Complete nucleotide sequence of a gene conferring polymyxin B resistance on yeast: Similarity of the predicted polypeptide to protein kinases

(membranes/receptors/phosphorylation)

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ABSTRACT Polymyxin B is an antibiotic that kills sensitive cells by disrupting their membranes. We have cloned a wild-type yeast gene that, when present on a high-copy-number plasmid, renders the cells resistant to the drug. The nucleotide sequence of this gene is presented. A single open reading frame within the sequence has the potential to encode a polypeptide (molecular mass of 77.5 kDa) that shows strong homologies to polypeptides of the protein kinase family. The gene, PBS2, located on chromosome X, is not allelic to the previously described PBS1 gene (where PBS signifies polymyxin B sensitivity). Although *pbs1* mutations confer resistance to high levels of polymyxin B, double mutants, pbs1 pbs2, are not resistant to the drug, indicating that PBS2 is essential for pbs1 activity. Models based on the proposed protein kinase activity of the PBS2 gene product are presented to explain the interaction between PBS1 and PBS2 gene products involved in conferring polymyxin B resistance on yeast cells.

Previous work from this laboratory demonstrated that the cells of *Saccharomyces cerevisiae* are killed by large doses (>0.5 mM) of polymyxin B, a polyamine peptide antibiotic (1). At low sublethal levels (<0.1 mM), polymyxin B renders cells permeable to various drugs, and it [and its nontoxic derivative, polymyxin B nonapeptide] completely abolishes mating (refs. 1 and 2; unpublished data). The latter is caused by interference with sexual agglutination (2). In addition, polymyxin B enhances the effect of mating pheromone MF α on *MATa STE2*⁺ cells but not on *MATa ste2* cells, indicating a degree of specificity of interaction with the MF α receptor (ref 2; unpublished data). These results suggest a complicated pattern of interactions between this antibiotic and the cells of *S. cerevisiae*.

Mutants resistant to high levels of polymyxin B (>1 mM) have been isolated (1). The *pbs* mutations are recessive, and all mutants tested thus far belong to a single complementation group. The *PBS1* gene has been mapped to the right arm of chromosome XV, about 10 centimorgans (cM) from the *ADE2* locus, proximal to the centromere (1).

The present communication describes the cloning, sequencing, and characterization of a yeast gene that confers resistance to polymyxin B when present on a high-copynumber plasmid. We shall refer to this gene as *PBS2* because the present study shows that it is not allelic to *PBS1* (see below). The examination of the sequence of the putative polypeptide encoded by the cloned DNA reveals regions of striking homology to a number of protein kinases from viral, mammalian, and yeast sources. We also show that the activity of the *PBS2* gene is essential for the expression of polymyxin B resistance in *pbs1* mutants and propose a model for the interaction between the *PBS1* and *PBS2* genes.[†]

MATERIALS AND METHODS

Yeast strains used in this work are listed in Table 1. The solid and liquid media, standard growth conditions, and genetic techniques have been described (4, 5). Yeast transformations were done by the method of Ito et al. (6). Procedures for plasmid isolation from Escherichia coli, restriction fragment analysis, subcloning, gel electrophoresis of DNA and RNA, and nucleic acid hybridizations have been described in detail (4). Total yeast RNA was prepared as described by Rose and Botstein (7) and analyzed by electrophoresis on formaldehyde/agarose gels (4). Total yeast DNA was obtained by the method of Nasmyth and Reed (8), extracted with phenol, and dissolved in Tris/EDTA buffer. The yeast DNA library in YEp24 (9) was prepared from DBY939 (PBS1⁺ PBS2⁺) and was kindly provided by D. Botstein (10). Polymyxin B was purchased from Sigma. Resistance to the drug was tested as described (1). DNA sequencing procedures were those of Sanger et al. (11) with modifications (12). More than 95% of the sequence was obtained by subcloning random sonication fragments (13) into M13mp18 or by the ordered deletion method of Dale et al. (14). The remaining portions of the sequence were determined by using internal primers (15).

RESULTS

Selection of Clones Resistant to Polymyxin B (PBS^r). The rationale of Rine et al. (16) was used in screening the yeast DNA library in YEp24 vector (10). The method relies on an increased dosage of a wild-type gene product to overcome the inhibitory effects of the drug. Yeast strain YNN27 was transformed with the DNA library to uracil prototrophy (Ura⁺). Approximately 13,000 Ura⁺ transformants were examined for resistance to the antibiotic (PBS^r) and 64 Ura⁺ PBS^r clones were identified. Linkage between the Ura⁺ phenotype and polymyxin B resistance was demonstrated by subjecting these clones to several cycles of growth to saturation under nonselective conditions. This regimen allowed the cells to segregate the plasmid and thus to become Ura⁻. Only those isolates that simultaneously became Ura⁻ and PBS sensitive (PBS^s) were retained. Six independent clones (Ura⁺ PBS^r) were obtained in this manner.

Characterization of Cloned DNA. The structure of one plasmid isolate named R5H is shown in Fig. 1. The plasmid consists of the YEp24 vector moiety and a 7.8-kilobase (kb) insert. The plasmid DNA can be subdivided into five *Cla* I fragments. The *Cla* I fragments 1 through 4 were subcloned

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Abbreviations: PBS, polymyxin B sensitivity; PBS^r, polymyxin B resistant; Ura⁺, uracil prototrophy; cM, centimorgan. *To whom reprint requests should be addressed.

