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Genetic Analysis of the *Escherichia coli* FtsZ·ZipA Interaction in the Yeast Two-hybrid System

CHARACTERIZATION OF FtsZ RESIDUES ESSENTIAL FOR THE INTERACTIONS WITH ZipA AND WITH FtsA*[S]

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The recruitment of ZipA to the septum by FtsZ is an early, essential step in cell division in *Escherichia coli*. We have used polymerase chain reaction-mediated random mutagenesis in the yeast two-hybrid system to analyze this interaction and have identified residues within a highly conserved sequence at the C terminus of FtsZ as the ZipA binding site. A search for suppressors of a mutation that causes a loss of interaction (*ftsZ*^{D373G}) identified eight different changes at two residues within this sequence. *In vitro*, wild type FtsZ interacted with ZipA with a high affinity in an enzyme-linked immunosorbent assay, whereas FtsZ^{D373G} failed to interact. Two mutant proteins examined restored this interaction significantly. *In vivo*, the alleles tested are significantly more toxic than the wild type *ftsZ* and cannot complement a deletion. We have shown that a fusion, which encodes the last 70 residues of FtsZ in the two-hybrid system, is sufficient for the interaction with FtsA and ZipA. However, when the wild type sequence is compared with one that encodes FtsZ^{D373G}, no interaction was seen with either protein. Mutations surrounding Asp-373 differentially affected the interactions of FtsZ with ZipA and FtsA, indicating that these proteins bind the C terminus of FtsZ differently.

In bacteria, the study of cell division has defined many genes active in the formation and cleavage of a septum (1–3). Currently, the earliest known step in the development of the septum is the formation of the Z-ring, a circular polymeric structure formed by the tubulin-like protein FtsZ (4). The cell division machinery assembles on the Z-ring in a sequential manner. Two proteins that act early in cell division, and directly on the Z-ring, are FtsA and ZipA (5–10). The structure of FtsA has been solved recently (11), and it supports previous indications that it is similar to actin (12, 13). FtsA may function by linking septum formation to peptidoglycan synthesis (8, 14). ZipA is an integral membrane protein that causes FtsZ fibers to bundle *in vitro* (15, 16). ZipA is thought to stabilize the FtsZ rings, in part because moderate overexpression of *zipA* can suppress the *ftsZ84* allele, which confers a defect in Z-ring formation (16). The structure of the highly conserved C-terminal fragment of ZipA has been solved by x-ray crystallography and NMR, and it shows conservation to several RNA-binding proteins (17, 18).

Genetic analyses of cell division have identified many genes as conditional alleles, but detailed genetic analysis describing an individual protein·protein interaction is difficult, because many interacting proteins are involved, often simultaneously (19–21). Because of this, it is advantageous to develop a surrogate system that allows the study of a protein·protein interaction through genetic analysis (*i.e.* the study of a protein·protein interaction through the identification and characterization of mutations). The two-hybrid system of yeast (Y2H)[1] is one such system, because of the wide range of genetic techniques that exist for yeast (22, 23) and because the two-hybrid system itself is a robust system for characterizing protein·protein interactions *in vivo* (24). Thus, it is a relatively simple extension of yeast genetic analysis to examine a two-hybrid interaction genetically. Previous work has indicated that the C terminus of FtsZ is important for the interaction with both ZipA and FtsA (9, 10, 15, 21, 25). To better define the interaction between FtsZ and ZipA, we have analyzed this interaction in yeast genetically. Using a mutation in *ftsZ* that reduces the interaction with *zipA* in the Y2H system, we have searched for intragenic suppressors (additional mutations within *ftsZ* that reverse the phenotype of the initial loss-of-function mutation). This search has identified mutations within a small segment of *ftsZ* that encodes a conserved sequence at the C terminus. We have characterized the effect of these mutations on the interactions with ZipA and FtsA and determined the effect of the mutations on cell division.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Oligonucleotides—All strains and plasmids used in this study are listed in Table 1s. Oligonucleotides are listed in Table 2s. Both tables are published as supplemental material in the online version of this paper.

Media and Reagents—Yeast and bacterial media were prepared by standard methods using materials readily available (22, 23). SC, YPD and other general yeast media are described in these references. YNB, BactoAgar, BactoTryptone, BactoPeptone, and yeast extract were purchased from Difco. Amino acid mixtures (CSM-LUTH, CSM-AHT), raffinose, glucose, and galactose were purchased from Bio101. Amino

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[S] The on-line version of this article (available at http://www.jbc.org) contains Tables 1s and 2s.

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[1] The abbreviations used are: Y2H, yeast two-hybrid system; FOA, 5′-fluoroorotic acid; YPD, yeast-peptone media with glucose; SC, synthetic complete media, YNB, yeast nitrogen base; AT, 3-amino triazole; bp, base pair(s); ELISA, enzyme-linked immunosorbent assay; IPTG, isopropyl-β-D-thiogalactopyranoside.

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acids, 5′-fluoroorotic acid (FOA), 4-methylumbelliferyl β-D-galactoside, and aminotriazole were purchased from Sigma Chemical Co. Zymolyase was purchased from ICN Biologicals.

Construction of Plasmids—All oligonucleotides used in this study are listed in Table 2s. FtsZ and ZipA were cloned by PCR amplification of genomic DNA from *Escherichia coli* strain MG1655. Wild type *ftsZ* cloned into pGAD424 was done by PCR amplification of the *ftsZ* gene from pDR3 using the oligos FtsZ-5′ and FtsZ-3′. The amplified PCR product was digested with *Mfe*I and *Sal*I and cloned into pGAD424 that was digested with *Eco*RI and *Bam*HI. *zipA* was amplified by PCR using oligos ZipA-5′ and ZipA-3′. The ZipA-5′ oligo results in a PCR product that deletes the membrane-spanning portion of the ZipA gene product. The resulting fragment was digested with *Eco*RI and *Sal*I and ligated into pLexA, to generate plasmid pSH47. This fragment was subsequently excised and cloned into pGAD424, which was digested identically to generate plasmid pSH230. The plasmid pSH228 was constructed by PCR amplification of plasmid pSH256 with oligos FtsZ-5′(311) and FtsZ-3′. The resulting fragment was digested with *Eco*RI and *Sal*I and cloned into pAS2-1. pSH229 was constructed in an identical manner, except that pSH41 was used as the template for the PCR reaction. pSH100 was constructed by PCR amplification of pAS1-*ftsA*, which was generously provided by Sandy Silverman, with oligos FtsA-5′ and ADHt. The resulting fragment was digested with *Eco*RI and *Sal*I and ligated into pAS2-1. Plasmid pSH232 was constructed by subcloning the *ftsA* gene into pGAD424, which had been digested with *Eco*RI and *Sal*I as well.

