# Changes in Mate Recognition Through Alterations of Pheromones and Receptors in the Multisexual Mushroom Fungus *Schizophyllum commune*

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ABSTRACT

*Schizophyllum commune* has thousands of mating types defined in part by numerous lipopeptide pheromones and their G-protein-coupled receptors. These molecules are encoded within multiple versions of two redundantly functioning B mating-type loci, Bα and Bβ. Compatible combinations of pheromones and receptors, produced by individuals of different B mating types, trigger a pathway of fertilization required for sexual development. Analysis of the Bβ2 mating-type locus revealed a large cluster of genes encoding a single pheromone receptor and eight different pheromones. Phenotypic effects of mutations within these genes indicated that small changes in both types of molecules could significantly alter their specificity of interaction. For example, a conservative amino acid substitution in a pheromone resulted in a gain of function toward one receptor and a loss of function with another. A two-amino-acid deletion from a receptor precluded the mutant pheromone from activating the mutant receptor, yet this receptor was activated by other pheromones. Sequence comparisons provided clues toward understanding how so many variants of these multigenic loci could have evolved through duplication and mutational divergence. A three-step model for the origin of new variants comparable to those found in nature is presented.

G-PROTEIN-COUPLED receptors (GPCR) are seven-transmembrane-domain proteins that constitute a large group of plasma membrane-spanning receptors used to sense cues from the external environment of a cell (for reviews, see Strader *et al.* 1994; Bockaert and Pin 1999). Many fungi employ GPCRs to detect small peptide pheromone ligands for recognition of nonself mating partners (Vaillancourt and Raper 1996). In both the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe*, each of two cell types expresses a pheromone receptor distinct for that cell type and a peptide pheromone recognized by the pheromone receptor of the alternate cell type. The one-to-one correspondence between pheromones and receptors of these yeast fungi is an arrangement also found in some hemibasidiomycetes and is predicted to be the case in filamentous ascomycetes in which mating pheromones and receptors, or their genes, were identified (Kronstad and Staben 1997, for review; Zhang *et al.* 1998; Shen *et al.* 1999; Pöggeler 2000). In homobasidiomycetes, commonly referred to as mushroom fungi, multiple mating types are determined in part by many genes encoding different isoprenylated peptide pheromones and pheromone-responsive GPCRs. A variety of such genes were identified for two model organisms, *Schizophyllum commune* and *Coprinus cinereus* (Wendland *et al.* 1995; Vaillancourt *et al.* 1997; O'Shea *et al.* 1998; Halsall *et al.* 2000). These genes lie within master regulatory complexes, called B in both systems, which control precise aspects of mate recognition and sexual development. Two linked, recombinable, and functionally redundant loci, Bα and Bβ, make up the B complex in *S. commune*. Each B locus has different sets of pheromone and receptor genes that define heterologous versions, or what we call specificities of a locus (Figure 1A). Nine different specificities of each B locus exist within the species. These specificities are currently designated Bα1, Bα2, . . ., Bα9 and Bβ1, Bβ2, . . ., Bβ9, where the previously designated Bα1′, Bα2′, Bβ1′, and Bβ2′ are now Bα8, Bα9, Bβ8, and Bβ9, respectively (Koltin *et al.* 1967; Stamberg and Koltin 1972). Two specificities of these loci, Bα1 and Bβ1, were partially characterized with molecular genetic tools and biological assays (Wendland *et al.* 1995; Specht 1996; Vaillancourt *et al.* 1997). Both specificities are multigenic; three different pheromone genes and a single pheromone receptor gene were identified in each. The four genes identified for each specificity do not account for B-dependent recognition of all known mates, thus the analyses of Bα1 and Bβ1 must be incomplete. Receptor genes and some of the pheromone genes from the Bα2 (Hegner *et al.* 1999) and Bα3 specificities (L. J. Vaillancourt and T. J. Fowler, unpublished results) were also isolated. The convention for naming these genes is, for example,

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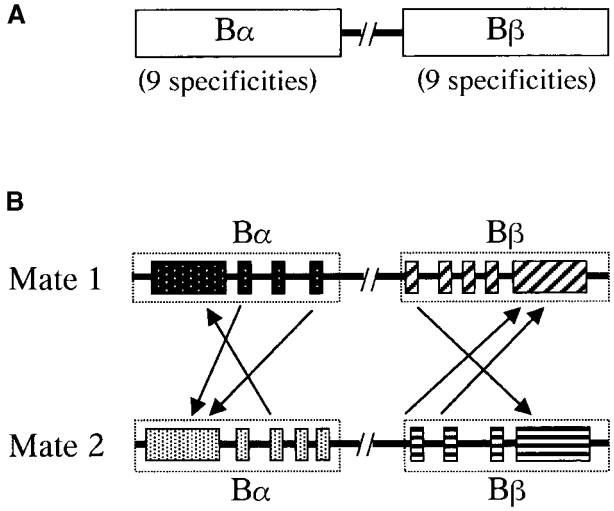


FIGURE 1.—Arrangement of the B mating-type loci of *Schizophyllum commune*. (A) Each of the loci Bα and Bβ has nine distinct heterologous versions called specificities (KOLTIN *et al.* 1967; STAMBERG and KOLTIN 1972). The two loci are linked by a recombinable region presumably of variable length (STAMBERG *et al.* 1977), allowing various combinations of the specificities of Bα and Bβ to arise. (B) Two hypothetical haploid individuals are represented by their B mating-type loci. Mate 1 and Mate 2 have different specificities of both Bα and Bβ. The B loci are multigenic, with the large rectangles representing pheromone receptor genes and the small rectangles representing pheromone genes. Each gene is different. The arrows indicate interactions between pheromones and receptors encoded at these loci that lead to activation of the B-regulated signaling pathway.

*bar1* for *B*α *r*eceptor of specificity *1* and *bbp2(4)* for the fourth *B*β *p*heromone of specificity *2*.

Each haploid individual of *S. commune* expresses pheromones and receptors encoded by genes in both Bα and Bβ, yet signal transduction in the haploid is not triggered by these molecules. Pheromones encoded by a given specificity do not activate the receptor encoded by the same specificity, nor is there any cross-activation between pheromones and receptors derived from linked Bα and Bβ specificities (Figure 1B). Strains containing any of the nine natural specificities of either Bα or Bβ can, by definition, activate strains containing any of the eight other specificities of the same locus. Thus, the interaction of a compatible pheromone and receptor, each produced from different specificities of the same B locus, is the requirement for activation of the B-regulated signaling pathway (Figure 1B). A surprising finding from analyses of Bα1 and Bβ1 specificities was that several pheromones with different predicted primary amino acid sequences were each able to activate the same receptor (WENDLAND *et al.* 1995; VAILLANCOURT *et al.* 1997). Furthermore, each of several pheromones was shown to activate more than one pheromone receptor. The different roles of pheromones and receptors in the initiation of sexual development were also elucidated in these experiments. Pheromones produced by an individual allow it to donate migrating nuclei to fertilize any mate with a receptor capable of activation by the pheromones. The receptors, therefore, act as gatekeepers for the signal transduction pathway that leads to the fertilization process, permitting fertilizing nuclei into the individual only if a compatible pheromone is presented by the mate. Because compatible mates in nature normally express both pheromones and receptors, the fertilization process is reciprocal (Figure 1B; WENDLAND *et al.* 1995; VAILLANCOURT *et al.* 1997).