[†]The sequence reported in this paper is being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J02946).

Strain	Relevant genotype	Source or ref.		
YNN27	MATα trp1-289 ura3-52	R. W. Davis*		
DBY703	MATα trp1-289 his3-1 ura3-52 [cir°]	D. Botstein [†]		
DBY939	MATa suc2-215am ade2-110	D. Botstein [†]		
KFY1-1	MATα leu2-3,112 his5 ura3-52 [cir°]	J. Broach [‡]		
KFY1-4	MATa leu2-3,112 trp1 his3 ura3-52 [cir ^o]	J. Broach [‡]		
GBH4	MATa his4-519 leu2-3,112 can1 pbs1-12	1		
GBH14	MATa trp1-289 can1 pbs1-12	1		
GBH17	MATa trp1-289 ura3-52 can1 pbs1-12	1		
GBH21	MATα trp1-289 ura3-52 pbs1-273	From YNN27 (2)		
GBD4	Diploid from cross GBH4 \times YNN27	1		
GBD7	Diploid from cross GBH4 \times GBH14	This work		
GBD13	Diploid from cross KFY1-1 \times KFY1-4	This work		
GBD16	Diploid from cross DBY939 × GBH17	This work		
GBD18	Diploid from cross KFY1-4 \times YNN27	This work		
K396-11A	MATa spoll ura3 adel hisl leul lys7 met3 trp5 [cir ⁺]	3		

Table	1	Veast	strains
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*Stanford University.

[†]Massachusetts Institute of Technology.

[‡]Princeton University.

separately into YEp24 and used to transform YNN27; however, none of the fragments could by itself confer resistance (not shown). Examination of RNA blot-hybridization patterns (Fig. 1) with a DNA probe prepared from *Cla* I fragment 3 revealed that this probe hybridized to two large RNAs (2.7 and 3.6 kb). The transcripts are present at low levels in untransformed yeast cells. Deletion analysis determined that a 3.2-kb *Dra* I-*Sac* I fragment (encoding the 2.7-kb RNA) is sufficient for the resistance phenotype (not shown).

Sequence of PBS2 Gene. The nucleotide sequence of the gene and the predicted polypeptide sequence are shown in Fig. 2. The single open reading frame (positions 1–2130) has the potential to code for a protein of 710 amino acids and molecular mass of 77.5 kDa. The predicted protein is strongly hydrophilic, with only a few hydrophobic pockets (e.g., the

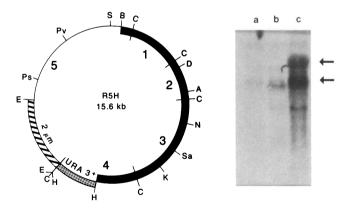


FIG. 1. Polymyxin B-resistance plasmid R5H. (Left) Essential features of R5H, the solid bar representing the cloned DNA. The numbers refer to Cla I fragments discussed in the text. The sizes of the Cla I fragments are as follows: 1, 1.45 kb; 2, 1.2 kb; 3, 4.15 kb; and 4, 2.1 kb. Fragment 5 (6.7 kb) consists of YEp24 sequences (except for the $URA3^+$ gene, which is part of fragment 4). The restriction sites are indicated as follows: A, Aat II; B, BamHI; C, Cla I; D, Dra I; E, EcoRI; H, HindIII; K, Kpn I; N, Nar I; Ps, Pst I; Pv, Pvu II; S, Sal I; and Sa, Sac I. In one experiment, the unique Kpn I site was replaced by a Sal I site to produce plasmid R5H.Sal. Note the absence of Pvu II sites in the cloned DNA (also see Fig. 5). (Right) Blot hybridization of total RNA from YNN27 [untransformed (lane a), transformed with YEp24 (lane b), and transformed with R5H (lane c)] probed with labeled Cla I fragment 3. The arrows show the two most abundant RNA species hybridizing to this probe. The lower species (2.7 kb) also hybridizes to Cla I fragment 2, and the upper RNA (3.6 kb) also hybridizes to Cla I fragment 4. The resistance gene is contained within the Dra I-Sac I segment of the cloned yeast DNA.

proline-leucine string at positions 94-99). Regions of a rather unusual amino acid composition within the deduced polypeptide include two threonine + serine sequences (amino acids 36-41 and 280-287), and a sequence rich in serine, glycine, and asparagine (positions 301-316). The polypeptide terminates in a short, strongly hydrophobic string of amino acids, reminiscent of yeast RAS1 and RAS2 proteins (18).

