detection of protein–protein interactions, 1 ml of the recovered post-mating cell suspension was inoculated into 9 ml of LB broth with appropriate antibiotics for selection of transconjugants and incubated, without shaking, overnight at 37°C. This was to permit outgrowth of transconjugants prior to the induction of hybrid protein expression, which was subsequently achieved by plating on selective media incorporating IPTG and X-gal to screen for protein–protein interactions.

Performing liquid mating in an automated 96 well format for detection of protein–protein interactions

Recipient SU202 strains carrying either pMS604 or pPC605 were inoculated into LB broth with appropriate antibiotic selection and incubated overnight at 37°C. These overnight cultures were then distributed in 150 µl aliquots into separate wells of a 96 well plate to prepare a matrix of recipient Prey clones that was stored at 4°C. The Prey matrix of recipient clones was replica inoculated into a 96 well plate with 50 µl of fresh LB broth in each well. The plate was then sealed using sterile pierceable adhesive aluminium foil and incubated overnight at 37°C on a microtitre plate shaker. A donor *E. coli* S17-1 strain carrying either pPC810 or pPC811 was inoculated into 5 ml of LB with appropriate antibiotic selection, and incubated overnight with shaking at 37°C. The overnight donor culture was then used to inoculate a fresh LB broth and incubated at 37°C until mid-log phase (OD₆₀₀ 0.3–0.5). Matings were prepared by transferring 50 µl of the donor culture to the wells of the Prey matrix of recipient clones. The 96 well mating plate was incubated at 37°C without shaking for 4 h, and then a 1 µl aliquot from each of the wells was transferred to the corresponding well in a fresh transconjugant selection plate. The transconjugant selection plate contained 150 µl of fresh LB media, with appropriate antibiotics for transconjugant selection (kanamycin, tetracycline and gentamycin for the selection of SU202 harbouring both a Prey and a Bait vector), in each well. The initial transconjugant selection plate was covered with Breatheasy sealing film (available from Greiner Ltd or Genetix Ltd) and then incubated overnight at 37°C with shaking. This initial transconjugant selection plate was used to replica inoculate a second fresh transconjugant selection plate to further purify transconjugants by transferring a 1 µl aliquot from each well of the initial transconjugant selection plate to its corresponding position in the second transconjugant selection plate. The media used in this round of selection also incorporated 1 mM IPTG to induce the expression of hybrid genes from the Prey and Bait vectors. Following incubation at 37°C overnight, a sample of culture from each well was spotted, using a autoclavable

polypropylene 96 well replication tool (available from Sigma Aldrich Ltd), onto the surface of indicator plates, which could be either LB agar incorporating appropriate antibiotic selection, IPTG and X-gal or MacConkey agar incorporating 10% lactose and IPTG to test for protein–protein interactions. Indicator plates were incubated at 37°C overnight and the following day the positions in the matrix in which the protein–protein interactions occurred were identified by inspection of culture spot colour (on LB agar incorporating IPTG and Xgal: white spots for protein–protein interactions and blue if there were no interactions; on MacConkey agar: white/pink for protein–protein interactions or magenta if there were no interactions). It was found to be important that cultures be grown in the presence of IPTG prior to spotting onto the final indicator media. In the LexA-based system, positive protein–protein interactions between hybrid proteins result in repression of a constitutively expressed *lacZ* reporter system, and SU202 reporter strain cells are therefore phenotypically Lac positive in the absence of hybrid gene expression. Culturing in IPTG allows degradation of any pre-existing β-galactosidase enzyme prior to spotting onto indicator plates to evaluate reporter gene expression and identify interactions. All of the above liquid transfer steps, mating setup and transconjugant selections, were carried out by the RoboAmp automated liquid handling system (available from MWG-Biotech Milton Keynes) but can be carried out manually using autoclavable 96 pin replication tools available from Genetix Ltd.

RESULTS AND DISCUSSION

Construction of novel mobilizable P2H system vectors: exploitation of bacterial conjugation for P2H system screening

In order to detect protein–protein interactions using the P2H systems described to date, both the Prey and Bait vectors for the systems have to be introduced into an appropriate *E. coli* reporter strain by bacterial transformation. The resulting transformants are then assayed for reporter gene activation/repression or a reconstituted enzyme activity in order to detect protein–protein interactions. We proposed that bacterial conjugation could be exploited as a technically simplified and more efficient means of introducing plasmids in combination to test for protein–protein interactions. The IncPα plasmid RP4 is a self-transmissible antibiotic resistance plasmid. It has been demonstrated that *E. coli* plasmids carrying a *cis*-acting mobilization (Mob) region can be transferred between *E. coli* strains, and into many other gram-negative bacteria, using the RP4-specific mobilization system with transfer efficiencies approaching unity when matings are carried out under optimal conditions (45,53). These mobilizable plasmids have mainly been used as suicide vectors for introducing transposons and cloned genes into non-*E. coli* hosts for mutagenesis and expression studies (49,54,55).

