

## ***PBS2*, a yeast gene encoding a putative protein kinase, interacts with the *RAS2* pathway and affects osmotic sensitivity of *Saccharomyces cerevisiae***

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The yeast gene *PBS2* encodes a presumed protein kinase. The gene is essential for manifestation of resistance to the antibiotic polymyxin B. Deletion of *PBS2* enables a *ras2-530* null mutant to grow on nonfermentable carbon sources; overexpression of *PBS2*<sup>+</sup> enhances viability of a *RAS2*<sup>Val19</sup> mutant. Overexpression of *PBS2*<sup>+</sup> also diminishes cellular response to mating pheromone MF $\alpha$ . These results suggest that the *PBS2* and *RAS2* genes affect a common pathway that may communicate with the pheromone response pathway. In addition, disruption of *PBS2* renders cells sensitive to high osmolarity: exposure to 0.9 M-NaCl causes growth arrest, appearance of bizarre morphological forms, and eventual death. A mutation suppressing *pbs2* deletion has been found. That mutation restores full polymyxin B resistance but only partially corrects the osmotic sensitivity defect. These observations indicate that *PBS2* is involved in diverse physiological pathways in yeast.

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### **Introduction**

Polymyxin B, a complex antibiotic produced by cells of *Bacillus polymyxa*, is very toxic to bacterial, fungal and animal cells. The drug exerts its effects by damaging the integrity of cell membranes (Storm *et al.*, 1977), and at high concentration it causes cell lysis.

Polymyxin B has several important uses in biological studies. First, high concentrations of the antibiotic, a positively charged molecule, displace calcium ions from anionic phospholipids and cause disorganization of cell membranes (Storm *et al.*, 1977). Thus, the topography of eukaryotic membranes, as well as the role of calcium ions in the maintenance of membrane structure and activity, can be elucidated with the help of this antibiotic (Bearer & Friend, 1980; Kubesch *et al.*, 1987). Second, the drug produces interesting pharmacological effects. For example, it is one of the most potent neuromuscular blocking agents known (Singh *et al.*, 1980), and it promotes the conversion of membrane acetylcholine receptors from the resting activatable state to the desensitized state (Brazil *et al.*, 1989; Brown & Taylor, 1983). Third, the antibiotic inhibits Ca<sup>2+</sup>-activated K<sup>+</sup> channels in mouse skeletal muscle (Weik & Lonnendonker, 1990) and in human red blood cells (Varecka *et al.*, 1987). In addition, polymyxin B interferes with

insulin-induced activation of hexose transport and metabolism in whole animals and in isolated muscle of mice and rats (Amir & Shechter, 1985; Amir *et al.*, 1987). In *Saccharomyces cerevisiae* polymyxin B is a powerful inhibitor of mating (Boguslawski, 1986).

Studies on polymyxin B resistance in yeast have revealed the existence of two genes, *PBS1* and *PBS2* (polymyxin B sensitivity), whose activities are essential for the resistance phenotype (Boguslawski, 1985; Boguslawski & Polazzi, 1987). The deduced amino acid sequence of the *PBS2* gene product bears a strong resemblance to polypeptides of the serine/threonine protein kinase family (Boguslawski & Polazzi, 1987; Hanks *et al.*, 1988), making it likely that the gene does encode a protein kinase. The *pbs1* mutation confers a high level of resistance to polymyxin B on yeast cells, but only if an intact *PBS2*<sup>+</sup> gene is present (Boguslawski & Polazzi, 1987). Disruption of this gene results in a drug-sensitive phenotype. Conversely, overexpression of *PBS2*<sup>+</sup> makes cells resistant to the drug even in the wild-type background.

The results discussed in the present communication show that, in addition to abolishing polymyxin B resistance, disruptions in the *PBS2* gene can suppress a recessive null mutation of the yeast *RAS2* gene, and that overexpression of *PBS2*<sup>+</sup> attenuates the effect of a dominant *RAS2*<sup>Val19</sup> mutation. Furthermore, enhanced expression of *PBS2*<sup>+</sup> diminishes the ability of cells to

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respond to a mating pheromone. These observations suggest that *PBS2* may be a part of the signal transduction circuitry in yeast.

## Methods

**Strains.** All yeast strains and plasmids used in this work are listed in Table 1. The media, growth conditions, and methods of genetic manipulation have been described by Sherman *et al.* (1983). YEPD medium contains 2% (w/v) glucose, 2% (w/v) Bacto peptone and 1% (w/v) yeast extract. Solid YEPD medium includes 2% (w/v) agar. All strains described here grow very well on this medium at 30 °C.