Prior to the discovery of pheromone and pheromone receptor genes in the B complex of *S. commune*, several mutagenesis experiments were carried out to alter the normal mate recognition process controlled by the B complex with an eye toward generating a new specificity of the Bβ locus. The first mutant, obtained by chemical mutagenesis of a Bα3-Bβ2 haploid strain, was self activated for B-regulated development and the lesion responsible for this phenotype was mapped within Bβ2 (PARAG 1962). Normally, mating of two individuals carrying different specificities of Bα or Bβ, or both, initiates B-regulated development as defined by the reciprocal migration of fertilizing nuclei into and throughout the mycelia of both mates. This process is concomitant with a distinct hyphal morphology called "flat" in which the hyphae grow submerged in agar medium and display characteristic branchiness and distortions of the hyphal walls. The unmated mutant had the flat phenotype with nuclei that migrated continuously from cell to cell. The mutant is alternatively called the primary B-on, B-constitutively on, or B-con mutant in the literature; we use the first term in this article. The primary B-on mutant was subjected to X-ray mutagenesis in later experiments (RAPER and RAPER 1973). New mutants were identified, which exhibited the fluffy wild-type hyphal phenotype and had no evidence of an activated pathway of B-regulated development. Mutants of this type with lesions that mapped to the B complex were designated as secondary mutants. While these mutants were selected for reversion from flat to normal haploid hyphal morphology and accordingly might have been suppressors, all but one were altered in mate recognition as compared to the wild-type grandprogenitor. The secondary mutants were divided into 11 classes according to their ability to switch on B-regulated development in matings with a comprehensive array of wild-type tester strains. None of the secondary mutants had the phenotype of a new specificity strictly comparable to the nine natural Bβ specificities. Analysis of these mutants, however, strongly suggested that the B complex encoded multiple functions, which we now recognize as the effects of pheromones and receptors.

In this study, we set out to determine how the products of the Bβ2 specificity had been changed by these mutations. We first characterized the wild-type Bβ2 specificity and then identified mutational changes in the primary B-on mutant and several secondary mutant classes. We

found that very limited changes in either a pheromone or a receptor can shift the spectrum of mates that are recognized as compatible. This study also provided significant clues as to how specificities of the B mating-type loci could have evolved.

## MATERIALS AND METHODS

**Strains, culturing, transformation, and test mating of *S. commune*:** *S. commune* was cultured and tested in matings according to Raper and Hoffman (1974). Activation of B-regulated development was identified by microscopic examination of the mating partners. Two phenotypes indicated B-regulated development had been turned on: the morphologically distinct submerged hyphal growth called flat, in the absence of development regulated by the A mating-type genes, and the formation of fused clamp connections at each hyphal septum, seen in the presence of A-regulated development (for representative photographs and diagrams, see Raper and Raper 1966). Strains used in this study were originally from the collection stored at the University of Vermont of J. R. Raper and colleagues or were derived from those strains. Protoplast preparation and subsequent transformations resulting in ectopic integration were done according to Specht *et al.* (1988), using Novozyme 234 (InterSpex Products, Foster City, CA) as the wall digesting enzyme, with modifications described by Horton and Raper (1991). Stable transformants were produced from the cotransformation of strains auxotrophic for tryptophan with two plasmids, one containing wild-type *trp1* and the other containing the DNA of interest from the mating-type locus. Transformants were identified as tryptophan prototrophs. On average, 30% of these prototrophs had also integrated the test DNA. A minimum of 12, but usually 25, independent prototrophic transformants were tested when a negative result for B-regulated development was reported. DNA from the Bβ loci was tested for the presence of active pheromone or receptor genes in one of two ways. In some instances, the DNA to be tested was integrated into nine strains, each representing a different natural Bβ specificity. These transformants were directly observed for B-regulated development (Vaillancourt *et al.* 1997). Most test DNAs were integrated into a B-null strain (see below) and the transformants were test mated with a set of strains representative of the nine natural Bβ specificities. Both partners of these matings were observed for B-regulated development.

**A B-null tryptophan auxotroph was developed:** A B-null strain of *S. commune* used as a transformation recipient (V153-21) was derived as follows from a secondary mutant originally designated Bβ2(1-8) by Raper and Raper (1973). This mutant shows no B-regulated development in matings with any wild-type strain. The Bβ2(1-8) null strain was forced to mate through confrontation with the primary B-on mutant. Heterokaryotic dikaryons were selected through complementing nutritional markers carried by the two mated strains. These dikaryons were fruited and offspring were isolated from single spores. Several offspring, including V153-21, were tryptophan auxotrophs that did not exhibit B-regulated development in test matings. Southern analyses of this functionally B-null strain using cloned DNA as probes indicated all Bβ2-specific DNA is deleted from the strain and all Bα3-specific DNA with pheromone and pheromone receptor function is deleted from the strain. DNA between the Bα3 pheromone receptor gene and the homologous flank adjacent to Bα was not isolated and tested (M. F. Mitton and T. J. Fowler, unpublished results).

**Libraries and genomic Southern analyses:** Standard methods for manipulation of DNA were used (Maniatis *et al.* 1982). A λ-bacteriophage genomic library of a wild-type Bα3-Bβ2 *S. commune* strain (4-8) was constructed by ligating size-selected *Sau*3AI restriction fragments into λDASH II predigested with *Bam*HI (Stratagene, La Jolla, CA). The library was subsequently amplified in *Escherichia coli* strain XL1-Blue. The amplified library was first screened with a probe derived from DNA flanking the Bβ1 locus. Subsequent probes were derived from clones isolated in the previous step of the chromosome walk. Overlapping clones were recognized initially by restriction enzyme site mapping and later confirmed by sequence analysis. At one position, no phage clone could be identified in the library to progress the walk. A 12-kb *Eco*RI-*Hin*dIII restriction fragment containing the next adjacent region of the Bβ2 locus was identified by genomic Southern analysis of strain 4-8. A subgenomic library of ~12-kb *Eco*RI-*Hin*dIII restriction fragments of this strain was constructed in pBluescript and the desired plasmid clone was identified. A total genomic library of a strain containing the Bβ2 primary B-on mutation (V15-34) was constructed and screened similarly to the wild-type genomic library. A wild-type *S. commune* cDNA library constructed from RNA produced during a mating of a Bβ1 strain with a Bβ2 strain (4-40 × 4-39), kindly provided by M. Raudaskoski, was also screened for this study. The phage vector for this library was λZAP II (Stratagene) and plasmid clones were released from the phage genome according to the manufacturer's instructions. *S. commune* genomic DNA was extracted by a cetyltrimethylammonium bromide (CTAB) method and digested as previously described (Fowler and Mitton 2000). Southern hybridization analyses were performed according to Maniatis *et al.* (1982). Low stringency Southern analyses were incubated with probe at 55° and washed at 55° in 2× saline sodium citrate with 0.1% SDS. DNA probes were labeled with [$^{32}$P]dCTP by the random hexamer labeling method (Feinberg and Vogelstein 1983).

**DNA sequences and sequence comparisons:** DNA was sequenced at the Vermont Cancer Center DNA Sequencing Facility, University of Vermont, using a dideoxynucleotide method and fluorescent labeling system (Perkin Elmer-Cetus, Norwalk, CT). DNA sequences were deposited in GenBank as follows: *bbr2*, AF378292; *bbp2(1)*, AF378297; *bbp2(2)*, AF378298; *bbp2(3)*, AF378295; *bbp2(5)*, AF378294; *bbp2(7)*, AF378296; *bbp2(8)*, AF378293. DNA and protein sequence comparisons were made using BLAST and BLAST 2 sequences programs located at www.ncbi.nlm.nih.gov (Altschul *et al.* 1997; Tatusova and Madden 1999).

**Site-specific mutagenesis of *bbp2(1)* and a heterologous assay in yeast:** Two groups of degenerate oligonucleotide primers (Genosys, The Woodlands, TX) were designed such that codons for all amino acids except valine were possible at the position four codons 5′ from the cysteine codon (eight codons 5′ from the stop codon) of *bbp2(1)*, a position we call the "Cys-4" position (Figure 3A). These primers were combined to include equimolar amounts of each in the mixture. A standard polymerase chain reaction (PCR) using the downstream degenerate primer mixture and a constant upstream primer amplified products from a *bbp2(1)* template. These products were subcloned into the *Eco*RI-*Bam*HI sites of pPGK (Kang *et al.* 1990), heterologously expressed in *S. cerevisiae,* and tested through a previously described mating assay (Fowler *et al.* 1999). Briefly, a pheromone gene construct was transformed into a *MAT***a** *HIS3 his4* yeast strain (SM2331) and a receptor gene construct was transformed into a *MAT*α *his3 HIS4* strain (SDK47). The two untransformed base strains SM2331 and SDK47 are prevented from mating by null mutations in the yeast pheromone **a**-factor genes and the **a**-factor receptor gene, respectively. Compatible *S. commune* pheromone and receptor pairs can substitute for the missing yeast **a**-factor

and **a**-factor receptor to produce a pheromone response and mating. Diploids from such a mating can be selected on medium lacking histidine.