To permit the exploitation of the RP4 plasmid mobilization system for P2H system screening procedures, we have constructed two novel mobilizable Bait vectors for use with the LexA repressor-based P2H system developed by Dmitrova and co-workers (39). A fragment of pDP804, carrying the entire *lexA408-jun* expression construct, was cloned into plasmid pJQ200-NS to form plasmid pPC810. We also constructed

a second plasmid, pPC811, in which the *jun* sequence was replaced with a MCS by cloning a PCR-amplified *lexA408-MCS* expression construct into plasmid pJQ200-NS. Plasmid pJQ200-NS was derived from pJQ200-sk and therefore carries an RP4 mobilizable element, the *mob* site. This site permits the efficient mobilization of this plasmid, and therefore the new Bait plasmids pPC810 and pPC811, from a donor strain of *E.coli* such as S17-1. This strain of *E.coli* carries a chromosomally integrated RP4 encoding all of the required transfer functions and its use ensures that once a plasmid has been mobilized to a recipient strain it cannot be further mobilized. An alternative strategy for mobilizing plasmids would be to use a helper plasmid, such as pRK600 (56). The helper plasmid also carries all of the transfer functions required to mobilize plasmids and can be used to mobilize plasmids out of any *E.coli* strain. However, in addition to mobilizing the desired plasmid, it is also transmissible. We preferred to use the S17-1 strain as a donor because it avoided any complications arising as a result of the helper plasmid entering the recipient.

The Bait plasmids pPC810 and pPC811 can be mobilized with high frequency into SU202 reporter cells already harbouring a Prey vector

Having constructed the novel mobilizable Bait vectors pPC810 and pPC811, *E.coli* surface matings were carried out to demonstrate that these plasmids could be mobilized into the *E.coli* SU202 reporter strain, already harbouring a Prey vector, to detect protein–protein interactions. Matings were prepared to mobilize pPC810 into SU202 harbouring either the pMS604 Prey plasmid (expressing a LexAWT-Fos hybrid protein) or the pPC605 plasmid (in which the *fos* sequence had been replaced by a MCS sequence and therefore only expressed a LexAWT DBD). The pPC811 plasmid was mated in the same manner to verify all four possible combinations of the Bait and Prey vectors. Surface matings were undertaken by mixing mid-log phase donor and recipient cultures in a ratio of 4:1 based on the culture OD₆₀₀ values. It was expected that the use of excess donor in this way would maximize the transfer frequencies ensuring that every recipient cell would receive a copy of a Bait vector. Matings were prepared in duplicate with one mating of each duplicate being removed and plated on selective media after 4 h incubation at 37°C, while the second mating was incubated overnight. Very high transfer efficiencies of $3\text{--}5 \times 10^{-1}$ were achieved for the 4 h matings. For the duplicate matings that had been incubated overnight, there appeared to be a significant reduction in the transfer efficiencies obtained compared with those observed for the 4 h matings. The explanation for this apparent drop in transfer frequency is that transconjugants initially grow considerably slower than either the recipient or donor strains, requiring a period of outgrowth to permit plasmid establishment after mating. From the observed transfer frequencies for the 4 h matings, it was obvious that not all recipient cells received a copy of a Bait plasmid. It is therefore likely that when matings are incubated for prolonged periods, the recipient cells outgrow the transconjugants and, as transfer frequency is calculated using total transconjugant and total recipient counts, there is therefore an apparent drop in the calculated transfer frequency. From this experiment, it was

concluded that a 4 h incubation was sufficient for carrying out highly efficient bacterial matings.

Having demonstrated that the novel Bait vectors could be mobilized into reporter SU202 cells already harbouring Prey vectors, we had to demonstrate that the resulting transconjugants displayed a β -galactosidase phenotype consistent with the combination of Prey and Bait plasmids they possessed. We found that when recovered mating cell suspensions were immediately plated on media selecting for both transconjugants and screening for protein–protein interactions simultaneously, growth and colour development, particularly of interaction negative and therefore Lac⁺ clones, required prolonged incubation periods of 2 days. We found that better results were obtained when selection of transconjugants and induction of hybrid protein expression for the detection of interactions was carried out sequentially. Recovered mating cell suspensions were therefore subjected to an outgrowth period in transconjugant selective LB broth lacking IPTG prior to plating on selective media incorporating IPTG to induce hybrid protein expression and X-gal to permit detection of protein–protein interactions. All of the transconjugants displayed the expected phenotype, i.e. those harbouring a combination of pPC810 with pMS604 and thus co-expressing both a LexA408-Jun and LexAWT-Fos hybrid proteins resulted in white colonies indicative of protein–protein interactions, while all other vector combinations gave blue colonies indicating no protein–protein interactions.