**Pheromone sensitivity assay.** Approximately  $10^7$  *MAT $\alpha$*  cells were suspended in 3 ml of 0.7% agar in water and poured on synthetic minimal medium plates containing the necessary nutrients. After the agar overlay had solidified, sterile filter disks (6 mm diameter) were placed on the surface, and synthetic mating pheromone (MF $\alpha_1$ , Sigma) was applied to each disk. The plates were incubated at 30 °C for 2–3 d until the zones of inhibition were easily observed.

**Viability assays.** The tests were performed essentially as described by Toda *et al.* (1985). Cells were grown at 30 °C to stationary phase in liquid minimal medium supplemented with the necessary nutrients, washed with water, and resuspended in starvation medium (2%, w/v, glucose and 0.17%, w/v, Difco yeast nitrogen base without nitrogen source or the nutrients) at  $1 \times 10^6$  cells ml<sup>-1</sup> (Toda *et al.*, 1985). After 96 h of shaking at 300 r.p.m. at 30 °C, aliquots were plated on solid YEPD, and viability was determined by counting colonies on each plate after 2 d.

**Heat shock.** Cells were patched on minimal medium plates supplemented with the necessary nutrients and grown to confluence. The plates were sealed with Parafilm and floated in a 55 °C water bath. After incubation, the plates were replicated onto YEPD and incubated at 30 °C for 24 h.

**Osmotic sensitivity.** Cells were grown in patches on solid YEPD and replica-printed onto YEPD containing 0.9 M-NaCl. The plate was incubated at 30 °C for 72 h and photographed. Survival of the cells was tested by incubating the patches on the 0.9 M-NaCl plate for 9 d and printing onto fresh YEPD.

**Phase-contrast and fluorescence microscopy.** Methods described by Adams & Pringle (1991) were used to assess the effect of high salt concentration on cell morphology. Cells were scraped from a plate of YEPD containing 0.9 M-NaCl, washed, fixed in formaldehyde, and examined microscopically. Rhodamine–phalloidin conjugate (Molecular Probes) was used to determine actin distribution.

**Plasmid constructions.** Two integrating plasmids were used in this study (Fig. 1). Plasmid YIp5-AN117 carries a 1.17 kb internal fragment (*Aar*II–*Nar*I) of the *PBS2* gene in the YIp5 vector (Boguslawski & Polazzi, 1987). To create a disruption, *pbs2-1*, the plasmid was digested with the restriction endonuclease *Cla*I. The linear molecule was integrated into the chromosomal site, disrupting the open reading frame at amino acid 141. Alternatively, a deletion, *pbs2-2*, was created by substituting the Tc<sup>r</sup>-*URA3*<sup>+</sup> portion of YIp5 (as a 3.2 kb *Cla*I–*Xmn*I fragment) for the 1.26 kb *Cla*I–*Eco*RV portion of the open reading frame of the gene. The resulting plasmid pGB5 was digested with *Aar*II, and the 4.1 kb fragment was integrated into the chromosome to create *pbs2-2* with the protein coding sequence truncated at amino acid 38. Southern analysis confirmed the nature of integration in each case (data not shown), and the two constructions displayed essentially identical phenotypes in the tests described in this communication.

For studies requiring overexpression of *PBS2*<sup>+</sup>, an episomal plasmid YEp24.*PBS2* was constructed. For this purpose, the parent plasmid R5H (Boguslawski & Polazzi, 1987) was digested with *Sac*I restriction endonuclease, the ends made flush with T4 phage DNA polymerase, and a *Sal*I linker added. After ligation and transformation of *Escherichia coli*, the plasmid was isolated, digested with *Dra*I and *Sal*I, and a 3.2 kb *Dra*I–*Sal*I fragment, containing the entire *PBS2* gene, was isolated by gel electrophoresis. The purified fragment was ligated into the YEp24 vector that had been digested with *Pvu*II and *Sal*I.