**PCR and site-specific changes in receptor and pheromone genes:** Several DNA fragments containing portions of the *bbr2* gene were generated from genomic DNA of strain V133-18 by PCR using oligonucleotide primers previously synthesized for *bbr2* sequencing. These PCR fragments were cloned into pBluescript (KS). V133-18 was derived from secondary mutant class Bβ2(1-3) in which Bβ2 receptor function has been altered (Raper and Raudaskoski 1968; Raper and Raper 1973). One set of oligonucleotide primers generated a DNA fragment with a 6-bp deletion relative to wild-type *bbr2*. A 508-bp *Sty*I-*Aat*II restriction fragment from the PCR product carrying the deletion was used to replace the comparable 514-bp *Sty*I-*Aat*II fragment in the cloned wild-type *bbr2*. The resulting plasmid was integrated into the B-null strain for testing. Site-specific changes in *bbr2* were made by overlap extension PCR (Ho *et al.* 1989) using oligonucleotides carrying the desired changes followed by replacement of the *Sty*I-*Aat*II fragment in *bbr2* as described above. DNA sequencing confirmed all changes.

The altered pheromone genes *bbp2(2-1)*, *bbp1(3-1)*, and *bbp1(1-1)* were constructed by PCR with oligonucleotide primers that contained the desired codon substitution at the Cys-4 codon. DNA sequencing confirmed that only the intended variation was present in each mutant gene. Transformants of the *S. commune* B-null strain were generated for each modified pheromone gene to determine the activity spectra of the altered pheromones.

## RESULTS

**Bβ2 has a single pheromone receptor gene and eight pheromone genes:** A chromosome walk was initiated in a genomic library constructed from a Bα3-Bβ2 strain of *S. commune* (Figure 2). The starting probe was derived from DNA flanking the Bβ1 specificity of the Bβ locus because Bβ1 genes do not cross-hybridize to any Bβ2 DNA on genomic Southern blots (Vaillancourt *et al.* 1997; M. F. Mitton and T. J. Fowler, unpublished results). The Bβ2 receptor gene, *bbr2*, was the first gene of the Bβ2 specificity to be identified functionally in transformation experiments, although it is not the gene closest to the homologous flank (Figure 2). Transformants of the B-null strain containing the *bbr2* transgene were induced for B-regulated development when mated with testers of the eight other Bβ specificities but not with a Bβ2 tester. B-regulated development occurred unilaterally, with fertilizing nuclei migrating into the mated transformant but never into the mated testers. Acceptance, but not donation, of fertilizing nuclei by the transformant suggested that only a receptor gene was present in the transformant. Sequence analysis of the genomic clone and corresponding cDNAs revealed a reading frame interrupted by five introns with a translation product predicted to fit within the GPCR superfamily. The predicted Bβ2 receptor, Bbr2, is 629 amino acids in length with seven hydrophobic regions located in the N-terminal half of the molecule. BLAST comparison of Bbr2 and the Bβ1 receptor, Bbr1 (Vaillancourt *et al.* 1997), showed 37% amino acid identity and 59% similarity over the 300 amino acids in their N termini, which is that portion of the receptors containing the regions predicted to be involved in ligand binding. The C termini of the two receptors have no regions of significant amino acid similarity.

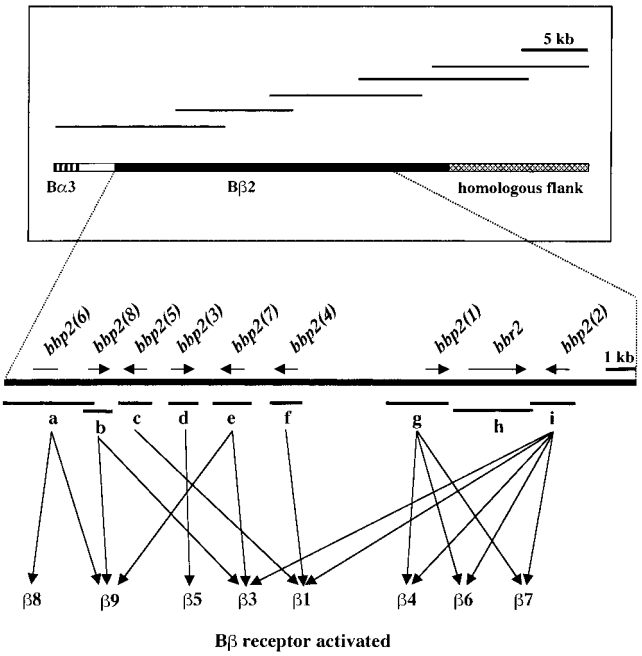


Figure 2.—The Bβ2 locus and mating activities conferred by its components. Five overlapping clones from a chromosome walk through the Bβ2 locus and surrounding regions are depicted in the boxed area. The crosshatched region is homologous to DNA flanking the Bβ1 locus and the striped region contains a Bα3 pheromone gene (T. J. Fowler, unpublished results). The unshaded area between Bβ2 and Bα3 does not have any identified mating function. The expanded view of the Bβ2 locus designates genes, with arrows pointing in the direction of transcription. A pheromone gene, preliminarily designated *bbp2(6)*, must be present in fragment a according to functional tests, but the gene sequence has not yet been deduced. The fragments designated a–i represent the smallest tested subclones of Bβ2 that retained specific mating activities. Transformants of the B-null strain containing fragment h accept fertilizing nuclei from strains of all Bβ specificities except Bβ2. Arrows extending from other fragments indicate the donation of fertilizing nuclei from transformants of the B-null strain containing that fragment to a strain of the indicated Bβ specificity with its unique Bβ receptor.

Two additional steps in the chromosome walk identified ~20 kb of DNA that spans the Bβ2 locus and the interlocus region between Bβ2 and Bα3 (Figure 2). A Bα3 pheromone gene was identified near the end of the last phage clone, indicating that the entire Bβ2 locus had been traversed and that the Bα3 locus had been entered. Plasmid subclones encompassing the entire region were tested in the B-null strain for their ability to induce B-regulated development in matings. Eight subclones induced Bβ-regulated migration of fertilizing nuclei into at least one of the test mates (Figure 2, fragments a–g and i). These B-null transformants do-

nated but did not accept fertilizing nuclei in matings, indicating the presence of pheromone transgenes.

DNA sequencing of seven active regions of Bβ2 led to the identification of the pheromone genes *bbp2(1)*, *bbp2(2)*, *bbp2(3)*, *bbp2(4)*, *bbp2(5)*, *bbp2(7)*, and *bbp2(8)*. The predicted polypeptide products of these seven genes are shown in Figure 3A. The sequence of *bbp2(4)* was reported previously (Fowler *et al.* 1999). While functional analysis of a clone containing the gene designated *bbp2(6)* clearly indicates a pheromone gene is present (Figure 2), the correct reading frame of the gene has yet to be discerned from DNA sequence analysis. cDNAs corresponding to *bbp2(1)* and *bbp2(4)* were isolated and compared to their respective genomic sequences, revealing the presence of a single intron interrupting the coding region of *bbp2(1)*. There are no introns interrupting the coding sequence of *bbp2(4)*.