Bacterial conjugation can be used for highly efficient library screening

It has been reported that when performed under optimal conditions, the frequency of transfer of RP4 mobilizable vectors can approach unity (53). In our mating experiments, where matings were carried out using log phase cultures of both donors and recipients, transfer frequencies of $\sim 3\text{--}5 \times 10^{-1}$ were routinely observed which equivocates to one in every two to three recipient cells successfully receiving a copy of a Bait vector. This means that the bacterial mating should permit the screening of complex libraries of pooled Prey clones to identify interacting partners for a defined Bait protein with an efficiency far greater than that attainable using bacterial transformation. Such mating strategies are also technically simpler to carry out, as cells do not have to be made competent and transformed.

In order to simulate a library screen, libraries of Prey clones were prepared. The OD₆₀₀ of two overnight cultures, SU202-pMS604 and SU202-pPC605, were measured and then the cells were pelleted and resuspended in saline solution to an OD₆₀₀ of 0.4. A set of serial dilutions of the SU202-pMS604 culture, down to 10^{-3} , was prepared using the SU202-pPC605 culture as the diluent. The resulting 10^{-3} dilution therefore represents a library of SU202 Prey clones, in which an interacting partner, LexAWT-Fos encoded by plasmid pMS604, occurs at a known frequency of 1:1000 among non-interacting species represented by clones harbouring the pPC605 plasmid and thus only expressing the LexAWT DBD. This library was screened by carrying out a surface mating with an S17-1 donor strain carrying plasmid pPC810. Recovered matings were initially plated to determine the transfer frequency for the mating and an optimal dilution

factor that would give ~500 transconjugants per plate. A 1 ml aliquot of the recovered mating was also inoculated into 9 ml of broth excluding IPTG to permit outgrowth of transconjugants. After the overnight outgrowth step, the transconjugant culture was diluted and a volume equivalent to 10 000 transconjugants plated over 20 plates of selective media to yield a plating density of ~500 colonies per plate and to screen for protein–protein interactions. White clones were recovered at a 5-fold higher frequency than expected. This was due to proliferation of transconjugants during the overnight outgrowth step evident from the higher than expected total transconjugant counts observed on plates. It was always observed that when plated on solid selective media to screen for protein–protein interactions, white clones, expressing interacting pairs of hybrid proteins, grew more rapidly than blue clones expressing non-interacting pairs of hybrid protein. This is likely to be due to the fact that in these clones high-level expression of β -galactosidase is repressed as a result of protein–protein interactions and they therefore express one less high molecular weight protein and have a growth advantage over Lac^+ clones in which no interactions occur. The overnight outgrowth step was carried out using broth excluding IPTG and one might therefore expect that, in the absence of hybrid protein expression, all transconjugants would proliferate in an unbiased manner and that the frequency of white to blue clones would therefore be unaffected by such proliferation. However, even in the absence of IPTG, there is hybrid gene expression (data not shown) and thus there would be some positive selection for clones harbouring interacting pairs of proteins during the outgrowth step resulting in the culture becoming enriched for such clones. The implication of the growth advantage exhibited by transconjugants harbouring positive protein–protein interactions is, as observed in the above experiment, that a disproportionate number of positive white colonies may be recovered when performing library screens due to amplification of a single transconjugant harbouring a single interacting protein pair. In addition, the extent of the growth advantage would probably depend on the strength of a particular protein–protein interaction, and therefore if a particular Bait protein used in a library screen had multiple interacting partners, there may be further enrichment for the strongest interacting pair. However, these effects could be very simply overcome by reducing the duration of the outgrowth step thus minimizing transconjugant amplification, and the above experiment clearly demonstrated that *E.coli* mating-based strategies using our novel mobilizable Bait vectors could be used to carry out efficient library screening for the identification of protein–protein interactions.