Table 1. *Saccharomyces cerevisiae* strains and plasmids used in this study

	Genotype or relevant markers	Source or reference
<b>Strains</b>		
YNN27	<i>MAT<math>\alpha</math> trp1-289 ura3-52</i>	R. W. Davis
GBH21	<i>MAT<math>\alpha</math> trp1-289 ura3-52 pbs1-273</i>	Boguslawski (1985)
GBH37	<i>MAT<math>\alpha</math> trp1-289 ura3-52 pbs1-273 pbs2-2</i>	This work, from GBH21
GBH38	<i>MAT<math>\alpha</math> trp1-289 ura3-52 pbs2-2</i>	This work, from YNN27
GBH46	<i>MAT<math>\alpha</math> trp1-289 ura3-52 pbs1-273 pbs2-2 PBS<sup>r</sup>*</i>	This work, from GBH37
JC482	<i>MAT<math>\alpha</math> his4 ura3 leu2</i>	Cannon <i>et al.</i> (1986)
JC302	<i>MAT<math>\alpha</math> ras2-530::LEU2<sup>+</sup> his4 ura3 leu2</i>	Cannon <i>et al.</i> (1986)
GBH74	<i>MAT<math>\alpha</math> ras2-530::LEU2<sup>+</sup> his4 ura3 leu2 pbs2-1</i>	This work, from JC302-26B
JC303-58	<i>MAT<math>\alpha</math> ras2-530::LEU2<sup>+</sup> his4 ura3 leu2 yak1(SRA7-14)</i>	Cannon <i>et al.</i> (1986)
X10	<i>MAT<math>\alpha</math> ras2-530::LEU2<sup>+</sup> his4 ura3 leu2 yak1(SRA7-14) pbs2-1</i>	This work, from JC303-58
SP1	<i>MAT<math>\alpha</math> leu2 his3 ade8 ura3 trp1 can1</i>	Toda <i>et al.</i> (1985)
GBH83	<i>MAT<math>\alpha</math> leu2 his3 ade8 ura3 trp1 can1 pbs2-2</i>	This work, from SP1
TK161-R2V	<i>MAT<math>\alpha</math> leu2 his3 ade8 ura3 trp1 can1 RAS2<sup>Val19</sup></i>	Toda <i>et al.</i> (1985), from SP1
GBH65	<i>MAT<math>\alpha</math> leu2 his3 ade8 ura3 trp1 can1 RAS2<sup>Val19</sup> pbs2-2</i>	This work, from TK161-R2V
<b>Plasmids</b>		
YEp24. <i>PBS2</i>	Episomal, Ap <sup>r</sup> , <i>URA3</i> <sup>+</sup> , complete <i>PBS2</i> <sup>+</sup> gene	This work
YIp5-AN117	Integrating, Ap <sup>r</sup> , <i>URA3</i> <sup>+</sup> (see Fig. 1)	Boguslawski & Polazzi (1987)
pGB5	Integrating, Ap <sup>r</sup> , Tc <sup>r</sup> , <i>URA3</i> <sup>+</sup> (see Fig. 1)	This work

\* Strain GBH46 carries a mutation that restores polymyxin B resistance (PBS<sup>r</sup>). The nature of this mutation has not been elucidated (see text for details).

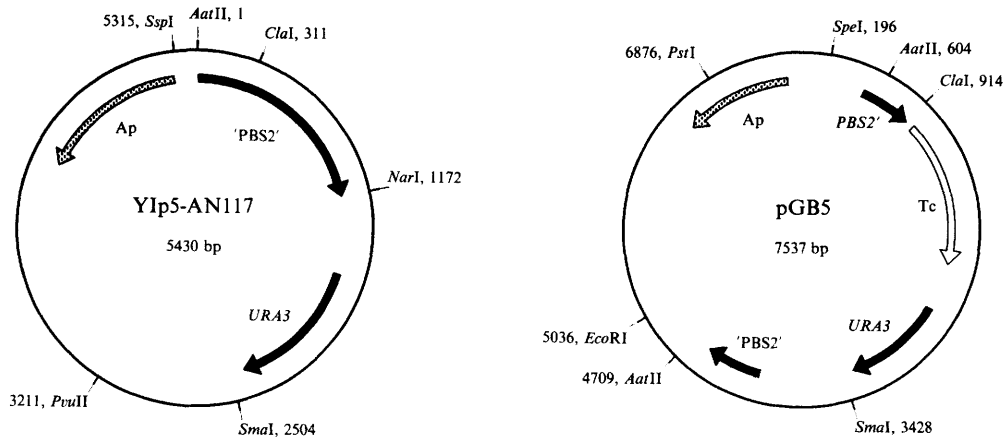


Fig. 1. Integrating plasmids YIp5-AN117 and pGB5. The details of plasmid construction are described in Methods. The *PBS2* sequences used to promote chromosomal integration are shown as filled arrows.