Amino Acid Sequence Homologies. Studies on the action of polymyxin B on mammalian cells have shown that this antibiotic is a strong inhibitor of the phospholipid/Ca²⁺dependent protein kinase C (19-21). This fact prompted us to compare the predicted sequence of PBS2 protein with several recently described protein kinases (22-25). The PBS2 protein indeed appears to be related to polypeptides of the protein kinase family (Fig. 3), including the oncogene proteintyrosine kinases and serine/threonine-protein kinases (27-29). The regions of relatedness include (in order) the sequences known to be essential for ATP binding [Gly-Xaa-Gly-Xaa-Xaa-Gly-(Xaa)14-Ala-Xaa-Lys-] and for phosphoreceptor activity [Arg-Asp-Val-(Xaa)₁₇-Asp-Phe-Gly-(Xaa)₂₀-Ala-Pro-Glu-(Xaa)₁₆-Asp-Xaa-Trp-Xaa-Xaa-Gly-] (26, 30) as well as other conserved regions shown in Fig. 3. Notable is the match between PBS2 and STE7 sequences (100 perfect matches and 25 additional highly conserved substitutions within the aligned domains of 252 and 263 amino acids, respectively).

Genetic Analysis. The cloned PBS2 gene confers polymyxin B resistance when present in high copy number. To determine the importance of copy number and the relationship with the previously described PBS1 gene (1), the cloned PBS2 DNA (a 6-kb BamHI-Sal I fragment from R5H.Sal; see Fig. 1) was ligated into a centromeric plasmid, YCp50 (9), to yield YCp50.PBS2. This plasmid is maintained at 1 or 2 copies per cell and does not confer polymyxin B resistance on wild-type cells (Fig. 4). A pbs1 mutant strain GBH21 was transformed with YCp50.PBS2, and the Ura⁺ transformants were tested for resistance. Since the *pbs1* mutation is recessive, the wild-type PBS2 gene on YCp50 plasmid should, if it were allelic, complement the mutation and restore the sensitive phenotype. However, as Fig. 4 shows, the strain remained resistant to the drug. Therefore, PBS2 gene is not functionally allelic with PBS1. Note that strain DBY939 from which PBS2 DNA was obtained is sensitive to polymyxin B (Fig. 4), so it is unlikely that a suppressor of *PBS1*⁺ would have been cloned by our procedure.

A gene-disruption experiment was carried out to determine whether the *PBS2* gene is essential for viability. A 1.17-kb *Aat* II-*Nar* I fragment (nucleotides 114-1285 in Fig. 2)