The *sacB* gene facilitates recovery and identification of Prey plasmids isolated during library screening and verification of protein–protein interactions

When clones exhibiting positive protein–protein interactions are obtained during library screening using Y2H systems, the identity of the cloned DNA on the Prey vector has to be determined. This often requires rescuing the Prey vector, via transformation into *E.coli*, and then sequencing the cloned DNA. The interaction is then verified again by re-introducing the recovered Prey vector into a fresh yeast reporter strain along with the Bait vector. Similar steps have to be taken when P2H systems are used for carrying out library screens

using transformation-based protocols. We proposed that the *sacB* gene present on our conjugative P2H system Bait vectors would facilitate the simplified rescue of Prey vectors after library screening and verification of identified protein–protein interactions.

To verify that the white clones retrieved in our library screen did in fact contain the pMS604 Prey plasmid, expressing the LexAWT-Fos protein, 12 random white clones were picked and cured of the pPC810 plasmid by exploiting the presence of the *sacB* gene on this plasmid. Three blue clones were also cured in the same way as controls. Clones were picked and streaked on media selecting only for the Prey vector and containing 5% sucrose to induce loss of the Bait vector. Loss of the Bait vector was verified on the basis of gentamycin sensitivity. Plasmid DNA was isolated from each of the clones and the identity of the plasmid in each clone verified by carrying out a BamHI–BglII double digest that permits the distinction of pMS604 from pPC605. All of the white clones were verified as having the pMS604 Prey plasmid and all of the blue clones were verified as having the pPC605 plasmid.

While curing in the manner described above provides a simple and fast way of obtaining the Prey vector in isolation for identification either by restriction analysis or by sequencing, it also permits verification of protein–protein interactions. If the observed Lac^- phenotype of a transconjugant clone is due to protein–protein interactions between hybrid proteins encoded by the Prey and Bait vectors, and not due to a mutational event in the *E.coli* cell, then curing the clone of the Bait vector should result in a reversion to a Lac^+ phenotype. This was demonstrated by streaking all of the cured clones on selective LB agar incorporating IPTG and X-gal and observing that all clones generated blue colonies. If further validation is required, cured clones can subsequently be re-mated to re-introduce the original Bait vector.

Bacterial conjugation can be used for exhaustive automated protein interaction mapping

In addition to permitting highly efficient and technically simplified screening of libraries of pooled Prey clones, the mobilizable Bait vectors should also permit the implementation of the LexA-based P2H system for automated exhaustive protein interaction mapping strategies analogous to those developed for Y2H systems. Libraries of Prey and Bait clones can be constructed, the Prey constructs being transformed into and maintained in the *E.coli* SU202 reporter strain and Bait constructs being transformed into and maintained in *E.coli* S17-1. Each of these clone libraries can be maintained in a matrix format, either in 96 well or 384 well formats, where the identity of each clone at each position in the matrices is known. Each clone in the Bait library matrix can then be mated with each clone in the Prey library in a matrix and the resulting transconjugants tested for reporter gene expression to detect protein–protein interactions (see Figure 3). This exhaustive screening in a matrix fashion reveals all possible interactions between proteins in the Prey and Bait libraries. To demonstrate the feasibility of this strategy, we prepared a matrix of Prey clones by distributing cultures of SU202 harbouring either pMS604 or pPC605 into individual wells of a 96 well plate. This master matrix plate was used to replica inoculate fresh

96 well plates for use in mating experiments. These simply involved the addition of logarithmic S17-1-pPC810 culture to each well of the Prey matrix to setup liquid matings. Following two rounds of transconjugant selection and purification, transconjugants were spotted onto the surface of indicator media, to screen for protein–protein interactions. These were identified according to the colour of colony spots after overnight incubation, and every occurrence within the matrix of SU202 clones harbouring the pMS604 plasmid (and thus expressing the LexA-Fos hybrid) was successfully identified (see photo insert in Figure 3).

In the above matrix experiment, liquid matings were employed with all of the steps being carried out using a RoboAmp liquid handling system. However, bacterial matings could in fact be carried out using a range of protocols including those developed for carrying out yeast matings. For example, in the protocol described by Cagney *et al.* (46) and employed by Uetz *et al.* (21), Bait clones were first spotted onto the surface of solid media and, having been allowed to dry, Preys were then spotted on top of the Baits and the plates incubated to allow mating. It is envisaged that carrying out bacterial matings in such a manner would further increase the efficiency and speed with which interaction mapping could be undertaken.

CONCLUSIONS

The Y2H system has been widely implemented for the detection and analysis of protein–protein interactions. Through the exploitation of yeast mating types, technically simple and automatable strategies have been developed, permitting its application to high-throughput and exhaustive proteomic interaction mapping. It has therefore become an important component technology in the field of functional genomics helping to further our understanding of complex biological processes, annotate genome sequences and facilitating the identification of novel therapeutic targets.