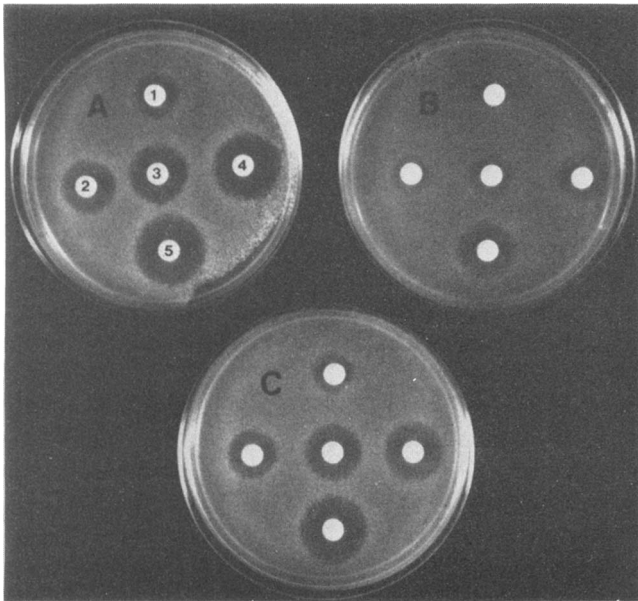


Fig. 2. Reduction of sensitivity to mating pheromone  $\alpha$  by overexpression of *PBS2*<sup>+</sup>. Increasing amounts of the synthetic pheromone MF $\alpha_1$  were applied to each filter disk (disk 1, 1.8  $\mu$ g; 2, 3.6  $\mu$ g; 3, 5.4  $\mu$ g; 4, 7.2  $\mu$ g; 5, 10.8  $\mu$ g). The plates were incubated for 2 d at 30 °C. A, GBH83, *pbs2-2*; B, SP1 transformed with YEp24.*PBS2*; C, SP1 transformed with YEp24 vector.

## Results

Previous studies revealed that polymyxin B is a potent inhibitor of mating in *Saccharomyces cerevisiae* (Boguslawski, 1986). This observation suggested that genes involved in polymyxin B resistance may interact with some elements of signal transduction pathways in yeast. As shown in Fig. 2, overexpression of *PBS2*<sup>+</sup> considerably reduces sensitivity of a cells to the mating

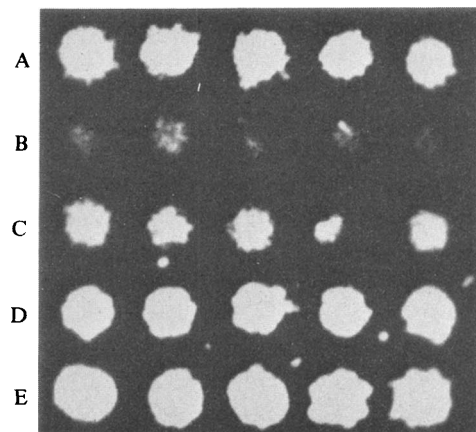


Fig. 3. Suppression of the *ras2-530* phenotype by disruption of the *PBS2* gene. Cells were grown at 30 °C on synthetic minimal medium plates with the necessary nutrients and replica-printed onto YPE medium (3% ethanol, 2% Bacto peptone, 1% yeast extract, 2% agar). The strains tested were as follows: A, JC482, wild-type; B, JC302-26B, *ras2-530*; C, GBH74, *ras2-530 pbs2-1*; D, JC303-58, *ras2-530 yak1/SRA7*; E, X10, *ras2-530 yak1/SRA7 pbs2-1*.

pheromone  $\alpha$  (disruption of *PBS2* may slightly enhance sensitivity). Therefore, a component of the yeast pheromone signalling pathway may be influenced by *PBS2*-dependent phosphorylation. However, although *PBS2* kinase belongs to the STE7 family of protein kinases (Hanks *et al.*, 1988), and the *STE7* gene is essential for mating in yeast (Teague *et al.*, 1986), neither disruption nor overexpression of *PBS2* can restore mating ability to cells lacking a functional *STE7* gene (data not shown). Variation in copy number of *PBS2* also has no effect on the activity of *FUS1* gene (Truehart *et al.*, 1987; data not shown).