**Pheromone and receptor are the only components of Bβ essential to migration of fertilizing nuclei:** The B-null strain does not exhibit any ability to activate B-regulated development in matings because of a deletion of the B complex (Raper and Raper 1973; see materials and methods). Thus the activities of individual B mating-type genes can be tested separately and in known combinations by introducing these genes into the B-null strain. Neither receptor gene *bbr1* nor *bbr2*, when transformed into the B-null strain, promoted activation of B-regulated development within the transformant in the absence of a compatible pheromone. However, each receptor gene promoted activation of B-regulated development within the transformant, as indicated by the acceptance of fertilizing nuclei by the transformant, in matings with any strain producing a compatible pheromone. The B-null strain was also transformed with single pheromone genes isolated from Bβ1 and Bβ2. As predicted, these transformants donated but did not accept fertilizing nuclei in matings with strains carrying compatible receptors. A mating of two B-null transformants, one transformed with a pheromone gene, *e.g.*, *bbp2(4)*, and the other transformed with a compatible receptor gene, *e.g.*, *bbr1*, activated B-regulated development only in the receptor transformant. Finally, when genes encoding this compatible pheromone and receptor pair were ectopically integrated into the B-null strain as part of a single DNA fragment, unmated transformants were activated for B-regulated development. These transformants were phenotypically indistinguishable from the primary B-on mutant. Such tests confirmed that pheromones and receptors have distinct functions in *S. commune* and that pheromone and receptor genes are the sole genes necessary for B-regulated development that reside within the Bβ mating-type locus. DNA surrounding pheromone and receptor genes in the locus is thought to be unique to each specificity, disallowing recombination within the locus when different specificities are paired at meiosis. The pheromone and receptor genes tested in this set of experiments were ectopically integrated into a genome where no other Bβ DNA exists, yet functional assays were consistent between transformants. Thus, the heterologous DNA context of the Bβ locus is not a requirement for gene expression leading to B-regulated development.

**The primary B-on mutant with self-activated B-regulated development has an altered pheromone:** The primary B-on mutant, in addition to being self activated for B-regulated development, was previously shown to be capable of donating fertilizing nuclei to its Bβ2 progenitor strain (Parag 1962; Raper and Raper 1973). In light of our current understanding of the roles of pheromones and receptors, the ability of this mutant to activate B-regulated development both in itself and in the wild-type progenitor Bβ2 strain would logically be conferred by a pheromone that was altered in such a way as to activate Bbr2 (Fowler *et al.* 1998). To test this idea, we decided to isolate and characterize the DNA responsible for conferring the mutant phenotype. A region of the mutant Bβ2 locus, which corresponded to a region in the wild-type Bβ2 containing the pheromone gene *bbp2(1)*, induced B-regulated development in Bβ2 transformants. This same DNA fragment was then integrated into the B-null strain. These transformants initiated B-regulated development in matings with Bβ2, Bβ6, and Bβ7 strains. DNA fragments containing wild-type *bbp2(1)*, when transformed into the B-null strain, promoted B-regulated development in matings with Bβ4, Bβ6, and Bβ7 strains (Figure 3B). The subclone from the mutant was sequenced and found to contain an allele of *bbp2(1)*, designated *bbp2(1-1)*, with two nucleotide differences compared to the wild-type allele. One nucleotide change was a missense mutation in the coding portion of the pheromone gene, leading to an alanine codon in place of a valine codon in the mutant pheromone gene (Figure 3B). The second nucleotide change was located in the 3′ untranslated region of the gene.

A point mutation in the 3′ flanking region is unlikely to affect pheromone specificity, but, to eliminate this possibility, the coding region of the *bbp2(1-1)* was tested separately in a heterologous expression system. We previously showed that *S. commune* pheromones and receptors, including Bbr2, could be expressed and were functional in the yeast *S. cerevisiae* (Fowler *et al.* 1999). Compatible pairs of *S. commune* pheromones and receptors activated the yeast pheromone response, which was monitored by reporter gene activity and a mating assay. For the current test, a single nucleotide change was introduced by PCR into a *bbp2(1)* cDNA to recapitulate the missense mutation of *bbp2(1-1)*. Yeast cells expressing the coding region of *bbp2(1-1)* were able to mate with cells expressing Bbr2 but not with cells expressing Bbr1 (Figure 3C). These experiments demonstrate that a valine-to-alanine change in a wild-type pheromone results in a mutant pheromone capable of Bbr2 activa-

**A**

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Bbp2(1)          MDSFDDLLDFLGAGTSRPAEASARNPLGSSSASSSSASLAASTSDLLSASPSSAPTSPDDVIMSILADAEHGYGGSNVHGWCVVA
Bbp2(2)                                  MDAQTTAPPTRPASPSPFAPSTAPTTVTPPASAAPPTNDTILALLANLEHEEDTDSNVHGWCIIA
Bbp2(3)                                                                  MDTFTYVDLAAVAAAAVADEVPRDFEDQITDYQSYCIIC
Bbp2(4)                                                        MDDFITLDFLEDTTPVFDFAPPTPNELTPEGYDEFMRMVANSDSPDGYFGGYCVVA
Bbp2(5)     MDRPHTKMAAFSARTSSPAPVASTIAFPSTRTKDTAPSTSGSSTPTRPASATPRAPDADLLRLLADARSAIARPPQDADEPDGYFAGYCVVM
Bbp2(7) MSTGLSDGAGECIAYRTRPAQQTSPSCSSLSRRNHRARPRGRGFHRSRAYGPKDKLTNPLGSNDRPSTKPADADVRRALASGDKPCGYGGGYCVVG
Bbp2(8)     MDSFTTLSLLDGTMPTFEDDMPVSFLDAALSLSGDCFSSQSSASSSRSTPFASPSPSSPSPNLLSSAKAATDPHLVVNADSPCGFGGGYCVVA
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**B**

| pheromone | predicted mature pheromone | Bβ receptors induced |
|---|---|---|
| Bbp2(1) | EHGYGGSN**V**HGWC | 4, 6,7 |
| Bbp2(1-1) | EHGYGGSN**A**HGWC | 2, 6,7 |
| Bbp2(2) | EHEEDTDSN**V**HGWC | 1, 3,4, 6,7 |
| Bbp2(2-1) | EHEEDTDSN**A**HGWC | 2, 6,7 |
| Bbp1(3) | EHTEAGEETT**A**RGWC | 2, 4,5,6,7 |
| Bbp1(3-1) | EHTEAGEETT**V**RGWC | 4,5,6 |
| Bbp1(1) | EHWRGGNTT**A**HGWC | 2, 4,5,6,7 |
| Bbp1(1-1) | EHWRGGNTT**V**HGWC | none detected |