- 340 - 310 - 360 -350 -330 - 320 TOG CAA TTT TTC CAG TTT TTT CCC TCT GOG TCC CGT TGC ACC TGA AAG GAT CTT TCT AAC -270 - 300 -290 -280 -260 -250 GTG TGT TGT CTA CTA GTG AGC GAT TTC GTG AGC CAT ACA CGT TCT ATA GAA AAT TGA ATA -200 -240 -230 -220 -210 -190 AAC TIT ACT TCA AAG GGA TCT GGA CAC AGA GAT AAC TGC TTA CCT GCT TGC CGG AAG AAA -140 -170 -160 -150 -180 -130 AGA ATT ACT AAA AAA GAA GAC AAG GGT AGC TGC TAT TGT GGG TAC ACG TTT CAC AGA ACT -80 -120 -110 -100 -90 -70 ACT TTT TCC TTG TCC TTC TCC AGA CAT CAA CGT CAT ACA ACT AAA ACT GAT AAA GTA CCC -50 -40 -30 -20 -60 -10 GTT TTT CCG TAC ATT TCT ATA GAT ACA TTA TTA TAT TAA GCA GAT CGA GAC GTT AAT TTC 10 20 30 40 1 50 TCA ANG ATG GAA GAC ANG TTT GCT ANC CTC AGT CTC CAT GAG AAA ACT GGT AAG TCA TCT Met Glu Asp Lys Phe Ala Asn Leu Ser Leu His Glu Lys Thr Gly Lys Ser Ser 60 70 80 90 100 110 ATC CAA TTA AAC GAG CAA ACA GGC TCA GAT AAT GGC TCT GCT GTC AAG AGA ACA Ile Gin Leu Asn Glu Gin Thr Gly Ser Asp Asn Gly Ser Ala Val Lys Arg Thr ACA TCT TCG 120 130 140 150 160 170 ACG TCC TCG CAC TAC AAT AAC ATC AAC GCT GAC CT CAT GCT CAT GTA AAA GCT TIT CAA Thr Ser Ser His Tyr Asn Asn Ile Asn Ala Asp Leu His Ala Arg Val Lys Ala Phe Gln 58 190 200 210 220 180 230 GAA CAA CGT GCA TTG AAA AGG TCT GCC AGC GTG GCC AGT AAT CAA AGC GAG CAA GAC AAA Glu Gln Arg Ala Leu Lys Arg Ser Ala Ser Val Gly Ser Asn Gln Ser Glu Gln Asp Lys 78 240 250 260 270 280 290 GCC AGT ICA CAA TCA CCT AAA CAT ATT CAG CAG TAT GTT AAT AAG CCA TTG CCG CCT CTT Gly Ser Ser Gln Ser Pro Lys His Ile Gln Gln Ile Val Asn Lys Pro Leu Pro Pro Leu 98 300 310 320 330 340 350 ccc GTA GCA GGA AGT TCT AAG GTT TCA CAA AGA ATG AGT AGC CAA GTC GTG CAG GGC TCC Pro Val Ala Gly Ser Ser Lys Val Ser Gln Arg Met Ser Ser Gln Val Val Gln Ala Ser 118 360 370 380 390 400 410 TCC ANG AGC ACT CTT ANG ANC GTT CTG GAC AAT CAA GAA ACA CAA AAC ATT ACC GAC GTA Ser Lys Ser Thr Leu Lys Asn Val Leu Asp Asn Gln Glu Thr Gln Asn Ile Thr Asp Val 138 420 430 440 450 460 * * * * 470 AAT ATT ACA ACC AAA ATT ACC GCC ACA ACA ATT GCT GTA AAT ACT GCC CTA Asn Ile Asn Ile Asp Thr Thr Lys Ile Thr Ala Thr Thr Ile Gly Val Asn Thr Gly Leu 480 490 500 510 520 530 158 CCT GCT ACT GAC ATT ACG CCG TCA GTT TCT AAT ACT GCA TCA GCA ACA CAT AAG GCG CAA Pro Ala Thr Asp Ile Thr Pro Ser Val Ser Asn Thr Ala Ser Ala Thr His Lys Ala Gln 178 540 550 560 570 580 5**9**0 TTG CTG AAT CCT AAC AGG AGG GCA CCA AGA AGG CCG CTT TCT ACC CAG CAC CCT AGA AGA Leu Leu Asn Pro Asn Arg Arg Ala Pro Arg Arg Pro Leu Ser Thr Gln His Pro Thr Arg 198 600 610 620 630 640 650 CA AN GTT GCC CCG CAT AAG GCC CCT GCT ATA ATC AAC ACA CCA AAA CAA AGT TTA AGT TPO ASR Val Ala Pro His Lys Ala Pro Ala Ile Ile Asn Thr Pro Lys Gin Ser Leu Ser 218 660 670 680 690 700 710 GCC CGT CGA GCG GTC AAA TTA CCA CCA GGA GGA ATG TCA TTA AAA ATG CCC ACT AAA Ala Arg Arg Ala Val Lys Leu Pro Pro Gly Gly Met Ser Leu Lys Met Pro Thr Lys 238 720 730 740 750 760 770 GCT CAA CAG CCG CAG CAG TTT GCC CCA AGC CCT TCA AAC AAA AAA CAT ATA GAA ACC TTA Ala Gln Gln Pro Gln Gln Phe Ala Pro Ser Pro Ser Asn Lys Lys His Ile Glu Thr Leu 258 780 790 800 810 820 830 TCA AAC AGC AAA GTI GTI GAA GGG AAA AGA TCG AAT CCG GGT TCT TTG ATA AAT GGT GTG Ser Asn Ser Lys Val Val Glu Gly Lys Arg Ser Asn Pro Gly Ser Leu Ile Asn Gly Val 278 840 850 860 870 880 * * * * * 890 CAA AGC ACA TCC ACC TCA TCA AGT ACC GAA GGC CCA CAT GAC ACT GTA GGC ACT ACA CCC Gin <u>Ser Thr Ser Thr Ser Ser Ser Thr</u> Glu Gly Pro His Asp Thr Val Gly Thr Thr Pro 298 910 920 930 940 * * * * 950 900 AGA ACT GGA AAC AGC AAC AAC TCT TCA AAT TCT GGT AGT AGT GGT GGT GGT GGT GGT CTT TTC Arg Thr Gly Asn Ser Asn Asn Ser Ser Asn Ser Gly Ser Ser Gly Gly Gly Gly Gly Leu Phe 318 960 970 980 990 1000 1010 GCA AAT TTC TCG AAA TAC GTG GAT ATC AAA TCC GGC TCT TTG AAT TTT GCA GGC AAA CTA Ala Asn Phe Ser Lys Tyr Val Asp Ile Lys Ser Gly Ser Leu Asn Phe Ala Gly Lys Leu 338 1030 1040 1050 1060 1070 1020 TOG CTA TOC TOT AAA GGA ATA GAT TTC AGC AAT GGT TOT AGT TOG AGA ATT ACA TTG GAC Ser Leu Ser Ser Lys Gly Ile Asp Phe Ser Asn Gly Ser Ser Arg Ile Thr Leu Asp 358