An important subset of signal transduction pathways is known to be regulated by two *RAS* genes which act as

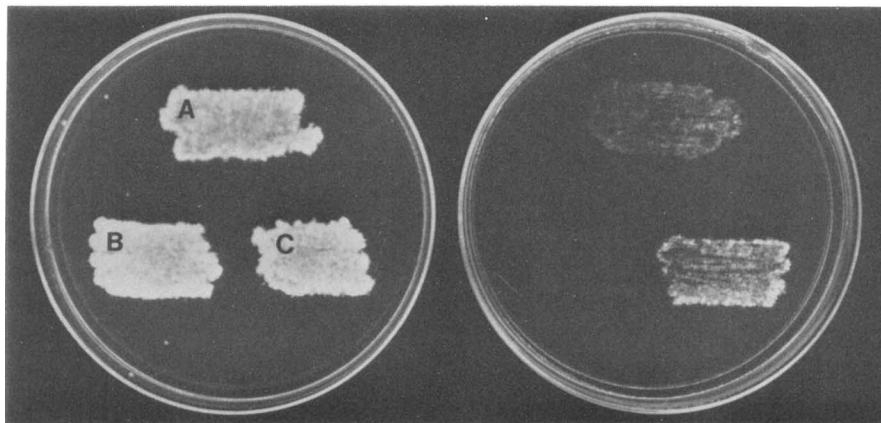


Fig. 4. Heat-shock sensitivity of *RAS2*<sup>Val19</sup> mutant. Cells were incubated at 55 °C for 40 min, replicated onto YEPD, and incubated at 30 °C for 24 h. The left panel shows control plate, with cell patches not subjected to heating. The plate on the right shows the patches that had been heat-shocked. The patches are: A, TK161-R2V transformed with YEpl24 vector; B, GBH65, *RAS2*<sup>Val19</sup> *pbs2*-2; C, TK161-R2V transformed with YEpl24.PBS2 plasmid.

modulators of adenylyl cyclase activity (Field *et al.*, 1990b; Toda *et al.*, 1985). Mutations in the *RAS2* gene confer pleiotropic effects on cells (Cannon *et al.*, 1986; Toda *et al.*, 1985). These effects include a decrease in cyclic AMP (cAMP) levels, enhanced sporulation in rich media, and accumulation of storage carbohydrates such as glycogen. In addition, *ras2* mutants are unable to use nonfermentable carbon sources such as ethanol for growth (Cannon *et al.*, 1986). However, these mutants grow very well on rich media such as YEPD that contain glucose as carbon source.

Disruption of the *PBS2* gene in the strain bearing a null allele of the *RAS2* gene (*ras2*-530::*LEU2*<sup>+</sup>) restores the ability of cells to grow on 3% ethanol (Fig. 3). However, the growth is not as robust as when a *ras2* suppressor mutation in another protein kinase gene *yak1* (Garrett & Broach, 1989) is present; this gene has been described as *SRA7* by Cannon *et al.* (1986). In contrast to *yak1/SRA7*, *pbs2* at 37 °C cannot suppress a *cyr1/cdc35* (adenylyl cyclase) temperature-sensitive defect (*cyr1*-2 and *cdc35*-10 alleles were tested) or a *ras1 ras2* double mutation (not shown). The disruption neither enhances nor diminishes growth of the two *cyr1* mutants at room temperature. Although these negative results may be explained by the weak suppressive effect of *pbs2* (Fig. 3), they do suggest a possibility that *PBS2* acts downstream of or in parallel to *RAS2* but not downstream of *CYR1*; they also indicate that the targets for *yak1* and *pbs2* suppression are probably not related.

A dominant missense mutation that substitutes valine for glycine at position 19 (*RAS2*<sup>Val19</sup>, activated *RAS2*) results in an increased level of cAMP in the cells, poor sporulation, lack of carbohydrate accumulation, and loss of viability upon starvation or heating (Toda *et al.*, 1985).