Construction of Yeast Strains—Strain SHy9 was generated by growing strain CG 1945 serially for two 10-ml overnight cultures, with about a 10^5 cell inoculum each, and plating on SC plates supplemented with 0.1% FOA, and colonies were allowed to grow for 5 days. Several colonies that grew were rechecked for all phenotypes, including loss of *GAL1p-lacZ* reporter activity. Strains SHy22 and SHy23 were generated by introducing pHO (kindly provided by Kim Arndt) into strain SHy9 and growing a transformant in 10 ml of SC-URA overnight. Cells were streaked onto a YPD plate and allowed to grow for 3 days. This plate was then replica printed onto an SC plate supplemented with 0.1% FOA. Colonies from this plate were patched onto a new YPD plate, grown overnight, and replica-printed onto an SPO plate. This plate was incubated at room temperature for 24 h and then at 30 °C for 5 days. Patches producing asci were then incubated with Zymolyase, and spores were separated by the random spores technique and plated on YPD (29). SHy22 and SHy23 are strains from spores of the same patch and differ only by mating type.

PCR-mediated Mutagenesis and Selection of Mutations—Mutations in FtsZ were generated by PCR amplification of pSH27 (pGAD424-*ftsZ*^{D45N,D373G}) using *Taq* DNA polymerase and reaction conditions that favored the incorporation of mutations. Oligonucleotides used as primers for amplification were GAL4ad and ADHt. These primers annealed to the GAL4 activation domain and the ADH terminator regions, respectively, and produced a PCR product that included about 300 bp of sequence on either side of the *ftsZ* gene. These regions of homology allowed for homologous recombination of the PCR fragment when cotransformed, with pGADGH vector DNA that had been linearized by digestion with *Eco*RI and *Bam*HI, into SHy63 (30). Recombinants were selected by leucine prototrophy. In this work, cotransformation with 100 ng of plasmid DNA and PCR fragment (each) resulted in about 1000 colonies, whereas transformation by either the vector or the PCR fragment alone resulted in zero to four colonies when plated onto LT plates. The interactions between the mutagenized *ftsZ* gene and *zipA* were tested by replica printing the transformants onto LHT plates supplemented with 0.5 mM AT. Colonies that grew after 3 days when incubated at 37 °C were scored as hits. Each colony was grown as a 5-ml culture and prepared using the Qiagen Qiaprep Turbo 8 miniprep kit as described by the manufacturer, except that 0.5 mg of Zymolyase was added per ml of buffer P1, and samples were incubated for 30 min at 30 °C in this solution. These preparations were used to transform *E. coli* strain KC8 by electroporation, and colonies were selected by growth on M9 plates that were supplemented with 50 mg/liter ampicillin, but lacked leucine, as described by Golemis *et al.* (31). Two to four colonies from each transformation plate were grown and prepared with the Qiagen miniprep kit again, this time without modification to the manufacturer's instructions. DNA from these preparations were analyzed by restriction analysis, and reconfirmation of the phenotypes was achieved by transforming into SHy23 again and rescoring the AT resistance.

Site-directed Mutagenesis of Plasmids for Expression in Yeast and Bacteria—Mutations identified in the previous section were further characterized by introducing the identified mutation into a pGAD424-*ftsZ* construct. This was accomplished by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit by Stratagene. Mutagenesis was performed as described by the manufacturer. Oligonucleotides used in this work were: D373G/T, D373G/B; D373S/T, D373S/B; D373G, P375L/T, D373G, P375L/B, as listed in Table 2s. For the alanine-scanning mutations, site-directed changes were introduced into pGAD-*ftsZ*^{D373G} (pSH-201). The mutation that results in a change from D to G at position 373 also results in a loss of the *Eco*RV restriction site. Each of the oligo pairs encode a change that restores this restriction site, in addition to the change at the codon to be changed to alanine. The oligos used were: D370A/T, D370A/B; Y371A/T, Y371A/B; L372A/T, L372A/B; F377A/T, F377A/B; L378A/, L378A/B; R379A/T, R379A/B; K380A/T, K380A/B; Q381A/T, Q381A/B. Candidate clones were screened for the reacquisition of the *Eco*RV site.

Purification of FtsZ and ZipA and Assay of the FtsZ·ZipA Interaction in Vitro—The wild type and mutant FtsZs were expressed with the N-terminal biotin tag MAGGLNDIFEAQKIEWH (32) to enable detection in an ELISA. The lysine in this sequence is biotinylated *in vivo* by the *E. coli* enzyme BirA. Plasmids were constructed by inserting the coding sequence for the biotin tag between the *Nco*I and *Nde*I sites of pET28 (Novagen) using the oligos BIOTAG/T and BIOTAG/B. In addition, *birA* was amplified from the plasmid pBIOTRX-BirA (26) by PCR using the oligos BirA 5′ and BirA 3′, digested with *Hin*D III and *Xho*I and ligated into the *Hin*dIII and *Xho*I sites of the same vector as the biotin tag to give the vector pETbio-birA. The genes *ftsZ*, *ftsZ*^{D373G}, *ftsZ*^{D373S}, and *ftsZ*^{D373G,P375L} were subcloned from the vectors pDB312, pEG028, pSH187, or pSH189, respectively, into the *Nde*I and *Hin*dIII sites of pETbio-birA to give plasmids pEG045, pEG051, pEG052, and pEG053.

Biotin-FtsZ and its mutants were expressed in the *E. coli* strain BL21(DE3)pLysS. Expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) once the A_{600} of the culture was between 0.5 and 1.0. At the same time, D-biotin was added to a final concentration of 0.1 mM. Cells were incubated at 37 °C for another 2–3 h, centrifuged, and resuspended in buffer A (50 mM Tris, pH 7.9, 50 mM KCl, 1 mM EDTA, and 10% glycerol) and stored at −70 °C. The proteins were then purified according to a previous report (33).