	1080		1090		1100			1110		1120		1130		
GAA	CTA GAA	TTT	TTG GAT	GAA	CTG GGT	CAT	GGT	**************************************	GGT	AAC GTC	TCA	AAG GTA	CTG	
GIU	Leu Giu 1140	rne	1150	Giu	Leu GIY 1160	HIS		Asn 1yr 1170	GIY	Asn Val 1180	Ser	Lys Val 1190	Leu	378
CAT	AAG CCC	ACA	AAT GTT	ATT	ATG GCG	ACG	AAG	GAA GTC	CGT	TTG GAG	CTA	GAT GAG	GCT	
His	Lys Pro 1200	Thr	Asn Val 1210	Ile	Met Ala 1220	Thr	Lys	Glu Val 1230	Arg	Leu Glu 1240	Leu	Asp Glu 1250	Ala	398
***	* TTT AGA	CAA	ATT TTA	ATG	GAA CTA	GAA	GTT	* TTG CAT	A A A	* TGC AAT	TCT	CCC TAT	ATT	
Lys	Phe Arg 1260	Gln	Ile Leu 1270	Met	Glu Leu 1280	Glu	Val	Leu His 1290	Lys	Cys Asn 1300	Ser	Pro Tyr 1310	Ile	418
CTC	ń	TAT	st.	TTC	*	GAG	•	*	TAC	ATG TGT	ATC	*	ATC	
Val	Asp Phe	Tyr	Gly Ala	Phe	Phe Ile	Glu	Gly	Ala Val	Tyr	Met Cys	Met	Glu Tyr	Met	438
	1320 *		1330 *		1340 *			1350 *		1360 *		1370 *		
GAT Asp	GGT GGT Gly Gly	TCC Ser	TTG GAT Leu Asp	AAA Lys	ATA TAC Ile Tyr	GAC Asp	GAA Glu	TCA TCT Ser Ser	GAA Glu	ATC GGC Ile Gly	GGC Gly	ATT GAT Ile Asp	GAA Glu	458
	1380 *		1390 *		1400			1410		1420		1430		
CCT	CAG CTA	GCG	TTT ATT Phe Ile	GCC Ala	AAT GCT	GTC Val		CAT GGA	CTA	AAA GAA Lys Glu	CTC	AAA GAG	CAG Gln	478
	1440		1450		1460			1470		1480		1490		
CAT	AAT ATC	ATA	CAC AGA	GAT	GTC AAA	CCA	ACA	AAT ATT	TTA	TGT TCA	GCC	AAC CAA	GGC	
HIS	1500	TTe	1510	Asp	1520	PTO	Inr	1530	Leu	Cys Ser 1540	AIA	ASN GIN 1550		498
ACC	GTA AAG	CTG	* TGC GAT	ттс	GGT GTT	TCT	GGT	AAT TTG	GTG	GCA TCT	TTA	GCG AAG	ACT	
Thr	Val Lys 1560	Leu	Cys Asp 1570	Phe	Gly Val 1580	Ser	Gly	Asn Leu 1590	Val	Ala Ser 1600	Leu	Ala Lys 1610	Thr	518
	s'r	TOT	*	TAC	*	CCT	CAA	*		TCG TTG	A A T	*		
Asn	Ile Gly	Cys	Gln Ser	Tyr	Met Ala	Pro	Glu	Arg Ile	Lys	Ser Leu	Asn	Pro Asp	Arg	538
	1620 *		1630 *		1640 *			1650 *		1660 *		1670 *		
GCC Ala	ACC TAT Thr Tyr	ACC Thr	GTA CAG Val Gln	TCA Ser	GAC ATC Asp Ile	TGG Trp	TCT Ser	TTA GGT Leu Gly	TTA Leu	AGC ATT Ser Ile	CTG Leu	GAA ATG Glu Met	GCA Ala	558
	1680 *		1690 *		1700 *			1710 *		1720 *		1730		
CTA Leu	GGT AGA	TAT Tvr	CCG TAT	CCA Pro	CCA GAA Pro Glu	ACA Thr	TAC Tvr	GAC AAC	ATT	TTC TCT Phe Ser	CAA Gln	TTG AGO	GCT	578
200	1740	-,-	1750		1760		-,-	1770		1780	0111	1790		570
ATT	GTT GAT	GGG	CCG CCA	CCG	AGA TTA	сст	TCA	GAT AAA	TTC	AGT TCT	GAC	GCA CAA	GAT	
116	val Asp 1800	GIY	1810	Pro	1820	Pro	Ser	Asp Lys 1830	Phe	Ser Ser 1840	Asp	Ala Gin 1850	Asp	598
TTT	GTT TCT	TTA	54	CAA	*	CCG	GAA	*	CCT	* ACA TAC	GCA	GCT TTA	ACA	
Phe	Val Ser 1860	Leu	Cys Leu 1870	Gln	Lys Ile 1880	Pro	Glu	Arg Arg 1890	Pro	Thr Tyr 1900	Ala	Ala Leu 1910	Thr	618
CAG	*	TCC	*		*		CAC	*	CAC	ATG AGT	C1C	*	ACT	
Glu	His Pro	Trp	Leu Val	Lys	Tyr Arg	Asn	Gln	Asp Val	His	Met Ser	Glu	Tyr Ile	Thr	638
	1920		1930 *		1940			1950 *		1960 *		1970 *		
GAA Glu	CGA TTA Arg Leu	GAA Glu	AGG CGC Arg Arg	AAC Asn	AAA ATC Lys Ile	TTA Leu	CGG Arg	GAA CGT Glu Arg	GGT Gly	GAG AAT Glu Asn	GGT Gly	TTA TCI Leu Ser	AAA Lys	658
	1980 *		1990 *		2000 *			2010 *		2020		2030		
AAT Asn	GTA CCG Val Pro	GCA Ala	TTA CAT Leu His	ATG Met	GGT GGT G1v G1v	TAT Tvr	AGC Ser	GTT AAT Val Asn	ATC Ile	CAA ATA Gln Ile	AAA Lys	GCA AAC	AGG	678
	2040		2050		2060	-,-		2070		2080	-,-	2090		
					AAA AAG			AAA AGC		TGT GGA				(00
HIS	2100	116	2110	Lys	2120 2120	GIN	Inr	2130	Tyr	Cys Gly 2140	ASN	ASP A18 2150		698
AAT	* ***	***	CCT TAC	ATA	* TAC ATA	TGŤ	TTA	TTG TAA	TAA	ACT TGC	ATT	ATA CTO	GTT	
Asn			Pro Tyr		-	-				2200		2210		710
ΑΤΑ	*		2170 *						GTC	TGA CTT				
	2220		2230		2240					2260		22.10		
ССТ	*	GGG	*	TTA					ATG	TTA TTT	CAA	GCA CCC	GCA	
	2280		2290		2300			2310		2320		2330		
AGT	sir.	ATG	*		* ATA TAT	TGT	GTT	*	TTA	TTG CTA	CGG	CCA TCO	ACT	
	2340		2350		2360			2370		2380		2390		
CCT	*	TAT	*	TCG	*		ACG	ŵ	AGA	*	GCT	CTG GAA	TTG	
	2400		2410		2420			2430		2440		2450		
TCT	GAT GGT	TTT	TCC GCT	стт	ĸ			* CTA GGA	AAG	*	AAG	TCC AA	ATC	
	2460		2470		2480			2490		2500		2510)	
ATC	GAG AAA	ATA	AAA GGT	GTT	TTG AAA		CAA	ATC CAC	GTT	ATT GAG	AGT	AGA TG	GGA	