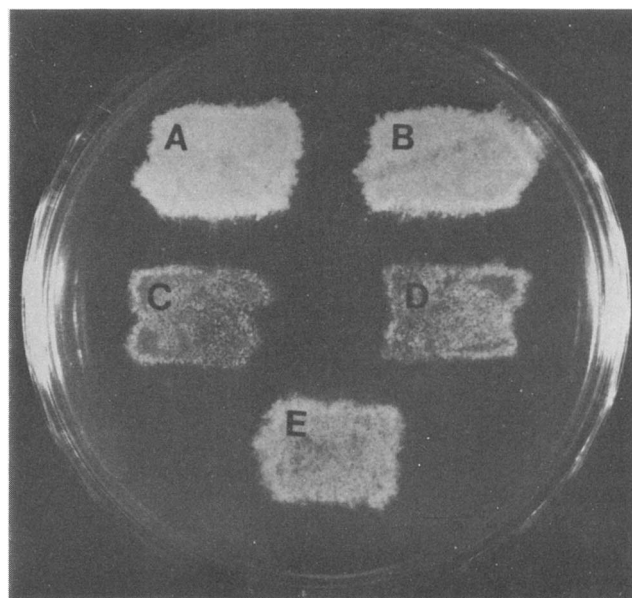


Fig. 5. Effect of disruption of the *PBS2* gene on the ability of yeast cells to grow on high osmolarity medium. Patches of cells were grown on YEPD and replicated onto YEPD containing 0.9 M-NaCl as described in Methods. The strains tested were as follows: A, YNN27 (wild-type); B, GBH21, *pbs1*-273, resistant to polymyxin B; C, GBH38, *pbs2*-2; D, GBH37, *pbs1*-273 *pbs2*-2; E, GBH46, *pbs1*-273 *pbs2*-2 *PBS*<sup>+</sup>, polymyxin B resistant revertant of strain GBH37 (see footnote to Table 1).

To assess the effects of changes in *PBS2* copy number on the viability of *RAS2*<sup>Val19</sup> mutants, the cells were transformed with either an integrating or an episomal plasmid (see Table 1) and subjected to a starvation regimen as described in Methods. The viability of the wild-type strain SP1 was excellent and appeared un-

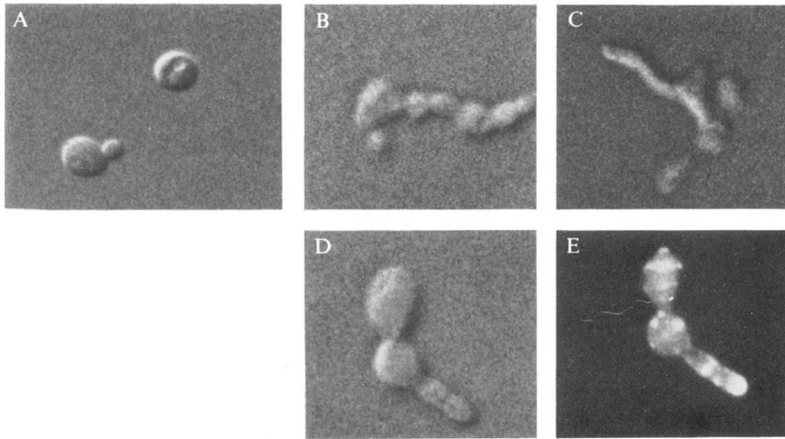


Fig. 6. Aberrant morphology of *pbs2* cells exposed to 0.9 M-NaCl. Cells from patches shown in Fig. 5 were fixed with formaldehyde and examined microscopically as described in Methods. A, wild-type cells (YNN27). B, C, D, a variety of aberrant morphological forms of *pbs2* cells (GBH38). E, the same cell as in D, but stained with rhodamine-phalloidin to reveal actin distribution.

affected by the condition of the *PBS2* allele (Table 2, unpublished data). In contrast, most cells of the activated *RAS2* mutant (strain TK161-R2V, quite unhealthy to begin with) died upon starvation. The survival of this strain was further depressed about fivefold by the *pbs2* deletion but was enhanced about threefold by overexpression of *PBS2*<sup>+</sup> (Table 2). These results are reinforced by the observation that *pbs2* disruption enhanced heat-shock sensitivity, whilst overexpression of *PBS2*<sup>+</sup> protected the cells from thermal death (Fig. 4).

Accumulation of glycogen by the various strains used in this study was examined by iodine staining (Cannon *et al.*, 1986). There was no effect either of overexpression of *PBS2*<sup>+</sup> or of *pbs2* disruption or deletion on the staining, and the pattern of glycogen accumulation followed that of the corresponding parent. Thus, regardless of the status of the *PBS2* allele, *ras2-530* colonies became dark brown when exposed to iodine vapour, while the *RAS2*<sup>Val19</sup> colonies remained straw yellow (data not shown). As with adenyl cyclase mutants (*cyr1*), the lack of effect may be related to the weak suppression (perhaps because the threshold of sensitivity is different for growth on ethanol and carbohydrate accumulation). Nevertheless, *PBS2* suppression, or lack of it, provides an additional means of discriminating between the several cellular effects of the *RAS2* gene.

