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, Bo 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 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 hemiascomycetes 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, recombiable, and functionally redundant loci, Bo 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 Ba1, Ba2, . . . , Ba9 and Bβ1, Bβ2, . . . , Bβ9, where the previously designated Ba1’, Ba2’, Bβ1’, and Bβ2’ are now Ba8, Ba9, Bβ8, and Bβ9, respectively (Koltin et al. 1967; Stambberg and Koltin 1972). Two specificities of these loci, Ba1 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 Ba1 and Bβ1 must be incomplete. Receptor genes and some of the pheromone genes from the Ba2 (Hegner et al. 1999) and Bβ3 specificities (L. J. Vaillancourt and T. J. Fowler, unpublished results) were also isolated. The convention for naming these genes is, for example,
bar1 for Bα receptor of specificity 1 and bbp2(4) for the fourth Bβ pheromone 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 \textit{S. commune}: \textit{S. commune} was cultured and tested in matings according to \textsc{Raper} and \textsc{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 \textsc{Raper} and \textsc{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 \textsc{Specht et al.} (1988), using Novozyme 234 (InterSpeX Products, Foster City, CA) as the wall digesting enzyme, with modifications described by \textsc{Horton} and \textsc{Raper} (1991). Stable transformants were produced from the cotransformation of strains auxotrophic for tryptophan with two plasmids, one containing wild-type \textit{trp1} and the other containing the DNA of interest from the mating-type locus. Transformants were identified as tryptophan prototrophs by growth on minimal medium plates lacking tryptophan. Transformants 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 B\textbeta 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 B\textbeta specificity. These transformants were directly observed for B-regulated development (\textsc{Vaillancourt et al.} 1997). Most test DNAs were integrated into a B-null strain (see below) and the transformants were tested mate with a set of strains representative of the nine natural B\textbeta specificities. Both partners of these matings were observed for B-regulated development.

A B-null tryptophan auxotroph was developed: A B-null strain of \textit{S. commune} used as a transformation recipient (V153-21) was derived as follows from a secondary mutant originally designated B\textbeta2(1-8) by \textsc{Raper} and \textsc{Raper} (1973). This mutant shows no B-regulated development in matings with any wild-type strain. The B\textbeta2(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 fruiting 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 B\textbeta2-specific DNA is deleted from the strain and all BaA3-specific DNA with pheromone and pheromone receptor function is deleted from the strain. DNA between the BaA3 pheromone receptor gene and the homologous flank adjacent to \textit{Ba} was not isolated and tested (M. F. \textsc{Mitton} and T. J. \textsc{Fowler}, unpublished results).

Libraries and genomic Southern analyses: Standard method

DNA sequences and sequence comparisons: DNA was sequenced at the Vermont Cancer Center DNA Sequencing Facility, University of Vermont, using a dyeoxydeoxynucleotide method and fluorescent labeling system (Perkin Elmer-Cetus, Norwalk, CT). DNA sequences were deposited in GenBank with the following accession numbers: \textit{hbr2}, AF378292; \textit{bbp2(1)}, AF378297; \textit{bbp2(2)}, AF378298; \textit{bbp2(3)}, AF378295; \textit{bbp2(5)}, AF378294; \textit{bbp2(7)}, AF378296; \textit{bbp2(8)}, AF378293. DNA and protein sequence comparisons were made using BLAST and BLAST 2 sequences programs located at www.ncbi.nlm.nih.gov (\textsc{Altschul et al.} 1997; \textsc{Tatusova} and \textsc{Madden} 1999).

Site-specific mutagenesis of \textit{bbp2(1)} and a heterologous assay in yeast: Two groups of degenerate oligonucleotide primers (\textsc{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 \textit{bbp2(1)}, a position we call the “Cys4” 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 \textit{bbp2(1)} template. These products were subcloned into the EcoRI-BamII sites of pPGK (\textsc{Kang et al.} 1999), heterologously expressed in \textit{S. cerevisiae}, and tested through a previously described mating assay (\textsc{Fowler et al.} 1999). Briefly, a pheromone gene construct was transformed into \textit{MATa HIS3 his4 yeast strain (SM2391)} and a receptor gene construct was transformed into \textit{MATa his3 HIS4 strain (SM2394)}. The two untransformed base strains SM2391 and SM2394 are prevented from mating by null mutations in the yeast pheromone \textalpha-factor genes and the \textalpha-factor receptor gene, respectively. Compatible \textit{S. commune} pheromone and receptor pairs can substitute for the missing yeast \textalpha-factor.
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 \( Bb\beta2(1-3) \) in which \( B\beta2 \) 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 \( Sfl\text{-}AatII \) restriction fragment from the PCR product carrying the deletion was used to replace the comparable 514-bp \( Sfl\text{-}AatII \) 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 \( Sfl\text{-}AatII \) 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

\( B\beta2 \) has a single pheromone receptor gene and eight pheromone genes: A chromosome walk was initiated in a genomic library constructed from a \( Bo3\text{-}B\beta2 \) strain of \( S.\ commune \) (Figure 2). The starting probe was derived from DNA flanking the \( B\beta1 \) specificity of the \( B\beta \) locus because \( B\beta1 \) genes do not cross-hybridize to any \( B\beta2 \) DNA on genomic Southern blots (Vaillancourt et al. 1997; M. F. Mitton and T. J. Fowler, unpublished results). The \( B\beta2 \) receptor gene, \( bbr2 \), was the first gene of the \( B\beta2 \) 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 \( B\beta \) specificities but not with a \( B\beta2 \) 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 \( B\beta2 \) 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 \( B\beta1 \) 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.