P2H systems present many advantages over yeast-based technologies, which largely derive from the ease with which *E.coli* can be genetically manipulated, the lack of cellular compartmentalization, its faster growth rate and the higher transformation efficiencies that are attainable permitting rapid and more efficient screening of complex libraries. The systems permit the investigation of prokaryotic protein–protein interactions in a prokaryotic genetic background, but they can also be used for the analysis of eukaryotic proteins. This may be particularly desirable in circumstances where homologous yeast proteins interfere with an interaction by interacting with and sequestering one of the interacting partners leading to false negatives, or by acting as a bridge between two proteins leading to false positives. The absence of such homologous eukaryotic proteins in *E.coli* may result in the observation of less false positives and negatives. Transcriptional activation in *E.coli* differs fundamentally from the mechanism in yeast and therefore false positives that occur with the Y2H system as a result of reporter gene activation by non-specific acidic activator sequences may not occur in *E.coli*-based P2H systems. *E.coli*-based systems can also permit analysis of eukaryotic proteins that are toxic when expressed in yeast because they interfere with the function of

yeast homologues. However, it is important to note that while P2H systems have many advantages, they do suffer from many of the same problems encountered by other hybrid technologies. Whenever fusion proteins are used for the detection of protein–protein interactions, there is the possibility that sites required for interaction may be occluded by the fused moieties. In addition, P2H systems may not be suitable for the analysis of all eukaryotic proteins due to problems associated with expression, stability and incorrect folding of eukaryotic proteins in bacterial cells. P2H systems should therefore be considered as a complementary technology to the Y2H system.

Despite their many proposed advantages, P2H systems have not yet found the same widespread application to large-scale protein interaction mapping as Y2H systems. One of the key advantages of *E.coli*-based systems is the higher transformation efficiencies attainable which promise more efficient library screening for identifying protein–protein interactions. However, although efficient, bacterial transformation is not particularly amenable to automated strategies. In an era where high-throughput automatable techniques are becoming ever more important in order to permit the full exploitation of the vast amounts of genomic sequence data, the technical simplicity of Y2H mating-based strategies has meant that the Y2H system has remained the preferred approach. We have constructed novel mobilizable P2H system Bait vectors for the LexA-based P2H system originally developed by Dmitrova and co-workers (39). We have developed *E.coli* mating-based strategies employing these vectors, demonstrating how they can be used for carrying out library screens to identify protein–protein interactions with an efficiency greater than that attainable with either bacterial transformation or yeast-mating strategies. Construction of similar mobilizable vectors for a P2H system permitting direct selection for protein–protein interactions (28,29,35) should permit even more efficient library screening than that possible with systems that employ genetic screening for interactions rather than selection where plating densities are restricted (30,33,39). The added benefit of the *sacB* gene on our Bait vectors permits simple recovery of Prey vectors for the identification of cloned insert DNA and the verification of identified protein–protein interactions. We have also demonstrated how the technical simplicity of *E.coli* mating-based P2H strategies permits their automation and facilitates exhaustive protein interaction mapping using matrices of Bait and Prey clones.

The P2H system developed by Joung *et al.* (31) permits the use of highly efficient phage transduction to combine Bait and Prey vectors to test for protein–protein interactions and could potentially be automated to facilitate high-throughput protein interaction mapping, although no such application has yet been reported. However, the fact that the strategies we have described here are analogous to those that have been developed for implementing Y2H mating-based strategies should give our conjugative LexA-based P2H system the added benefit of permitting its implementation for high-throughput proteomic interaction mapping using similar automated platforms and protocols to those already developed for carrying out automated Y2H mating-based strategies. In addition, while the vectors we have constructed are for use with the LexA-based P2H system, the work reported here demonstrates how through the simple incorporation of a *mob* element similar mobilizable vectors could be constructed

for any of the previously described P2H systems broadening their applicability by permitting their implementation for high-throughput protein interaction mapping in a similar manner. Such applications of P2H systems will permit further evaluation of the potential strengths and weaknesses of each of these systems. The high efficiency of bacterial mobilization could be further exploited to facilitate the screening of highly complex peptide aptamer libraries to identify peptides capable of specifically disrupting designated protein–protein interactions in a similar manner to that described by Colas *et al.* (2). Ultimately, the use of mobilizable vectors and *E.coli* mating-based strategies may finally allow P2H systems and all of the proposed advantages of *E.coli*-based P2H systems to be fully exploited helping to increase the speed and efficiency with which protein interaction mapping can be undertaken for the determination of protein functions and the identification of novel therapeutic targets.

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