FIG. 2. Nucleotide sequence of the *PBS2* gene and the predicted amino acid sequence of the product. Nucleotides are numbered above the sequence; amino acids are numbered on the right side of the sequence. Regions rich in serine and threonine, and the Gly-Asn-Ser stretch (amino acids 301–316) are underlined. A potential stem–loop sequence at the 3' untranslated region is doubly underlined. A possible transcription termination sequence (TAG/TATGA/TTT) of the kind observed by Zaret and Sherman (17) is found between nucleotides 2157 and 2262. The black dots at nucleotides 114, 424, and 1285 indicate the locations of *Aat* II, *Cla* I, and *Nar* I sites, respectively, used in the subcloning of an internal fragment of *PBS2* and subsequent gene disruption experiments (see the text).

spanning the *Cla* I site between fragments 2 and 3 (see Fig. 1) was subcloned into an integrating plasmid, YIp5 (9), digested with *Aat* II and *Nar* I (plasmid YIp5-AN117; 5.4 kb). The DNA was cut with *Cla* I, and a diploid GBD13 was transformed to uracil prototrophy. The diploid was sporulated, and 10 tetrads were dissected. All tetrads yielded four viable spores (2 Ura⁺: 2 Ura⁻), showing that *PBS2* is not

essential for viability. The site of integration was outside of the *ura3* locus (31). Identical results were obtained when a haploid strain YNN27 was transformed with YIp5-AN117. Southern blot analysis of the URA⁺ transformants showed that the *PBS2* gene was disrupted by integration of a single copy of the plasmid (Fig. 5) and that the gene was not duplicated elsewhere in the genome.

Genetics: Boguslawski and Polazzi

Υ		
355 ITLDELEFLDELSHGNYGNVSKVLHKPTNVIMATKE 186 I-L-DL51G-GN-G-V-K-LH-PI-A-K- 52 I4-G-G-G-G-V-K-LH-PI-A-K- 5 L5-LE-VG-G-YG-V-K-L2RPSVVK- 334 V-L-0FL-LGKG-FG-VK-T-L-A-K-		PBS2 STE7 SNF1 CDC28 PKC
391 VRLELDEAKFRQILMELEVLHKCNSPYIVDFYGAFF 222 I-VE 8 QLV-EL-LVK 7 II-FYGAYY 86 IV 1AK 3 QE-EI 0 SOYL 20 42 IRLE2DEG 18 NIVDIVH 6 YLVOFF 2 370 LK-DV 14 ROVL 9 LLH-C 0 F2VD 2 YF *		FBS2 STE7 SNF1 CDC118 PKC
427 IEGAVYMCMFYMDGGSLDKIYDESSEIGGIDEPQLA 271 11 MEY-D-GSLDKI 13 SSEI- 128 I-MEY0AG 2 LFD 15 93 LDY0ME 8 GA-I-K-F 2 QL 416 V 0 MEYGG-L2HI 2 VGEFQ		FBSC STE7 SNF1 CDCC8 PKC
463 FIANAVIHGLKELKEGHNIHHEDVKPTNILCGANQG 309 -IAVL-GLL-QIIHEDIKF-NVLG 151 F 3 II-AVHKOVHRULKF-NVL 5 N 0 122 1 KGI 3 H-ILHRDLKF-NLL 5 N 0 442 F-AI-G-GLLH 3 II-RDLKF-NV 5 G		FBS2 STE7 SNF1 CDC28 PKC
439 TVKLCDEGV2GNLVASLAKTNIGCQSYMAPERIK3L 345 -1KLCDEGVSL1-SIA+T-VGYM-PERI 191 VKL-DEGL20NI 11 GY-APE-I 150 LKL-DEGL 7 L-A 5 V 3 Y-APE-L 476 LKL-DEG 8 G0V2RTGY-APE-I 2 *** **		PBS2 STE7 SNF1 CDC28 PKC
835 NPDRATYTVQSDIWSLGLSILEMALGRYPYPFETYD 381 4 Y-1DVWSLGL HE G FP 5 D 381 4 Y = 1DVWSLGL HE G FP 5 D 381 4 Y = 5 DUWS GV-LMR-FFE 189 5 YD-WSIGEM 2 R-F 0 513 Q 1 YD-W-GV-L EMG -FFED		PBS2 STE7 SNF1 CDC28 FKC
571 NIFSQLSAIVDGPPPRLPSDKFSSDAQDFVSLCLQKI 421 11 IV-FP-PRLP-12YS-EDFVCK 259 -LF-NIS 6 PKS G 5 L-V2LNRI 211 IFSS-I 7 RV 5 EA 6YL 546 -LF1 7 PKS 7 S EA 0 VSIC10RL	607 449 295 242 581	STE7 UNF1