Protein concentrations of biotin-FtsZ and its mutants were determined by the Bradford method. The extent of incorporation of biotin was determined by measuring the displacement of 2-(4′-hydroxyazobenzene)benzoic acid from avidin. In short, 40 μl of protein sample or buffer was mixed with 360 μl of 0.5 mg/ml avidin and 0.3 mM 2-(4′-hydroxyazobenzene)benzoic acid in 100 mM sodium phosphate, 150 mM NaCl, pH 7.2. The decrease in absorbance at 500 nM was measured, and the concentration of biotin was determined using $\Delta\epsilon_{500} \times 10^{-3} = 34$. The biotin-tagged FtsZs were between 50% and 75% biotinylated.

ZipA-(23–328) was overexpressed from the plasmid pDB348 in BL21(DE3)plysS. Expression was induced as for biotin-FtsZ, above, and the cells were centrifuged, resuspended, and stored similarly. At the time of purification, the cells were thawed, phenylmethylsulfonyl fluoride was added to 1 mM, and the cells were lysed by passage through a French press. The cell extract was clarified by centrifugation at 100,000 × *g* for 1 h, and ZipA-(23–328) was precipitated by adding ammonium sulfate to 35% saturation. The ammonium sulfate pellet was dissolved in buffer A and dialyzed against buffer A overnight. ZipA-(23–328) was purified to homogeneity by passage over a Mono-Q column (Amersham Pharmacia Biotech) and elution with a 50–230 mM gradient of KCl in buffer A. The protein concentration of ZipA-(23–328) was determined as described according to Gill and von Hippel (34).

The interaction between ZipA-(23–328) and biotin-FtsZ and its mutants was assayed in an ELISA format. ZipA-(23–328) was immobilized in the wells of an Immulon 4HBX 96-well plate in 50 mM Tris, pH 8.5, 100 mM NaCl at 1 μg/ml overnight at 4 °C. Unbound ZipA-(23–328) was removed, and the wells were blocked with blocking buffer (0.2% bovine serum albumin in PBS-T (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.5, 140 mM NaCl, 2.7 mM KCl, 0.05% Tween 20)). After two washes with PBS-T, biotin-FtsZ and its mutants were added at various concentrations in blocking buffer for 1 h at room temperature. Unbound FtsZ was removed, and the wells were washed three times with PBS-T. Next, 0.1 μg/ml streptavidin-horseradish peroxidase conjugate in blocking buffer was added and incubated at room temperature for 1 h. The wells were washed four times after the removal of the conjugate. The horseradish peroxidase substrate *o*-phenylenediamine was then added in sodium phosphate-citric acid buffer, color development was stopped after a few minutes with 1.3 N H_2SO_4, and the absorbance at 490 nm was measured.

Cell Biology Methods—*E. coli* morphology was determined by phase contrast microscopy of *E. coli* cells as described previously (5). Experi-

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TABLE I
Complementation of a ftsZ deletion by alleles of ftsZ expressed from a regulated promoter

Plasmids were transformed to strain PB143/pCX41 [*dadR*$^-$ *trpE*$^-$ *trpA*$^-$ *tna*$^-$ *recA*::*Tn10 ftsZ*^{o}/*repA*^{ts} *ftsZ*^{t}], and transformants were grown overnight at 30 °C in LB + Ap (50 μg/ml) + Cam (50 μg/ml) + glucose (0.1%). Cultures were diluted in LB to $A_{600} = 1 \times 10^{-7}$, and 0.1-ml aliquots were spread on four plates. One plate containing LB + Ap (50 μg/ml) + glucose (0.2%) was incubated at 30 °C. The other plates were incubated at 42 °C. These plates contained LB + Ap (50 μg/ml) supplemented with no. 5, or 10 mM IPTG. For each plate, the number of colony forming units was determined. Plasmids used in this experiment were: vector, pMLB1113; wild type *ftsZ*, pDR3; *ftsZ*^{D373G}, pSH179; *ftsZ*^{D373S}, pSH181; *ftsZ*^{D373G,P375L}, pSH183.

Plasmid	Colony forming units, temperature/IPTG			
	30/0	42/0	42/5	42/10
		°C/μM		
Vector	787	0	0	0
Wild type *ftsZ*	729	8	554	516
ftsZ^{D373G}	642	0	0	0
ftsZ^{D373S}	605	0	0	0
ftsZ^{D373G,P375L}	550	0	0	0

mental conditions are described in the legends for Tables I and II.

β-Galactosidase Assay—Cultures to be tested were grown for 36 h in 5 ml of SC-Leu-Trp media. New cultures were inoculated with 100 ml of the overnight cultures, and the new cultures were grown for 16 h. Cells densities were between 0.8 and 1.0 A_{600} for these strains. Samples of these cultures were assayed for LacZ activity in quadruplicate, in a 96-well microtiter plate (100 μl per well). Samples were mixed with 100 μl of lysis buffer and substrate (40 μl of Promega cell lysis buffer, 40 μl of 0.125 mg/ml 4-methylumbelliferyl β-D-galactoside (Sigma), and 20 μl of 10× β-galactosidase assay salts). Samples were incubated at 30 °C for 4–8 h with shaking and read on a Victor II fluorescence plate reader from Wallac. Fluorescence intensity increased with time, and after 8 h, negative control wells showed about 400 units, whereas positive control wells for the ZipA·FtsZ interaction showed about 50,000 units and for the FtsA·FtsZ interaction showed about 10,000 units.

RESULTS

A Two-hybrid System That Allows for the Selection of Mutations in ftsZ, which Affect the FtsZ·ZipA Interaction—Our interest in developing a genetic system to characterize the FtsZ·ZipA interaction was piqued by the observation that a mutation in *ftsZ* resulted in a reduced interaction with *zipA*, when studied in the yeast two-hybrid system. This observation was made when one of several clones derived by PCR amplification of *E. coli* genomic DNA was subcloned into the two standard two-hybrid systems. When *ftsZ*^{N45D,D373G} was expressed as a fusion to the B42 activation domain, an interaction was seen with *zipA* expressed as a fusion to the LexA DNA binding domain. This interaction could clearly be scored in standard analyses, such as on indicator plates containing 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal), and by leucine prototrophy, but was significantly less robust than a fusion to wild type *ftsZ* (Fig. 1). No interaction was seen when the mutated *ftsZ* allele was examined in the galactose system (Fig. 2*B*, and results not shown). An examination of the mutations introduced during cloning suggested that one of the mutations could be important for the FtsZ·ZipA interaction. The first change was N45D, which has been characterized by Wang *et al.* (35) as one that affects the GTPase activity of FtsZ. This change was discounted as the cause of the altered interaction with ZipA by several experiments (Ref. 15, and results not shown). This evidence includes the observation that ZipA binding to FtsZ is not affected by guanine nucleotides and that deletion analysis of FtsZ had already shown that the GTPase domain of FtsZ was not involved in binding to ZipA. The other mutation, D373G, resulted in a change in a highly conserved region of the C terminus of FtsZ (discussed below). At the time this project began, the role of this region in cell division had not been characterized. This mutation provided us with an opportunity to study how a mutation with diminished function could be used to characterize this interaction.