**C**

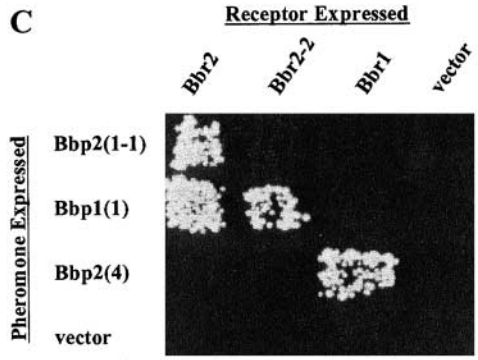



FIGURE 3.—Sequence and mating activity of wild-type and mutant Bβ2 pheromones. (A) Predicted amino acid sequence of seven Bβ2 pheromone precursors. A post-translational modification signal at the C terminus of each is underlined (cysteine-aliphatic-aliphatic, one of several amino acids). By analogy to other pheromones with this signal, this cysteine is modified by isoprenylation, probably with a farnesyl group, followed by cleavage of the three C-terminal amino acid residues and methylation of the cysteine (ANDEREGG *et al.* 1988). The precise N-terminal cleavage site for the *S. commune* pheromones is unknown, but mature pheromones of 11–14 amino acids are predicted (shown in boldface type) on the basis of similar pheromones from other basidiomycetes that have been characterized biochemically (SAKAGAMI *et al.* 1981; OLESNICKY *et al.* 1999; KOSTED *et al.* 2000). Data from *C. cinereus* led to a proposed recognition site of two consecutive charged amino acids, usually an acidic-basic pair such as EH or EK, for the N-terminal cleavage reaction (OLESNICKY *et al.* 1999). This motif can be identified in some, but not all, of the *S. commune* sequences. (B) Predicted mature pheromone sequences are shown pairwise for four wild-type pheromones and their mutant counterparts. Bbp1(3) and Bbp1(1) were previously reported in VAILLANCOURT *et al.* (1997). Variations at the position four amino acids toward the N terminus from the C-terminal cysteine (Cys-4) are shown in boldface type. Each pheromone precursor gene was transformed into the B-null strain and several independent transformants of each gene were mated to strains representing all nine Bβ specificities. (C) A missense mutation in the coding region of *bbp2(1-1)* was separated from a point mutation in the 3′ untranslated region of the gene and tested for the effect of the missense mutation on the *bbp2(1-1)* pheromone in a heterologous expression system (FOWLER *et al.* 1999). *S. cerevisiae* strains with complementing auxotrophic markers were tested for pheromone response and mating via *S. commune* pheromones and receptors. Overlapping horizontal and vertical streaks of yeast strains expressing the coding regions of various *S. commune* pheromones or receptors were grown on nonselective medium to allow diploid formation. The yeast were then replica plated onto selective medium on which only diploids could grow (shown). Bbp1(1)/Bbr2 and Bbp2(4)/Bbr1 combinations are positive controls for expression and interaction; Bbp1(1)/Bbr1 and Bbp2(4)/Bbr2 are negative controls for interaction. Bbr2-2 is a mutant receptor with a two-amino-acid loss that discriminates between Bbp2(1-1) and Bbp1(1) (see also Figure 4C).

tion and that it is the sole mutation necessary for the altered pheromone/receptor recognition.

**Activation of the Bβ2 receptor by the mutant pheromone appears to require the alanine substitution:** The importance of alanine at four residues toward the N terminus from the C-terminal cysteine (Cys-4, Figure 3B) in the predicted mature form of the mutant pheromone Bbp2(1-1) was tested by substituting other amino acids into the Cys-4 position. Our hypothesis was that the alanine residue, while biochemically similar to valine, provides a less bulky side group and perhaps allows the pheromone to interact positively with Bbr2 as a result. We tested substitutions of glycine and serine in place of alanine because the side groups of glycine and serine, while biochemically distinct, are also smaller than that of valine. A substitution of leucine was included in the experiment as an additional test of the hypothesis since leucine has a hydrophobic side group larger than that of valine. An oligonucleotide primer incorporating the desired codon was used in a PCR to generate the coding region of a pheromone gene with each of these changes. Yeast cells containing these altered genes did not elicit a mating response from cells expressing the Bbr2 receptor. We then considered whether substituting any amino acid residue other than alanine for valine at the Cys-4 position of Bbp2(1) would result in a pheromone capable of activating the Bβ2 receptor. Degenerate oligonucleotide primers were designed to substitute a codon(s) for each amino acid except valine into the Cys-4 position. PCR products generated with the degenerate oligonucleotides were cloned for expression in yeast and tested by the mating assay. A total of 8% (13) of the

transformants were able to mate with the yeast strain expressing Bbr2. The pheromone transgenes from 5 of these transformants were sequenced. All five genes had a codon for alanine at the Cys-4 position.

**The Cys-4 position in several pheromones is crucial for specificity of interaction with the natural array of pheromone receptors:** Comparison of several pheromones by their predicted mature amino acid sequences and their spectra of receptor activations showed a positive correlation between an alanine residue in the Cys-4 and the ability to activate Bbr2 (Figure 3B). To test the strength of this correlation, an alanine codon was substituted for the valine codon at the Cys-4 position in another Bβ2 pheromone gene, *bbp2(2)*, and a valine codon was substituted for the alanine codon at Cys-4 in two pheromone genes of Bβ1, *bbp1(3)* and *bbp1(1)*, to create mutant pheromone genes *bbp2(2-1)*, *bbp1(3-1)*, and *bbp1(1-1)*, respectively (Figure 3B). Bbp2(2-1), with alanine replacing valine at Cys-4, maintained the wild-type capability of Bbp2(2) for activating B-regulated development in Bβ6 and Bβ7 strains, and, like the comparable mutant Bbp2(1-1), gained the ability to activate Bbr2; Bbp2(2-1) lost the ability of its wild-type counterpart to activate receptors in Bβ1, Bβ3, and Bβ4 strains (Figure 3B). Pheromone Bbp1(3-1), with an alanine-to-valine substitution at Cys-4, maintained the ability to induce B-regulated development in test mates carrying Bβ4, Bβ5, and Bβ6 receptors but unlike Bbp3(1) was unable to activate receptors in mates carrying Bβ2 and Bβ7. The substitution of valine for alanine at Cys-4 in Bbp1(1-1) led to a defect such that no pheromone activity could be detected in mating assays. In each case, the change between alanine and valine at the Cys-4 position resulted in a shift in the spectrum of receptors that could be activated, and all of the pheromones tested that had alanine at the Cys-4 position could activate Bbr2.

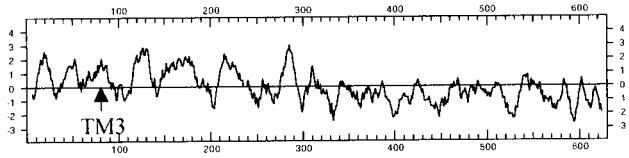
**A two-amino-acid loss in the Bβ2 receptor leads to a change in recognition of pheromones:** The secondary mutants of Bβ2 included one class of mutants, Bβ2(1-3), that retained abilities to donate fertilizing nuclei to the grandprogenitor Bβ2 strain and accept fertilizing nuclei from other Bβ specificities, but this class did not accept fertilizing nuclei from the primary B-on mutant (Raper and Raudaskoski 1968; Raper and Raper 1973). We examined one of the mutants in this class. Its ability to donate fertilizing nuclei to a Bβ2 tester suggested that the mutant pheromone gene *bbp2(1-1)* was present, but its fluffy hyphal phenotype suggested that its Bβ2 receptor could not respond to the mutant pheromone. Since fertilizing nuclei were accepted from other Bβ specificities, the Bβ2 receptor in this mutant was competent to recognize other pheromones and to transduce a signal. Isolation of a portion of the *bbr2* gene from the mutant led to identification of a six-nucleotide deletion relative to wild-type *bbr2*. The deletion eliminated two consecutive codons from the receptor gene. The receptor produced from this mutant gene would be deficient for a lysine-leucine pair (Lys75-Leu76) in a region predicted to encode the third transmembrane helix (Figure 4, A and B). An equivalent deletion was incorporated in an otherwise wild-type *bbr2* gene by exchanging a restriction fragment having the deletion with its wild-type counterpart. The reconstituted mutant receptor gene, *bbr2-2*, was integrated into the B-null strain. Bbr2-2 was tested through matings of the transformants to each wild-type Bβ specificity as well as a strain expressing the mutant pheromone Bbp2(1-1) (Figure 4C). Bbr2-2 was activated by pheromones from strains with Bβ specificities other than Bβ2; however, it did not respond to Bbp2(1-1), indicating that the 6-bp deletion is responsible for the change of receptor activation with respect to Bbp2(1-1). Bbr2-2 is not completely equivalent to the receptor in the original Bβ2(1-3) mutant, however, because Bbr2-2 could not respond to Bβ4 or Bβ7 testers, whereas the original mutant could mate with strains with these specificities (Raper and Raper 1973).