FIG. 3. Homologies between the predicted sequence of PBS2 protein and some other members of the protein kinase family: the single-letter amino acid code is used. The sequences were aligned by eye. The following equivalences were assumed for the purpose of the present alignment: 1=L=V, F=Y=W, G=A, K=H=R, E=D, and Q=N. Dashes indicate identical spacing but different amino acids, while small numbers within open gaps show distances between indicated amino acids in the corresponding sequences. Asterisks show positions that are either identical or highly conserved in all five sequences. An arrowhead points to the conserved lysine residue that is postulated to form a part of the catalytic site of kinases (26). The sequences compared to *PBS2* were taken from references 25 (*STE7*), 23 (*SNF1*), 22 (*CDC28*), and 24 (*PKC*). The *STE7* gene product has not been shown yet to possess protein kinase activity, but it is included here for its exceptionally high degree of homology to *PBS2*.

When the *PBS2* gene in *pbs1* strain GBH21 was disrupted by integration of plasmid YIp5-AN117 (Fig. 5), the resulting double mutant was also viable and grew well on standard media. However, the disruption resulted in the disappearance of resistance (Fig. 6), even though GBH21 is normally resistant to polymyxin B concentration as high as 1.5 mM.

We used the 2- μ m plasmid method of Falco and Botstein (32) to assign the PBS2 gene to a chromosome. A plasmid, YEp24-4.15, carrying Cla I fragment 3 (see Fig. 1) was linearized with Kpn I and used to transform strain DBY703 [cir⁰]. The strain with integrated plasmid was then crossed to a mapping strain K396-11A[cir⁺] (3). The presence of $2-\mu m$ DNA in the resulting diploid induced instability of the chromosome with the integrated YEp24-4.15 and unmasked a met3 mutation, thus locating the PBS2 gene on chromosome X. This assignment was confirmed by hybridization of Cla I fragment 3 to the chromosome X band resolved by orthogonal field-alternating gel electrophoresis (ref. 33; data not shown). Three-factor crosses demonstrated linkage of PBS2 to INO1 and ARG3 genes (this work; John Cannon, University of Pennsylvania, personal communication). Among 108 tetrads tested for ARG3-PBS2 linkage, 55 were parental ditype, 51 were tetratype, and 2 were nonparental ditype. For the PBS2-INO1 pair, we observed 69 parental

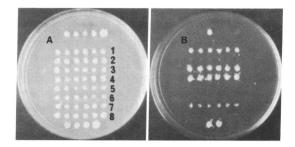


FIG. 4. Effect of the copy number of the *PBS2* gene on the expression of resistance to polymyxin B. Yeast strains were transformed to Ura⁺ with YCp50 (top and bottom rows) or with R5H (odd-numbered rows) or YCp50.PBS2 (even-numbered rows). The control plate (*A*) was printed onto a yeast extract-peptone-glucose plate containing 1.0 mM polymyxin B (*B*) and incubated 48 hr at 30°C. Rows: top (unnumbered), from left to right: YNN27, GBH21, GBD13, GBD18, and GBD16; 1 and 2, YNN27; 3 and 4, GBH21; 5 and 6, GBD13; 7 and 8, GBD18; bottom (unnumbered), from left to right: DBY939, GBH17, GBD7, and GBD4. Note that some "wild-type" strains are more sensitive to polymyxin B than others (e.g., compare GBD13, row 5, and GBD18, row 7). Even though the cells are transformed with the same plasmid R5H, GBD13 remains sensitive while GBD18 acquires resistance. The reason for this variability is unknown.

ditype and 39 tetratype asci; for the *INOI-ARG3* pair, we found 31 parental ditype, 73 tetratype, and 4 nonparental ditype asci. From these results, the calculated map distances are 18.0 cM for *INOI-PBS2*, 29.1 cM for *PBS2-ARG3*, and 44.9 cM for *INOI-ARG3*, and the order of the genes is *INOI-PBS2-ARG3* as determined from the double crossover (lowest frequency) class of recombinant spores and from the distances calculated above.