The lack of growth in the galactose system provided a clear strategy for determining which residues in FtsZ facilitate binding to ZipA, through the selection of intragenic suppressors. Selection of suppressors that restored the FtsZ·ZipA interaction was achieved by PCR mutagenesis of the whole *ftsZ* gene as cloned into pGAD424, using primers that annealed to the *GAL4* activation domain fragment and the *ADH* terminator. The primers allowed ~300-bp extensions to both ends of the *ftsZ* gene. These extensions provided regions of homology that allowed the PCR products to be cloned by recombination (Fig. 2*A*) (30). The PCR products were transformed directly into strain SHy63 (a derivative of the Gal-Y2H strain CG1945 that had been previously transformed with pAS2-1-*zipA*), with the pGADGH vector that had been linearized, and is therefore not stable in yeast unless it has been repaired. Repair could be achieved by homologous recombination with the ends of the PCR products containing portions of the *GAL4* activation domain gene, and the *ADHt* terminator. Transformation into strain SHy63 allowed the selection of recombinant plasmids expressing *ftsZ* alleles. Alleles interacting with the *Gal4*$_{bd\text{-}zipA}$ fusion were identified through the activation of the *GAL1p-HIS3* reporter. 73 colonies grew on plates that lacked histidine and were supplemented with 1 mM aminotriazole, two of which are shown in Fig. 2*B*. Plasmids were recovered and retested in yeast, and 12 plasmids were purified that could be recovered, had normal restriction analysis patterns, and conferred plasmid-dependent phenotypes (histidine prototrophy only when introduced into a strain that carried pAS2-1-*zipA*). The *ftsZ* genes in these 12 plasmids were sequenced to identify any mutations. Eight of the 12 plasmids contain mutations in the conserved C terminus region. The remaining four plasmids do not contain mutations that result in amino acid changes in FtsZ are presumed to contain mutations that affect the copy number of the plasmid or the expression of the Gal4·FtsZ hybrid protein and have not been characterized further. The eight mutations identify five different residue changes from the original plasmid. Two mutations are reversions to the wild type aspartate residue at position 373, two mutations change the Asp-373 residue to serine, and one changes this glycine to cysteine. The remaining suppressors change the highly conserved proline residue at position 375 to leucine (twice) and to serine. These mutations are indicated in Fig. 3. The residues that comprise the C terminus of the *E. coli* FtsZ are shown. Residues in *capital letters* show conservation among FtsZ proteins from prokaryotes and plants. The mutations identified in yeast that are critical for the interaction of FtsZ with ZipA map within this sequence, and no mutations from other regions of *ftsZ* were identified, indicating that these residues may comprise the principal region of interaction with ZipA.

The suggestion that these mutations have significant effects on the interaction of FtsZ with ZipA was confirmed by introducing some of them into an unmutagenized pGAD424-*ftsZ* plasmid, and rechecking the phenotypes. Wild type *ftsZ* was compared with *ftsZ*^{D373G}, *ftsZ*^{D373S}, and *ftsZ*$^{D373G,\ P375L}$. We checked the interaction with *zipA* and *ftsA* in the galactose Y2H system (Fig. 4). The latter interaction was examined, because other work has suggested that FtsA also interacts with FtsZ at its C terminus, and we were therefore interested in characterizing this interaction (10, 21, 35). As can be seen in Fig. 4, the FtsZ·FtsA interaction is also highly sensitive to mutations in the C terminus of FtsZ. All strains tested show good growth on plates supplemented with histidine (Fig. 4*A*). The interaction of ZipA with FtsZ is best scored on plates

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TABLE II

Dominant effects of ftsZ mutations expressed in E. coli

Plasmids were transformed to strain CH3 [*dadR*$^-$ *trpE*$^-$ *trpA*$^-$ *tna*$^-$ *recA*::*Tn10*], and transformants were grown overnight at 37 °C in LB + Ap (50 μg/ml) + glucose (0.1%). Cultures were diluted 200-fold in LB + Ap (50 μg/ml) supplemented with the indicated concentration of IPTG, and growth was continued at 37 °C for 4–5 h until A_{600} = 0.8–1.0. Division phenotypes were determined phase microscopy. Plasmids used in this experiment are described in Table I.

Plasmid	Cell division phenotype, [IPTG]				
	0	25	50	100	250
			μM		
Vector	WT[a]	WT	WT	WT	WT
Wild type *ftsZ*	WT/Min$^-$	WT/Min$^-$	WT/Min$^-$	Min$^-$/Sep$^-$	Sep$^-$
ftsZ^{D373G}	WT/Min$^-$	Min$^-$/Sep$^-$	Sep$^-$	Sep$^-$	Sep$^-$
ftsZ^{D373S}	WT/Min$^-$	WT/Min$^-$	WT/Min$^-$	Sep$^-$	Sep$^-$
ftsZ^{D373G,P375L}	WT/Min$^-$	Sep$^-$	Sep$^-$	Sep$^-$	Sep$^-$

[a] WT, wild type; WT/Min$^-$, mixture of short rods and minicells, typical *ftsZ*-overexpression phenotype; Min$^-$/Sep$^-$, mixture of minicells, normal rods, and filaments; Sep$^-$, long filaments, very few division septa present.