**Other alterations in the third transmembrane region of Bbr2 can change pheromone recognition:** To try to understand why the Lys75-Leu76 loss has an effect on receptor/pheromone interactions, other changes affecting the third transmembrane region of Bbr2 were made. The effect of substituting an alanine residue for either Lys75 (*bbr2-3*) or Leu76 (*bbr2-4*) was assayed first. Neither change in the receptor led to a difference in B-regulated developmental response when compared with Bbr2 (Figure 4C). As with the wild-type version of Bbr2, one or more pheromones produced by each Bβ specificity, except Bβ2, activated the altered receptors. In addition, each receptor could respond to the mutant pheromone Bbp2(1-1).

Several other alleles of *bbr2* with site-directed mutations were constructed (Figure 4C): codons for Lys75 and Leu76 were switched in their order to Leu75-Lys76 (*bbr2-5*); Leu76 alone was deleted (*bbr2-6*); and Leu77-Leu78 were deleted (*bbr2-7*). An unintended variation occurred in a PCR amplification of *bbr2* in an unrelated experiment to change Pro85 to Ser85 within the third transmembrane domain (*bbr2-8*). Various deficiencies in the initiation of B-regulated development were identified in test matings of transformants expressing these altered receptors, but each receptor was still able to respond to wild-type pheromones from several Bβ specificities (Figure 4C). None of these latter mutant receptors were able to initiate B-regulated development in response to Bbp2(1-1).

**Receptor null mutants and pheromone gene alterations lead to deficiencies in nuclear movement during fertilization:** Several phenotypic classes of the original secondary mutants generated by X-irradiation are incapable of accepting fertilizing nuclei in Bβ dependent matings with testers of any Bβ specificity (Raper and Raper 1973). The largest such class was termed Bβ2(1-

A




B

68 75 76 77 78 85 91
IWCDISS**KL**LLGVSIGIPASGLCI

C

| Allele | TM3 composition | Bβ Specificity of Test Mate β1 | β3 | β5 | β8 | β9 | β6 | β4 | β7 | B-null + *bbp2(1-1)* | β2 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *bbr2* | K75, L76 (wt) | + | + | + | + | + | + | + | + | + | - |
| *bbr2-3* | K75A | + | + | + | + | + | + | + | + | + | - |
| *bbr2-4* | L76A | + | + | + | + | + | + | + | + | + | - |
| *bbr2-8* | P85S | + | + | + | + | + | + | + | + | - | - |
| *bbr2-5* | K75L, L76K | + | + | + | + | + | + | + | + | - | - |
| *bbr2-7* | L77Δ, L78Δ | + | + | + | + | + | + | + | + | - | - |
| *bbr2-2* | K75Δ, L76Δ | + | + | + | + | + | + | - | - | - | - |
| *bbr2-6* | L76Δ | + | + | + | + | + | - | - | - | - | - |

FIGURE 4.—Mutations in the third transmembrane helix of Bbr2 can alter pheromone recognition necessary for B-regulated development. (A) A Kyte-Doolittle hydropathy plot of Bbr2 showing seven hydrophobic regions (above the zero line) between the N terminus and amino acid 300, each of sufficient length to span a membrane. By analogy to other GPCRs, the N terminus on the far left is extracellular and the C terminus is intracellular. The third transmembrane region, where mutations relevant to this study were identified, is indicated. (B) Sequence of the predicted third transmembrane helix of wild-type Bbr2 with the two-amino-acid residues missing from the Bβ2(1-3) mutant receptor underlined. (C) Results of Bβ-dependent mating assays to monitor B-regulated development in a B-null-strain transformed with variants of the Bβ2 receptor. The test mate named "B-null + *bbp2(1-1)*" expresses only the mutant pheromone Bbp2(1-1). The other test mates are wild-type strains of the indicated Bβ specificity presumed to express all their natural Bβ pheromones. −, no B-regulated development; +, B-regulated development equivalent to wild-type Bbr2; +, B-regulated development either slower or less pervasive than wild-type Bbr2.

1). The inability of one member of this class to accept nuclei is the result of a disruption of the coding region of *bbr2* by a transposon called *scooter*, leading to the null allele, *bbr2-1* (Figure 5; FOWLER and MITTON 2000). Class Bβ2(1-1) mutants can donate fertilizing nuclei to strains of all Bβ specificities, including Bβ2, indicating the mutant pheromone gene, *bbp2(1-1)*, of the primary B-on mutant is retained in this secondary mutant class as well as at least a minimal group of wild-type Bβ2 pheromone genes.

Members of another smaller class of secondary mutants, Bβ2(1-6), are also incapable of accepting fertilizing nuclei from any Bβ tester. Unlike the class Bβ2(1-1) mutants, Bβ2(1-6) mutants donate fertilizing nuclei to testers of all Bβ specificities *except* Bβ4 (Figure 5; RAPER and RAPER 1973). We examined one of these mutants to determine the lesion or lesions responsible for its phenotype. Genomic Southern hybridization analyses of the mutant and a wild-type Bβ2 strain, using *bbr2* as a probe, indicated the *bbr2* gene is severely truncated in the mutant (Figure 6A). This result suggests that the inability of the mutant to respond to pheromone signals from any strain differing at Bβ, but not Bβα, is because the truncation results in a null allele of *bbr2*. A probe to *bbp2(1)* and *bbp2(1-1)* revealed no length polymorphism between the wild-type and mutant strains for the restriction fragments containing the *bbp2(1)* and *bbp2(1-1)* alleles, respectively (Figure 6B). This result, along with the previous functional analysis showing B-regulated development is initiated in a Bβ2 strain when mated with the class Bβ2(1-6) mutant (RAPER and RAPER 1973), suggests that *bbp2(1-1)* remained intact in this mutant strain. Southern analysis also showed that *bbp2(2)* had been deleted from this secondary mutant (Figure 6C). This deletion would knock out pheromone Bbp2(2), which normally activates the Bβ4 receptor and several other receptors (Figure 2). Although the wild-type pheromone Bbp2(1) can

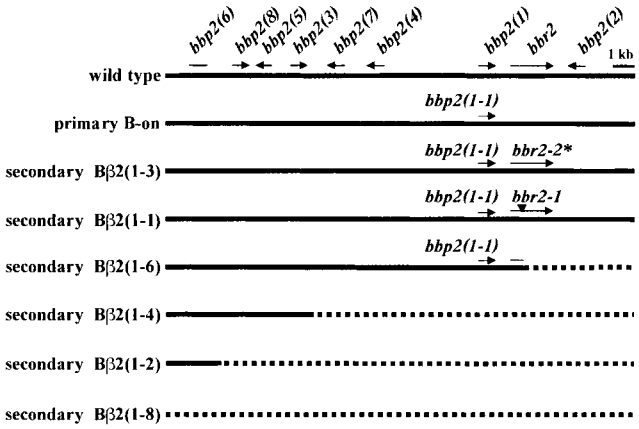



FIGURE 5.—Interpretation of the Bβ2 locus in primary B-on and secondary mutants. The mutant B loci are arranged in descending order according to the extent of their mating deficiencies. Altered genes in the mutant loci are indicated and deletions are marked by the dashed lines. Each secondary mutant was derived from the primary B-on mutant by X-ray mutagenesis (see text; RAPER and RAPER 1973). Bbp2(1-1) is capable of activating the resident receptor Bbr2. Bbr2-2* has a two-amino-acid deletion that confers discrimination against activation by Bbp2(1-1) confirmed by the reconstructed receptor Bbr2-2 (Figure 4C), but Bbr2-2* must have an additional undetermined difference(s) with Bbr2-2 based on mating tests (see text). *bbr2-1* is a null allele due to insertion of a transposon, indicated by an arrowhead (FOWLER and MITTON 2000). Deletion points in the mutant loci are inferred from the loss of pheromone or receptor functions in the mutants as determined by matings (RAPER and RAPER 1973), in conjunction with Southern hybridization analyses using regions of the Bβ2 locus as probes (T. J. FOWLER, unpublished results).