DISCUSSION

We have identified a yeast gene that confers PBS^r phenotype when propagated on a high-copy-number plasmid. The *PBS2*

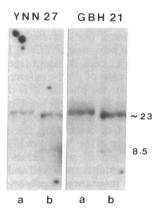


FIG. 5. Southern blot hybridization analysis of PBS2 gene and pbs2 disruption. Total DNA was isolated from yeast strains YNN27 (PBS1+) and GBH21 (pbs1-273), digested with Pvu II, and fractionated on 0.8% agarose gel. DNA was transferred onto nitrocellulose membrane and hybridized [2× SSPE (1× = 0.15 M NaCl/10 mM sodium phosphate, pH 7.4/2 mM EDTA, pH 7.4) at 37°C for 20 hr] to a probe prepared from the Cla I fragment 3 (see Fig. 1) subcloned into pBR322 deleted for the HindIII-Pvu II DNA. The membrane was washed three times at room temperature with $0.8 \times$ SSPE containing 0.1% NaDodSO4 and then incubated in the same solution for 30 min at 65°C. Lanes: a, DNA without disruption; b, DNA from transformants obtained by integration of YIp5-AN117 plasmid. Since there are no Pvu II sites within the PBS2 gene (see Fig. 1) and only one in the YIp5 moiety, cutting the chromosomal DNA with Pvu II generates one hybridizing fragment in the absence of integration and two smaller fragments when a single plasmid integration has occurred. Numbers in the margin show the approximate sizes (kb) of the fragments generated by Pvu II digestion of the disrupted DNA.

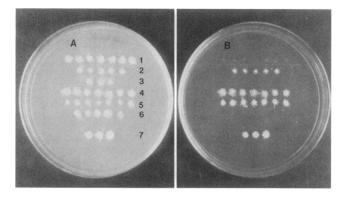


FIG. 6. Effect of PBS2 gene disruption on polymyxin B resistance. Strains YNN27 (rows 1-3) and GBH21 (rows 4-7) were transformed to Ura⁺ with the following plasmids: YEp24-BN445 (rows 1 and 4), R5H (rows 2 and 5), YIp5-AN117 linearized with Cla I (rows 3 and 6), and YEp24 (row 7). YEp24-BN445 carries a fragment from R5H that extends from the unique BamHI site just upstream of the cloned yeast DNA to the Nar I site at nucleotide position 1285 (see Figs. 1 and 2). The control plate (A) was printed onto a yeast extract-peptone-glucose plate containing 0.9 mM polymyxin B (B) and incubated 24 hr at 30°C. See the text for further details.

gene is located on chromosome X and is not allelic to the previously described PBS1 gene (1), nor does its high dosage appear to make *pbs1* mutants more resistant to the drug (not shown). Gene disruption experiments and Southern blot analysis show that PBS2 exists in a single copy in the yeast genome and is not essential for viability. However, the inactivation of the PBS2 gene in a pbs1 background leads to a complete loss of resistance to polymyxin B. This result demonstrates that PBS2 is necessary for the expression of pbs1 activity.

How do cells become resistant to polymyxin B? The resistance observed when cells are transformed with R5H multicopy plasmid is most likely due to titration of the inhibitor by the excess of PBS2 gene product. It is not known how many copies of $PBS2^+$ gene have to be present for the cell to acquire resistance, but the number must be greater than three because the presence of YCp50.PBS2 in a diploid is not sufficient to make cells resistant (see Fig. 4, GBD18, rows 7 and 8). In the case of *pbs1*-conferred resistance, the simplest explanation for the phenotype is that a mutation in a membrane component prevents the antibiotic from reaching its site(s) of action. (This interpretation implies that PBSI gene product is a part of or controls the synthesis of that component.) A mutation of this kind would be expected to be recessive, as indeed are all *pbs1* alleles tested thus far. However, since *pbs1* mutants grow well and have no obvious phenotype except for resistance to polymyxin B, the mutation could not have caused a dramatic functional or structural change in the membrane.

The deduced amino acid sequence (Figs. 2 and 3) strongly suggests a protein kinase function for the putative PBS2 polypeptide, and deletions of portions of the single open reading frame from plasmid R5H abolish the PBS2-mediated resistance (Fig. 6). Therefore, although biochemical characterization of the PBS2 gene product is still lacking, it seems reasonable to assume that it is a protein, most likely a protein kinase. Consequently, one model that can be suggested is that the PBS2 kinase could be involved in phosphorylation of a membrane component (a receptor?) that is affected by polymyxin B and that is modified by the *pbs1* mutation. In wild-type cells, polymyxin B may prevent efficient phosphorylation and thus cause inhibition of growth. The drug also may cross the membrane and inhibit the kinase directly. In contrast, the *pbs1* mutant cells may have undergone modification in the receptor so as to exclude polymyxin B because of a much increased affinity for phosphate groups donated by the kinase and because of the resulting conformational change in the receptor. When PBS2 gene is disrupted, no phosphorylation of the receptor can occur, polymyxin B is able to bind, and inhibition of growth is seen. Alternatively, one could postulate that physical binding of the PBS2 kinase to the *pbs1* receptor precedes (or follows) phosphorylation and is essential for resistance. Again, in the absence of the kinase protein, the resistance would be lost. The definitive test of these models awaits the isolation and characterization of the postulated protein kinase and the cloning and characterization of the PBS1 gene. Whether PBS2 gene encodes a yeast equivalent of protein kinase C, the kinase specifically inhibited by polymyxin B in mammalian cells (19-21), also remains to be seen.

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