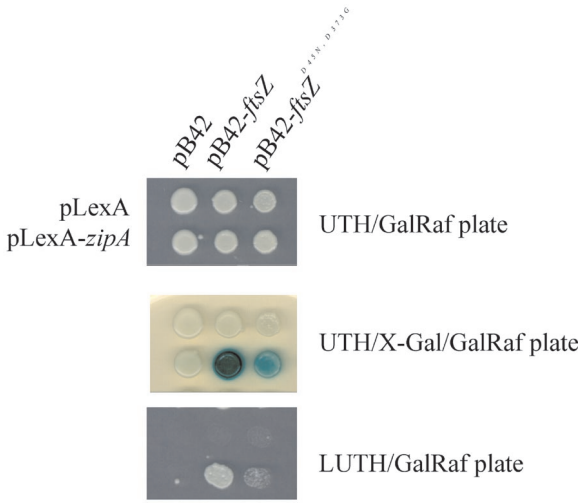

FIG. 1. **The interaction of *ftsZ* with *zipA* in the yeast two-hybrid system is specific and sensitive to mutations in *ftsZ*.** Diploid strains were constructed by mating EGY48 containing either pLexA or pSH47 (pLexA-*zipA*) with YM4271 containing pB42, pSH256 (pB42-*ftsZ*, wild type), or pSH48 (pB42-*ftsZ* ^{D39N,D373G}). Overnight cultures were spotted into a microtiter plate containing 100 μl of media per well, and 5-μl spots were applied onto plates using a pin arrayer, as indicated in the figure. Plates were incubated at 30 °C for 4 days.

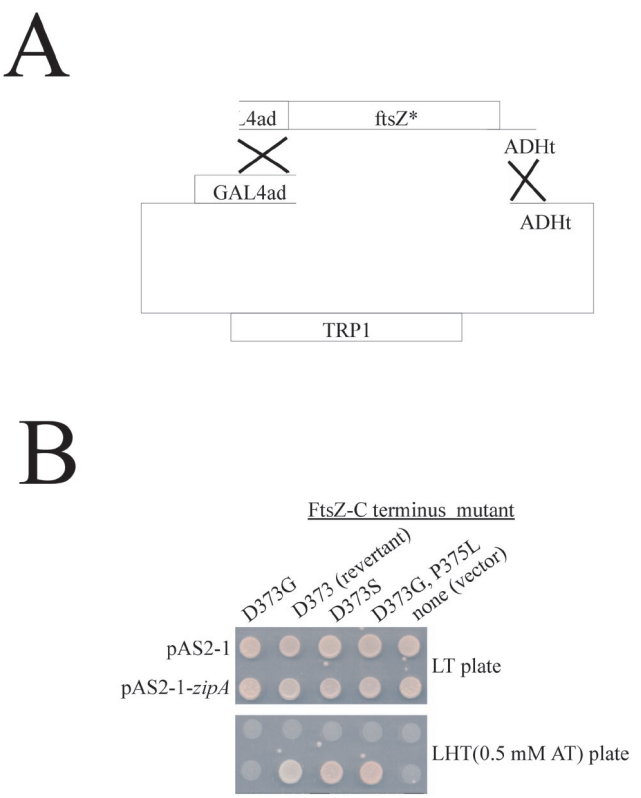

FIG. 2. **Isolation of intragenic suppressors of the *ftsZ*^{D373G} mutation.** *A*, mutations in *ftsZ* were generated by mutagenic PCR amplification of the *ftsZ*^{D373G} allele as a construct in pGAD424. Primers for the amplification were to sequences about 300 bp away from the multiple cloning site of the vector, allowing for cloning by *in vivo* recombination and expression of clones carrying mutations that restored the interaction of *ftsZ* with *zipA*. *B*, growth phenotypes of pGADGH-*ftsZ* plasmids recovered from the screen. Plasmids were transformed into yeast strain CG1945, along with either plasmid pAS2-1 or plasmid pSH227 (pAS2-1-*zipA*), as indicated, grown overnight, and spotted onto the indicated plates.

containing 0.5 mM AT (Fig. 4*B*), where robust growth is seen for the wild type FtsZ, and for the two suppressors identified in Fig. 2. Expression of the *GAL4-zipA* fusion confers a higher background than either the vector control or the *GAL4-ftsA* fusion, giving growth on plates without AT, regardless of which $GAL4_{AD}$ construct is expressed. In the case of *ftsA*, the interaction is more sensitive, and its interactions with *ftsZ* can be scored on plates lacking histidine and AT (Fig. 4*B*). In this case, *ftsA* interacts well with wild type *ftsZ*, and with the *ftsZ*^{D373S} allele. It does not interact with either allele that changes the Asp-373 residue to glycine.

Further characterization of the interaction between ZipA and FtsZ was performed *in vitro* with purified proteins. ZipA-(23–328), the soluble form of the protein, which lacks the N-terminal membrane-spanning domain, and FtsZ proteins, expressed from the alleles examined in Fig. 3, were purified from *E. coli* to homogeneity. The interactions were tested in an ELISA, as shown in Fig. 5. Wild type FtsZ shows a high affinity for ZipA, having an apparent dissociation constant of 0.19 (±0.009) μM. The affinity of the FtsZ^{D373G} mutant for ZipA was too low to be quantified in this assay, despite the protein being otherwise well behaved, indicating that the mutation has a significant impact on the interaction of these two proteins. Two suppressors were analyzed as well, FtsZ^{D373S} and FtsZ^{D373G,P375L}, and were shown to have dissociation constants of 6.2 (± 0.9) μM and 1.3 (± 0.2) μM, respectively. Both proteins show greatly improved interactions with ZipA, although neither protein interacts with ZipA as well as wild type FtsZ.

FtsA Also Binds to the Conserved Carboxyl Terminus of FtsZ, but in a Manner Different from ZipA—The results in Fig. 4, as well as work by others (9, 10, 15, 21, 35), strongly implicate the C terminus of FtsZ as playing a critical role in the interaction of FtsA, in addition to ZipA. We were interested in examining the role of additional residues in the conserved C terminus of FtsZ in binding to FtsA as well as ZipA. In Fig. 6, we examined the role of the C terminus in binding to ZipA and FtsA in the Y2H system. In this experiment, we expressed a segment of the

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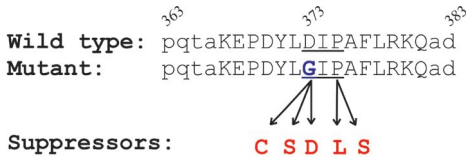

FIG. 3. **Summary of mutations isolated by PCR mutagenesis in the two-hybrid system.** The FtsZ C terminus is shown as both the wild type sequence (*top line*), and with the D to G mutation that was encoded by the template DNA for this work (*second line*). Suppressors isolated from this DNA are indicated in the third line. Residues Asp-373 to Pro-375 that comprise the signature DIP sequence are *underlined*. FtsZ residues are *numbered*. Conserved residues are capitalized.