Polymyxin B is a membrane-active antibiotic, and genes controlling cellular responses to the drug may be logically thought to participate in membrane functions. One manifestation of membrane activity is osmoregulation or the ability of cells to cope with the increased salt concentration in the growth medium. As shown in Fig. 5, the wild-type cells and *pbs1* (polymyxin B resistant) mutant grew well when 0.9 M-NaCl was present. In contrast, a *pbs2* mutant and *pbs1 pbs2* double mutants were unable to grow under such conditions (Fig. 5, C and D), and continued incubation resulted in death of these

Table 2. Viability of several yeast strains upon starvation in liquid medium

Strain	Relevant genotype	Survival (%)
SP1	Wild-type	95.3
TK161-R2V	<i>RAS2</i> <sup>Val19</sup>	0.37
TK161-R2V(YEp24.PBS2)	<i>RAS2</i> <sup>Val19</sup> <i>PBS2</i> <sup>+</sup> *	1.20
GBH65	<i>RAS2</i> <sup>Val19</sup> <i>pbs2-2</i>	0.07

\* *PBS2*<sup>+</sup> is overexpressed in cells transformed with YEp24.PBS2 plasmid.

cells. Microscopic examination of cells exposed to 0.9 M-NaCl revealed (Fig. 6) that *pbs2* mutant cells became morphologically aberrant, unable to form buds, but instead producing elongated, almost hypha-like extensions. However, as in normal cells, actin appeared to accumulate in the growing tip (Fig. 6, E). Cells grown in the absence of NaCl displayed normal morphology and viability (not shown). Interestingly, a strain selected as a polymyxin B resistant derivative of *pbs1 pbs2* (i.e. bearing a suppressor of *pbs2* disruption) showed only weak growth on 0.9 M-NaCl (Fig. 5, E), even though its level of resistance to polymyxin B equalled that of the *pbs1* parent. The suppressor strain, although clearly inhibited by the high salt concentration, was morphologically normal (data not shown). These results may be interpreted as meaning that the aberrant morphology is only partially related to osmotic sensitivity of the *pbs2* disruptant, and that involvement of *PBS2* kinase in antibiotic resistance could be separated from its role in osmoregulation. The above observations, coupled with suppression of *RAS2* mutations, suggest that *PBS2* is involved in several diverse physiological processes, all of which may relate to cell membrane function.

## Discussion

The results of this study indicate that the *RAS2* and *PBS2* genes may be involved in a common pathway. The fact that deletions of *PBS2* suppress the null *ras2* phenotype of JC302-26B suggests that *PBS2* acts (negatively) on an element downstream of *RAS2*; it also implies an antagonism between the products of the two genes. In addition, overexpression of *PBS2* weakens the effect of the enhanced *RAS2* activity of TK161-R2V, thus confirming the antagonistic relationship between *PBS2* and *RAS2*. It is possible that *RAS2* is a negative regulator of *PBS2* activity; another possibility is that *RAS2* overcomes the negative effect of *PBS2* on a component downstream from these two genes (for example, by promoting dephosphorylation of that component). Alternatively or simultaneously, deletion of *PBS2* may allow derepression of *RAS1* gene that is normally repressed in the presence of nonfermentable carbon sources (Breviario *et al.*, 1986), thus permitting growth of the *ras2 pbs2* double mutant on ethanol. It is important to note that the suppression is not very strong, and that neither overexpression nor deletion of *PBS2* allows cells to bypass other *RAS* functions or to restore a completely wild-type phenotype.

One cellular component directly downstream from *RAS2* is the adenylyl cyclase complex (encoded by *CYR1*, *CAP*, *IRA1*, and perhaps other genes; Fedor-Chaikin *et al.*, 1990; Field *et al.*, 1990a; Mitts *et al.*, 1992; Vojtek *et al.*, 1991). The *RAS* proteins control the activity of this complex (Fedor-Chaikin *et al.*, 1990; Field *et al.*, 1990a; Toda *et al.*, 1985; Vojtek *et al.*, 1991). Since *PBS2* deletion or overexpression cannot suppress mutations either in the *CYR1* (*CDC35*) gene or in a double mutant *ras1 ras2* (data not shown), changes in *PBS2* do not allow cells to bypass the cyclase step. Nevertheless, a specific interaction can be envisioned. It is possible that the antagonistic relationship between *RAS2* and *PBS2* is reflected, in part, in the phosphorylation–dephosphorylation state of the adenylyl cyclase complex: *PBS2* kinase may down-modulate the complex activity, while *RAS2* could promote dephosphorylation. If so, in a *ras2* background, deletion of *PBS2* should decrease the amount of a phosphorylated complex, thereby enhancing its basal activity, and thus permitting the *ras2* mutant to grow on ethanol (Fig. 3). In *RAS2<sup>Val19</sup>* cells, in which constitutive activation of the adenylyl cyclase is the presumed reason for loss of viability upon starvation (Toda *et al.*, 1985), diminished phosphorylation would lead to an increased cyclase activity (already enhanced by the activated *RAS2* protein) and further depression of viability. Conversely, overexpression of *PBS2* kinase would be expected to enhance cell survival (Table 2, Fig. 4), because it would counteract the

overstimulation of the complex by the *RAS2<sup>Val19</sup>* protein.