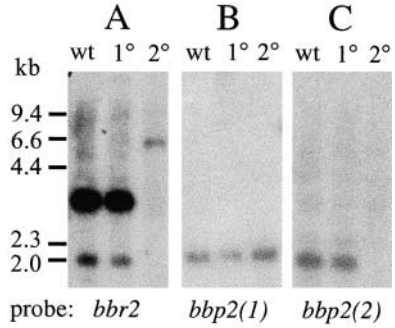



FIGURE 6.—Southern hybidization analysis of the Bβ2(1-6) secondary mutant. Genomic DNA from wild-type Bβ2 strain 4-8 (wt), primary B-on mutant strain V15-34 (1°), and Bβ2(1-6) secondary mutant strain V134-7 (2°) were compared. (A) DNA was digested with *Bam*HI and *Nsi*I and probed with a *bbr2* specific probe. Digests with additional restriction enzymes indicate that the deletion point is located within the first 1.2 kb of *bbr2*, truncating the coding region by at least 50% (not shown). (B) DNA was digested with *Eco*RI and *Xba*I and probed with a 2-kb cloned DNA fragment containing *bbp2(1)*, which hybridizes to both *bbp2(1)* and *bbp2(1-1)*. (C) DNA was digested with *Bam*HI and *Nsi*I and probed with a 2-kb cloned DNA fragment containing *bbp2(2)*. Approximately 1 μg of DNA was loaded in each lane.

activate the Bβ4 receptor, the mutant Bbp2(1-1) pheromone is incapable of activating the Bβ4 receptor (Figure 3B). In the Bβ2(1-1) secondary mutant class mentioned above, the deficiency of Bbp2(1-1) with regard to activation of the Bβ4 receptor is hidden by the redundant function of Bbp2(2). In the Bβ2(1-6) secondary mutant, it is the combination of the mutated pheromone Bbp2 (1-1) and complete loss of the pheromone Bbp2(2) that precludes donation of fertilizing nuclei in a mating with a Bβ4 strain. In total, mutations in two pheromone genes and a receptor gene were required to produce the peculiar mating phenotype of this secondary mutant strain.

## DISCUSSION

The origins of the research described here date back to genetic experiments with *S. commune* that started in the early 1960s and extended for more than a decade (PARAG 1962; KOLTIN and RAPER 1966; RAPER and RAUDASKOSKI 1968; RAPER and RAPER 1973). The underlying purpose of those earlier experiments was to elucidate the process by which new specificities of a B mating-type locus, comparable to those found in nature, could have arisen. These earlier studies encompassed an extensive mutational analysis of the Bβ2 locus. The results indicated that the locus controlled at least three mating functions: specificity of compatible mate recognition, donation of migrant fertilizing nuclei, and acceptance of migrant fertilizing nuclei. Molecular analysis of this same Bβ specificity and of another variant, Bβ1, demonstrated that the locus contains multiple genes predicted to encode molecules of two types: lipopeptide pheromones and G-protein-coupled receptors. Southern hybridization experiments and functional tests to date indicated that each of the nine specificities of the Bβ locus extant in nature is composed of a unique combination of pheromone and receptor genes and that donation of migrant nuclei is regulated by pheromones while acceptance of migrant nuclei is regulated by pheromone receptors (VAILLANCOURT *et al.* 1997; FOWLER *et al.* 1998; this article). It is the effective coupling of pheromones encoded by the Bβ genes of one specificity with compatible receptors produced by another specificity that triggers the pathway of nuclear migration during fertilization.

Previously, partial characterization of the Bβ1 mating-type locus revealed a single pheromone receptor-encoding gene and three pheromone-encoding genes (VAILLANCOURT *et al.* 1997). Our current analysis of Bβ2 also identified a single unique pheromone receptor gene and, surprisingly, eight pheromone genes. If we assume accordingly that each of the nine versions of the Bβ locus contains four to five genes encoding pheromones and one gene encoding a receptor, then ~350 different combinations of these two types of molecules are possible for the products of the Bβ locus alone. Functional

tests of individual genes examined to date indicate that only about one-third to one-half of these combinations are capable of triggering B-regulated development. Therefore we estimate that 120–180 active couplings of Bβ pheromones and receptors exist within the natural population of *S. commune*. A similar estimate might be made for the Bα locus.

While eight pheromone-encoding genes exist in Bβ2, the minimal set of pheromones required for effective interaction with the receptors encoded by all eight other Bβ specificities is encoded by just three genes: *bbp2(2)*, *bbp2(3)*, and *bbp2(6)* (Figure 2). The pheromones encoded by the remaining Bβ2 genes are functionally redundant with this minimal set. From our observations, however, there is some indication of differential effects on the efficiency and intensity of the response induced by the interaction of different pheromone/receptor pairs (Figure 4C; Wendland *et al.* 1995; Vaillancourt *et al.* 1997). The functional redundancy observed may therefore have some selective advantage within the natural population.

Molecular genetic analyses of both the Bα locus (Wendland *et al.* 1995; Wendland and Kothe 1996; Hegner *et al.* 1999) and the Bβ locus (Vaillancourt *et al.* 1997) in *S. commune* have begun to provide clues toward understanding how so many different specificities of these two B mating-type loci might have evolved. A hypothesis that Bα and Bβ may have evolved from a common ancestral locus through a large duplication and inversion was proposed from evidence found within sequence comparisons of Bα1 and Bβ1 (Vaillancourt *et al.* 1997). The genes contained within each locus may then have diverged through successive point mutations, as demonstrated by comparison of *bar1* genes from Bα1 strains isolated from different geographical regions (Wendland and Kothe 1996), and through small insertions or deletions comparable to those in the Bβ2 locus described in this article. Furthermore, there is evidence of gene duplications within Bβ2. The genes *bbp2(4)*, *bbp2(5)*, *bbp2(7)*, and *bbp2(8)* encode precursor pheromones with very similar amino acid sequences at their C termini, the portions presumed to be modified and processed to mature functional peptides of perhaps 11–14 amino acid residues (Figure 3A; Anderegg *et al.* 1988; Olesnicky *et al.* 1999). Closer comparisons of identities in the predicted mature pheromone sequences show that these pheromones form two subsets: Bbp2(4) with Bbp2(5), both of which activate only the Bβ1 receptor, and Bbp2(7) with Bbp2(8), both of which activate only the Bβ3 and Bβ9 receptors (Figure 3A, Figure 2). Only two amino acid positions in the predicted mature pheromones, at Cys-4 and Cys-6, consistently differ between the two subsets, suggesting that specificity for their respective receptor partners is determined through one or both of these positions. In the pheromones Bbp2(1) and Bbp2(1-1), the Cys-4 position was critical for activation of the Bb2 receptor (Figure 3B). The next challenge is to understand how these amino acid differences effect pheromone discrimination among receptors at the structural level.

The pheromone receptors of *S. commune* are clearly related, but their lineage is as yet obscure. Bbr2 is significantly more similar to the characterized Bα receptors of *S. commune*, Bar1, Bar2, and Bar3 (Wendland *et al.* 1995; Hegner *et al.* 1999; L. J. Vaillancourt, unpublished results), than it is to the only other member of the Bβ series examined to date, Bbr1 (Vaillancourt *et al.* 1997). Bbr2 has at least 50% amino acid identity with the N-terminal halves of these Bα receptors, which include the transmembrane helices, but only 37% identity over the N-terminal half of Bbr1. Furthermore Bbr2 has more identity with several *C. cinereus* receptors (O'Shea *et al.* 1998; Halsall *et al.* 2000) than it does with Bbr1. Genomic Southern blot experiments showed previously that at least six of the other eight Bα receptor genes shared enough DNA similarity with the Bα1 receptor gene, *bar1*, to hybridize to a *bar1* probe under conditions of relatively high stringency (Laddison 1995). We showed by a genomic Southern blot of strains representing the nine Bβ specificities that, even at lowered stringency, *bbr1* hybridizes uniquely to a restriction fragment in the Bβ1 strain. In an equivalent test, *bbr2* hybridizes only to a single restriction fragment from a Bβ2 strain (M. F. Mitton, unpublished result). From these results, we infer that the Bβ1 and Bβ2 receptors have relatively distant origins and are less related to the other six Bβ receptors extant in nature than are the receptors of the Bα series related to each other.