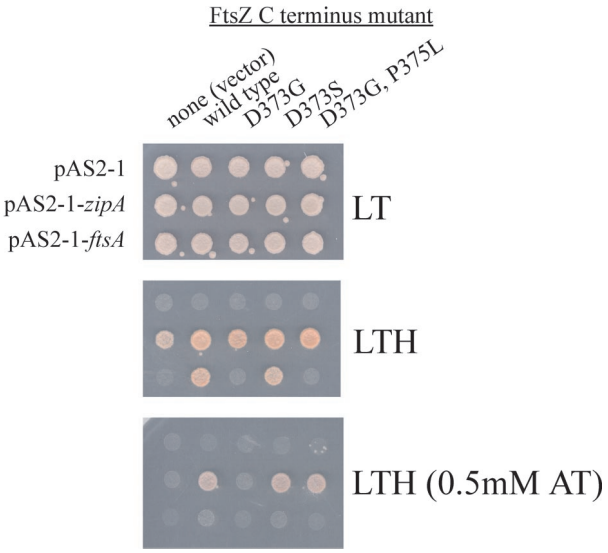

FIG. 4. **Interaction of intragenic suppressors of the *ftsZ*^{D373G} mutation with *zipA* and *ftsA* in the two-hybrid system.** Yeast diploid strains resulting from crosses of yeast strain SHy22 containing pAS2-1, pSH227 (pAS2-1-*zipA*), or pSH 100 (pAS2-1-*ftsA*) with yeast strain SHy23 strains containing pGAD424-*ftsZ*-based plasmids as indicated in the figure. Diploid strains for testing are grown overnight and spotted, as described in Fig. 1, onto the plates indicated in the figure.

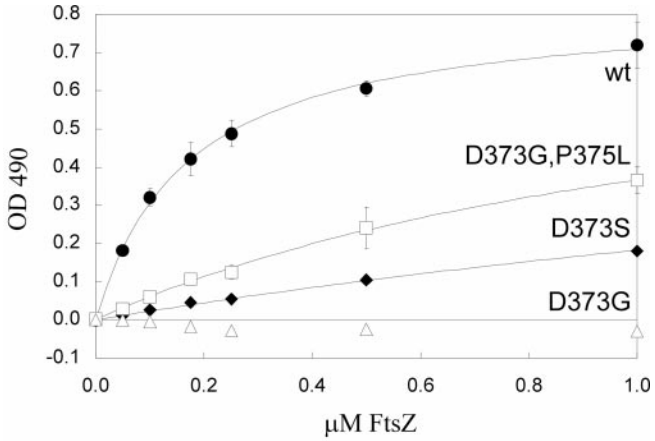

FIG. 5. **Determination of the dissociation constants for the binding of FtsZ and FtsZ mutants by ZipA.** The interaction was assayed as described under "Experimental Procedures," and the data (●, biotin-FtsZ; □, biotin-FtsZ$^{D373G,\ P375L}$; ♦, biotin-FtsZ^{D373S}; and △, biotin-FtsZ^{D373G}) were fit by linear regression with a steady-state affinity model. Each data point is an average of three repeats.

ftsZ gene, which encodes the last 70 residues of FtsZ as a fusion to the *GAL4*$_{bd}$, rather than to the *GAL4*$_{ad}$, as has been done in previous experiments. The *ftsZ*^{D373G} mutation, expressed in a similar construct, clearly indicates that this residue plays a key role in the interaction of both fusions assayed.

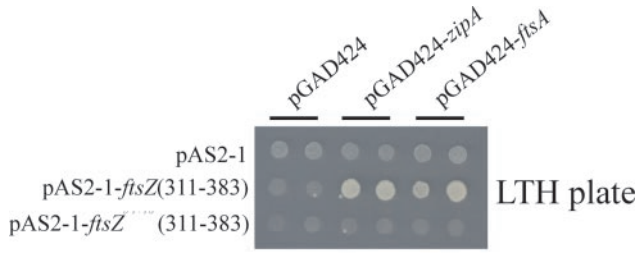

FIG. 6. ***zipA* and *ftsA* interact with the C terminus of *ftsZ* in the yeast two-hybrid system.** Strains were constructed as described in Fig. 4 using strain CG1945 instead of strain SHy22 for the bait plasmids (pAS2-1, pSH228 (pAS2-1-*ftsZ*-(311–383)), and pSH229 (pAS2-1-*ftsZ*^{D373G}-(311–383)). Prey plasmids (pGAD424, pSH230 (pGAD424-*zipA*), and pSH232 (pGAD424-*ftsA*)) were transformed into strain SHy23 to obtain strains for mating. Controls were performed as described in Fig. 4, but are omitted from the figure for clarity. *Duplicate spots* represent overnight cultures of two independent transformants.

Next, we sought to determine whether ZipA and FtsA interact with identical residues in the FtsZ C terminus. Eight additional residues within this conserved sequence were mutated individually to alanine. Strains expressing derivatives of pGAD424-*ftsZ* that have specific residues changed to alanine are indicated in Fig. 7. The strains were characterized for histidine prototrophy and for β-galactosidase activity, as shown in the figure. In this experiment, strain CG1945 was used for the bait plasmids. In these diploids, background growth of the strains containing pAS2-1-*zipA* were more comparable to that of the strains containing pAS2-1-*ftsA*, so only the SC-LHT plate is shown. Robust growth on plates containing 0.5 mM AT was only seen with the *zipA*·*ftsZ* interactions (data not shown).

Phenotypic Consequences of Mutations That Change Residues in the FtsZ C Terminus—The mutations described above were assayed in *E. coli* to determine whether there were any biological consequences associated with them. The mutations were introduced into pDR3, a plasmid that expresses *ftsZ* under the control of the *lac* promoter, and can complement a *ftsZ* deletion when induced by IPTG. Two assays were performed. In the first, *ftsZ* alleles expressed under a regulated promoter were characterized for their ability to complement an *ftsZ* deletion. The results are presented in Table I. pDR3 efficiently complements in the presence of IPTG, whereas none of the mutants can. Additional effects can be seen in Table II, where the expression of *ftsZ* from the same plasmids as those in Table I are examined for dominant effects. Expression of extrachromosomal *ftsZ* is toxic at high levels, as can be seen when a strain carrying pDR3 is exposed to high concentrations of IPTG, which expresses the plasmid copy of the *FtsZ* gene to high levels.