At least two additional cellular elements could be the targets for the *RAS2*–*PBS2* pathway or for *PBS2* alone. First, as shown earlier (Fig. 2), overexpression of *PBS2<sup>+</sup>* diminishes mating factor sensitivity. It may be significant that the pheromone response pathway in yeast is mediated by G proteins that share some structural and biochemical features with *RAS* proteins (Blumer & Thorner, 1991). An independent suggestion of ‘cross-talk’ between the pheromone response elements and the *RAS*–adenylyl cyclase pathway has been recently presented by Arkinstall *et al.* (1991).

Second, as already pointed out, *PBS2* is essential for the expression of polymyxin B resistance of *pbs1* mutants (Boguslawski & Polazzi, 1987). Thus, it is likely that the *PBS1* gene product is another substrate for *PBS2* kinase, and that resistance requires phosphorylation of the *PBS1* product. However, other interpretations are possible. For example, it could be that the mutation in *PBS1* results in an increase in *PBS2* expression and, thus, resistance. Two pieces of evidence argue against this possibility. First, there is no difference in the amount of *PBS2* mRNA detectable in either wild-type or *pbs1* cells (data not shown), and previous results indicate that at least four copies of intact *PBS2<sup>+</sup>* are necessary to confer polymyxin B resistance in the wild-type, *PBS1<sup>+</sup>*, background (Boguslawski & Polazzi, 1987). Therefore, at least fourfold (and probably higher) derepression of *PBS2* would have to result from *pbs1* mutation. Second, the resistance due to overexpression of *PBS2<sup>+</sup>* is not observed at 37 °C, whilst that due to *pbs1* mutation is equally strong at 25 °C and 37 °C (not shown). This means that the biochemical basis for resistance must be different in these two cases even though *PBS2* kinase (single gene copy) is essential for expression of the *pbs1*-conferred resistant phenotype.

*pbs1* mutants are deficient in net potassium uptake (R. Gaber and G. Boguslawski, unpublished data). This observation opens an attractive (but speculative) prospect that *PBS1* encodes a (component of) a potassium channel that is dependent on the activities of the *RAS2* and *PBS2* genes. Such a possibility is supported by the evidence that animal *RAS* and other G proteins are linked to eukaryotic potassium channel activity and regulation (Birnbauer *et al.*, 1990; Colin *et al.*, 1990; Rane, 1991), that phosphorylation state affects potassium channel functioning (Rudy, 1988), and that polymyxin B inhibits calcium-activated potassium channels in mammalian cells (Varecka *et al.*, 1987; Weik & Lonnendonker, 1990). A very recent report (Schultz *et al.*, 1992) demonstrates that potassium flux regulates adenylyl cyclase activity in *Paramecium*. The cloning and characterization of *PBS1* will be necessary to determine

whether and how the *RAS2* gene product might interact with potassium channels and with the molecules involved in polymyxin B resistance in yeast.

**Addendum.** While this work was in progress, I was informed by Dr Richard Kahn of the National Cancer Institute, Bethesda, MD, USA, that the previously reported sequence of *PBS2* (Boguslawski & Polazzi, 1987) is in error. The corrections are as follows: nucleotide 665 (C) is replaced by a G, and nucleotide 667 (G) is replaced by a C. These replacements change amino acids Ala222 and Val223 to Gly222 and Leu223, respectively. Further, an additional T residue is found in the nucleotide sequence at the position 2002 that results in a replacement of Tyr668 by Leu668 and in the appearance of a nonsense codon TAG immediately after the Leu668 residue. Therefore, the predicted *PBS2* polypeptide contains 668 amino acids rather than the previously calculated 710 amino acids. The changes in the sequence have no bearing on the previous and current genetic data or on the surmised membership of the *PBS2* product in the protein kinase family.

This paper is dedicated to the memory of my mother.

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