Several aspects of this study illustrate potential for the functional divergence of pheromones and receptors by mutation during evolution to generate a system with many related, yet distinct, components. A single amino acid exchange between valine and alanine at one specific site of several different pheromones resulted in significant alteration of their spectra of interaction with the array of pheromone receptors extant in the natural population of *S. commune* (Figure 3B). Site-directed mutations in Bbr2 also show that one or two amino acid substitutions or deletions can change the spectrum of pheromone recognition of a receptor (Figure 4C). This evidence also indicated that neither the Lys75 nor Leu76 residues of the receptor Bbr2 are likely to be in direct contact with the mutant pheromone Bbp2(1-1), because each could be replaced by alanine without effect. The effects of several different deletions and the Pro85-to-Ser85 mutation on the third transmembrane helix, however, suggest that the receptor's conformation has been compromised, perhaps due to the inability of the altered helix to interact normally with its protein or lipid surroundings. Comparable results were seen in *C. cinereus*, where one or two conservative amino acid changes in a pheromone were shown to alter its ability to interact with the pheromone receptors tested and a single conservative amino acid change in a pheromone

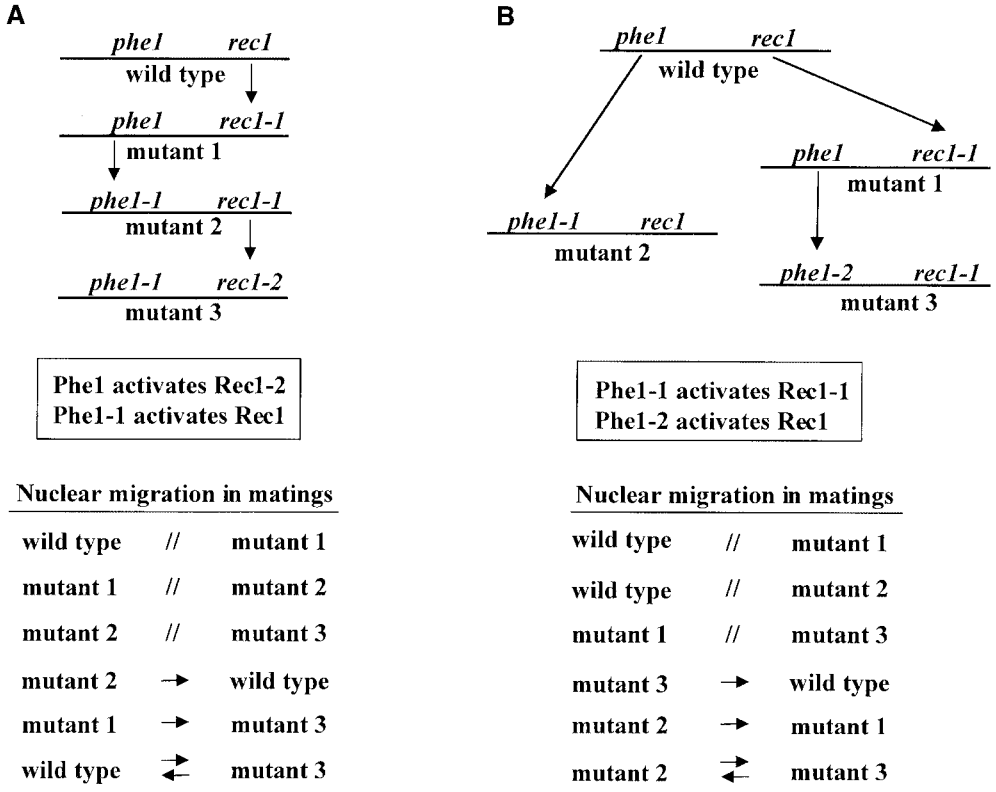



FIGURE 7.—Two models for the evolution of an additional specificity of a B locus in three mutational steps. A hypothetical locus minimized to a single pheromone gene, *phe1*, and a single receptor gene, *rec1*, can be altered in three mutational steps to produce a new specificity having bilateral nuclear migration. Self-activating mutations are not allowed because they are thought to severely reduce fitness in nature. We assume in matings with other specificities of the locus that bilateral nuclear migration is unaffected by the proposed mutations. (A) Three mutations occur consecutively, leading to three mutant types. If Phe1 can activate only Rec1-2 and Phe1-1 can activate only Rec1, then mutant 3 has a new specificity allowing bilateral fertilization when mated with the original wild type. Mutant 1 and mutant 2 can mate unilaterally, but not bilaterally, with other strains in the scheme and thus are not equivalent to a new specificity. One new specificity was gained in this scheme. (B) Some mutations occur in different individuals of the population, producing three mutant types. If Phe1-1 can activate only Rec1-1 and Phe1-2 can activate only Rec1, then mutant 2 and mutant 3 can exchange fertilizing nuclei in a bilateral fashion, indicating each has a new fully functional specificity. In this scheme, the wild-type locus is no longer considered a complete specificity because the wild-type strain cannot exchange nuclei with mutant 2 and only unilaterally accepts nuclei from mutant 3. The result of this scheme is a net gain of one additional specificity because even though two new specificities are produced in mutant 2 and mutant 3, the original wild-type specificity is no longer a complete specificity. //, no nuclear migration; single arrow, unilateral migration of fertilizing nuclei; double arrow, bilateral migration of fertilizing nuclei.

receptor allowed the mutant receptor to recognize a previously incompatible pheromone (OLESNICKY *et al.* 2000). Thus, in both organisms, the natural pheromone-receptor interactions are finely tuned and can be altered significantly, even by conservative amino acid substitutions or small deletions in either partner.

Earlier attempts, through mutagenesis, to produce a new specificity of Bβ equivalent in all functional aspects to wild-type specificities did not succeed (PARAG 1962; RAPER and RAPER 1973; RAUDASKOSKI *et al.* 1976). Now that molecular characterization of several B specificities has revealed the multigenic nature of these loci, it is apparent that several mutations of limited scope would be required to achieve the original goal. We hypothesize that a minimum of three mutations would be necessary to produce a new specificity from an existing specificity. Figure 7 depicts two schemes for such a conversion, starting from a generic specificity. We limit the mutations to those that do not elicit self-activation of B-regulated development. Individuals with the primary B-on mutant phenotype grow poorly, fertilize unilaterally rather than reciprocally in matings, and are shown to bear a high energy cost relative to wild type due to the mutation (HOFFMAN and RAPER 1971). These attributes almost certainly lead to low fitness of B-on mutants in a natural environment. Thus, according to our current models, the original genetic screens used to search for new B specificities would not mimic a path leading to a new specificity. Our hypothetical models would be difficult to test directly through laboratory studies because some of the required infrequent mutations could be identified only through a series of crosses, and other mutations would not show a new mating phenotype until subsequent mutations occurred to produce a compatible receptor or pheromone partner. These types of mutations could well exist within a population and eventually be incorporated into a new specificity. Once generated, a new specificity would begin to accumulate additional changes that do not alter function but provide heterology at the locus. Otherwise, the rare new specificity might be lost through recombination events with its progenitor specificity because B-on recombinants could be produced in these matings. In *S. commune*, survival of a new specificity of a B mating-type locus is likely because the widespread distribution of multiple specificities provides a significant chance that

potential mates are not of the progenitor specificity. Once established enough to avoid loss by genetic drift, a new specificity may thrive until it reaches an equilibrium frequency in the population. This would be similar to an immigrant specificity becoming established and maintained in a population through balancing selection (James *et al.* 1999).

*S. commune* depends solely on sexual spores for dispersal. Perhaps it is not surprising, therefore, that this species optimized its outbreeding potential by generating numerous compatible mating partners. A benefit to the investigating scientist is the opportunity to understand how so many variants of the loci can be created and how discrimination among so many receptors and ligands is achieved.

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