Dominant effects can be seen for all of the mutated alleles, with strong effects seen with the *ftsZ*^{D373G} allele and the *ftsZ*^{D373G,P375L} allele. In these cases, an Sep$^-$ phenotype is seen at substantially lower concentrations of IPTG. The same is true for the *ftsZ*^{D373S} allele, which has a more modest phenotype relative to the other mutations in this assay as well, but is still substantially more toxic than the wild type gene expressed on a plasmid. Thus, a comparison of the *ftsZ* alleles with the wild type *ftsZ* indicates that all of the mutations examined in Tables I and II show profound cell division defects.

DISCUSSION

Recently, the bacterial cell division proteins FtsZ, ZipA, and FtsA have been structurally characterized, as well as the ZipA·FtsZ interaction (11, 17, 18, 36). In addition to these structural characterizations, functional and biological studies, such as those described in this report, will allow us to under-

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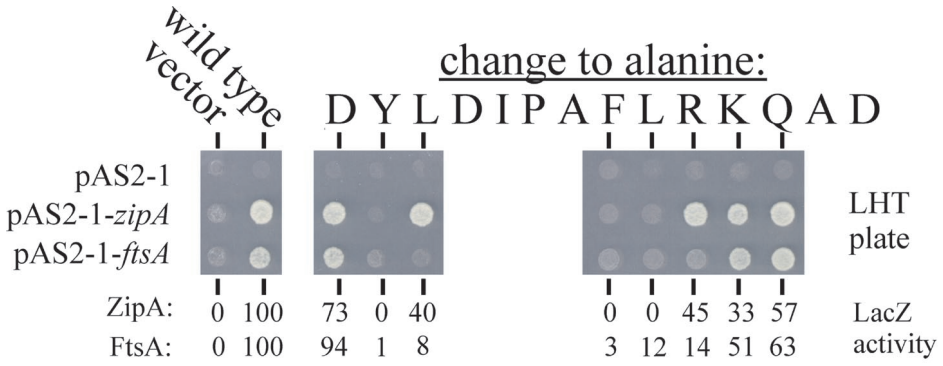

FIG. 7. **Comparison of the interaction of *ftsA* and *zipA* with alanine-scanning mutations in other conserved residues of the *ftsZ* C terminus.** Strains were constructed as described in Fig. 6.

stand how biologically important residues function in these interactions. Results presented here show that several residues within the conserved C terminus of FtsZ play essential roles in cell division, and minor changes within this sequence can have lethal consequences.

The results presented in this study that characterize the role of individual residues in the biological context of the Y2H system are in general agreement with the *in vitro* characterization of Mosyak *et al.* (17). One area where differences were seen is the first group of conserved residues: Asp-370, Tyr-371, and Leu-372. All three residues were shown to contribute important binding energy in the BIAcore system, but only the Y371A change was shown to be important in the Y2H system. The L372A change functioned well in the Y2H system, despite being the most important of the three *in vitro*. It is possible that the FtsZ binding pocket of ZipA can accommodate the D370A and L372A changes in the Y2H system more effectively than it can *in vitro* or that it can accommodate the Y371A change in the *in vitro* system better than in the Y2H system. Two mutations, L372A and R379A, affected the interaction with *ftsA* to a greater extent than the interaction with *zipA*. The severe effect of the R379A change is especially interesting, because structural data show that the arginine residue is solvent-exposed when bound by ZipA, which is consistent with the modest effect with *zipA* in yeast. These results clearly indicate that, although both proteins bind the same C terminus core of FtsZ, they bind to different residues within this core. Residues that were identified as playing a major role in the interaction *in vitro* showed similarly significant roles in the experiments presented here.

This work was initiated through an exhaustive search for suppressors of the loss-of-function allele, *ftsZ*^{D373G}. We identified only residues in the conserved C terminus, despite the fact that the entire *ftsZ* gene was subjected to mutagenic PCR. As these results were first obtained, it was surprising that second-site intragenic suppressors were obtained at only one residue, Pro-375. If this conserved segment was the site of the interaction with ZipA, then one might expect that mutations that increase the affinity of ZipA for the D373G mutant of FtsZ would be restricted to this region, but the presence of suppressors at a single residue was not anticipated. Structural studies have shed some light on our results (17, 18). Although the Asp-373 residue plays a critical role in the interaction between FtsZ and ZipA, it is not directly involved in the interaction with ZipA *per se*. Rather, it plays a critical role in defining the structure of the residues that contact ZipA significantly. This is illustrated in Fig. 8. This figure shows the FtsZ peptide bound by the C-terminal domain of ZipA, as determined by x-ray crystallography, and is depicted using the program RIBBONS (37). The Asp-373 side chain contributes a hydrogen bond to the α-carbon backbone, which helps define the transition from β-sheet structure to α-helix. The change to glycine introduces a significant structural change, including the removal of this

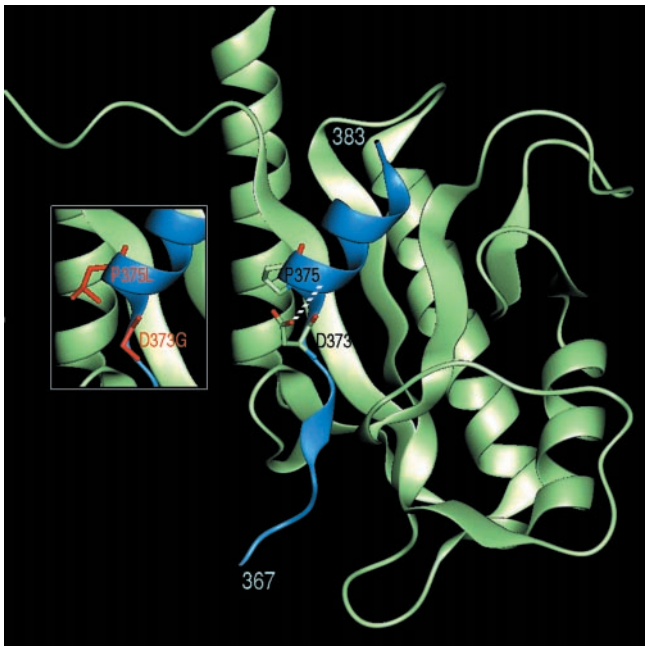

FIG. 8. **Structure of the FtsZ·ZipA complex.** The interacting residues of FtsZ are indicated in *blue*, with the Asp-373 and Pro-375 side chains shown. The ZipA structure is shown in *green*. *Numbers* indicate residues of the FtsZ peptide. The hydrogen bond between Asp-373 and the main chain of FtsZ is indicated by the *white dashed line*. In the *inset*, the D373G and P375L changes are modeled into the ZipA·FtsZ structure. Representations were done using the program RIBBONS (37).