Two additional steps in the chromosome walk identified ~20 kb of DNA that spans the \( B\beta2 \) locus and the interlocus region between \( B\beta2 \) and \( Bo3 \) (Figure 2). A \( Bo3 \) pheromone gene was identified near the end of the last phage clone, indicating that the entire \( B\beta2 \) locus had been traversed and that the \( Bo3 \) 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 \( B\beta \)-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 BB2 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 BB 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 BB1 and BB2. 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 BB 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 BB DNA exists, yet functional assays were consistent between transformants. Thus, the heterologous DNA context of the BB 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 BB2 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 BB2 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 BB2 locus, which corresponded to a region in the wild-type BB2 containing the pheromone gene bbp2(1), induced B-regulated development in BB2 transformants. This same DNA fragment was then integrated into the B-null strain. These transformants initiated B-regulated development in matings with BB2, BB6, and BB7 strains. DNA fragments containing wild-type bbp2(1), when transformed into the B-null strain, promoted B-regulated development in matings with BB4, BB6, and BB7 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-
Figure 3.—Sequence and mating activity of wild-type and mutant B\(\beta\)2 pheromones. (A) Predicted amino acid sequence of seven B\(\beta\)2 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 B\(\beta\)/H9252 specificities. (C) A missense mutation in the coding region of Bbp2(1-1) was separated from a point mutation in the 3\('/\)H11032 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).

Activation of the B\(\beta\)2 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 B\(\beta\)2 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, bhp2(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 bhp2(2-1), bhp1(3-1), and bhp1(1-1), respectively (Figure 3B). Bhp2(2-1), with alanine replacing valine at Cys-4, maintained the wild-type capability of Bhp2(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; Bhp2(2-1) lost the ability of its wild-type counterpart to activate receptors in Bβ1, Bβ3, and Bβ4 strains (Figure 3B). Pheromone Bhp1(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 Bhp3(1) was unable to activate receptors in mates carrying Bβ2 and Bβ7. The substitution of valine for alanine at Cys-4 in Bhp1(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 bhp2(1-1) was present, but its fluffy hyphal phenotype suggested it was not the Bβ2 receptor. 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 6bp 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 tests, 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-
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 B/H9252/2(1-3) mutant receptor underlined. (C) Results of B/H9252- dependent mating assays to monitor B-regulated development in a B-null-strain transformed with variants of the B/H9252/2 receptor. The test mate named "B-null/H11002" expresses only the mutant pheromone Bbp2(1-1). The other test mates are wild-type strains of the indicated B/H9252 specificity presumed to express all their natural B/H9252 pheromones.

Members of another smaller class of secondary mutants, Bβ(2)(1-6), are also incapable of accepting fertilizing nuclei from any Bβ tester. Unlike the class Bβ(2)(1-1) mutants, Bβ(2)(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, is because the truncation results in a null allele of bbr2. A probe to bhp2(1) and bhp2(1-1) revealed no length polymorphism between the wild-type and mutant strains for the restriction fragments containing the bhp2(1) and bhp2(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 bhp2(1) remained intact in this mutant strain. Southern analysis also showed that bhp2(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...
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; Koezin 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 Ba 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), bbr2(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; VAILLCHANT et al. 1997). The functional redundancy observed may therefore have some selective advantage within the natural population.

Molecular genetic analyses of both the Ba locus (WENDLAND et al. 1995; WENDLAND and KOTHE 1996; HEGNER et al. 1999) and the Bβ locus (VAILLCHANT 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 Ba 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 Ba1 and Bβ1 (VAILLCHANT 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 Ba1 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), bbr2(5), bbb2(7), and bbr2(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 Ba receptors of *S. commune*, Bar1, Bar2, and Bar3 (WENDLAND et al. 1995; HEGNER et al. 1999; L. J. VAILLANTCOURT, unpublished results), than it is to the only other member of the Bβ series examined to date, Bbr1 (VAILLCHANT et al. 1997). Bbr2 has at least 50% amino acid identity with the N-terminal halves of these Ba 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 Ba receptor genes shared enough DNA similarity with the Ba1 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, *bb2* hybridizes only to a single restriction fragment from a Bβ2 strain (M. F. MURTON, 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 Ba 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
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 B9 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

**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.
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).

*Schizophyllum 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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**LITERATURE CITED**


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