critical hydrogen bond. The Pro-375 residue also plays a structural role in defining the ZipA binding site. The residues that directly interact with ZipA are interspersed through the sequence that includes the Asp-373 and Pro-375 residues, in particular, residues Ile-374, Phe-377, and Leu-378 (17). Therefore, the structure of the FtsZ C terminus is critical because of the number of interactions that occur along the α-carbon chain of FtsZ (17, 18). The best explanation that can be offered from the structural data is that the D373G change perturbs the orientation of the interacting residues, and this perturbation is alleviated by the P375L mutation. In the *inset* of Fig. 8, the D373G and P375L mutations are modeled into the structure. The *inset* shows where the hydrogen bond is lost, and where the leucine side chain would interact with ZipA. Further structural studies will be required to determine whether the P375L mutation provides an additional hydrophobic interaction with ZipA or whether it allows hydrophobic capping of the FtsZ peptide to restore the conformation of the interacting residues. Finally, although the number of mutations that can negatively affect an interaction is fairly large, the number of possible changes that can *increase* the strength of the interaction seems to be small.

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Although none of the recent studies can conclude that the conserved C terminus residues of FtsZ comprise the complete target sequence for ZipA, it is certainly true that the region plays a critical role in the interaction with ZipA. The interaction of the FtsZ C terminus with ZipA has been characterized extensively (5, 9, 17, 18, 21). Additionally, the results of Fig. 6 indicate the C-terminal tail of FtsZ is sufficient for the interaction of FtsZ with both ZipA and FtsA. The portion of FtsZ encoded by the Y2H fusion plasmid in this figure consists of the last 72 residues of FtsZ. The interaction of ZipA with a MBP·FtsZ-(311–383) fusion protein, with the wild type protein, and the 17-residue peptide are all similar, as measured in a BIAcore biosensor assay.[2] Therefore, it is possible to conclude that the mutations recovered in this study were limited to the conserved residues of the FtsZ C terminus, because this region defines the principal ZipA interaction site. Thus, we have met a goal of this study, which was to use a random genetic approach to help define a protein·protein interaction.

The suppressors identified in this study provide information about the sensitivity of the cell division machinery to changes in the interactions of its components. The *FtsZ*^{D373G} allele has profound effects on cell division and viability, as well as conferring on the mutant protein a greatly reduced affinity for ZipA and for FtsA. For these proteins, these results are consistent with the importance of their proper interaction with FtsZ. The suppressors show that these interactions are extremely sensitive. Neither of the alleles characterized in detail in this study (FtsZ$^{D373G,\ P375L}$ or FtsZ^{D373S}) complement a deletion. In the case of the double mutation, this is less informative about the relation between affinity for ZipA and function, because it shows a dramatically reduced affinity for FtsA, which complicates our determination of why this mutation fails to complement. The other allele, *ftsZ*^{D373S} causes more modest changes on the interaction with *ftsA*. No difference from wild type could be seen between *ftsA* and *ftsZ*^{D373S} by Y2H assay, indicating that any change is relatively minor. The data, including Y2H and the *in vitro* analysis in Fig. 5, suggests that it is more likely that this allele fails to complement because of altered interactions with *zipA*. Although it is possible that another protein also interacts with FtsZ at this site, the simplest conclusion to draw at this time is that these proteins cannot tolerate even moderate changes in their interactions.

If the interaction characterized in this study represents an important antibacterial target, it is necessary to show that the interaction between ZipA and FtsZ is not just essential but is very sensitive to interference. Although it is true that many protein·protein interactions are essential, their identification as promising antibacterial targets depends on the interaction being sensitive to interference. In some cases, inhibition of expression or activity by 50% can be lethal. For many essential genes, reducing expression by 95% or more can result in no observable defect. Several genes have been characterized that are functional when they have activity at 1%, or less, of their wild type levels. Classic examples include nonsense mutations, when their phenotypes can be alleviated by suppressor tRNAs (38, 39). The strength of a protein·protein interaction, or an activity, as a pharmaceutical target can be evaluated by such data. Specifically, if reducing a protein·protein interaction moderately (50–80%) has phenotypic consequences, then it could be regarded as a strong pharmaceutical target. If reducing the interaction by 100-fold or greater is required to inhibit growth, it may be problematic to find a drug that can achieve this level of inhibition through its specific activity and pharmacokinetics, in a true *in vivo* situation. One of the reasons that cell division is considered an important area of antibacterial research is that many of its steps are very tightly controlled, and are very sensitive to changes in expression levels. Cell division is sensitive to changes of 2- to 4-fold in the expression of *ftsZ*, *zipA*, and other genes (3, 6, 40). If the FtsZ·ZipA interaction itself is as sensitive, then this interaction has potential as a target. The results presented here argue that this is the case.

It is clear that the yeast two-hybrid system is a powerful system for the study of protein·protein interactions (24, 41–45). In this report, we have taken advantage of commonly used techniques for classical genetic analysis in yeast (*i.e.* the characterization of gene function through the identification of mutations) and applied them to a protein·protein interaction of *E. coli*. The need for such methods rests on the observation that most important cellular processes (both eukaryotic and prokaryotic) are as dependent on protein·protein interactions as they are on enzymological functions. Although inhibition of enzyme function is well understood as a means of developing therapeutics, developing compounds that function through the inhibition of protein·protein interactions is much less well understood (46). Two clear examples of drugs that inhibit a protein·protein interaction are that of FK506, which inhibits the interaction of type I transforming growth factor-β receptors with FKBP12 (47), and the peptidomimetic compound BILD 1263, which inhibits the interaction of the herpes simplex virus ribonucleotide reductase subunits (48). The polymerization of tubulin by taxol is another interaction affected by a chemotherapeutic, but in this case taxol functions by stabilizing the tubulin dimer associations (49, 50). Protein·protein interactions may comprise large surface areas, and those that do would be much less favorable as drug targets. Applying the yeast two-hybrid system as a vehicle for yeast genetic analysis provides a fairly rapid and general method for determining the nature of a protein·protein interaction.

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