

Secretion of FK506/FK520 and rapamycin by *Streptomyces* inhibits the growth of competing *Saccharomyces cerevisiae* and *Cryptococcus neoformans*

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FK506 and rapamycin are immunosuppressants that inhibit signalling cascades required for T-cell activation, yet both are natural products of *Streptomyces* that live in the soil. FK506 and rapamycin also have potent antimicrobial activity against yeast and pathogenic fungi, suggesting a natural role in inhibiting growth of competing micro-organisms. The immunosuppressive and antimicrobial activities of FK506 and rapamycin are mediated by binding to the FKBP12 prolyl isomerase and the resulting FKBP12/FK506 and FKBP12/rapamycin complexes inhibit conserved protein targets, either the phosphatase calcineurin or the TOR (target of rapamycin) kinases, respectively. *Streptomyces* sp., '*Streptomyces hygrosopicus* subsp. *ascomyceticus*' and *Streptomyces hygrosopicus*, which produce FK506, FK520 (also known as ascomycin, a C21 ethyl derivative of FK506) and rapamycin, respectively, produced toxins that inhibited the growth of competing cells of the yeast *Saccharomyces cerevisiae* and the pathogenic fungus *Cryptococcus neoformans*. Yeast and fungal mutants lacking FKBP12 or expressing dominant drug-resistant calcineurin or TOR mutants were resistant to FK506 and rapamycin, and to the toxins produced by *Streptomyces*. *Streptomyces* strains with mutations in the FK506 or rapamycin biosynthetic enzymes were impaired in toxin production. Finally, the toxins secreted by '*S. hygrosopicus* subsp. *ascomyceticus*' and *S. hygrosopicus* promoted formation of FKBP12/calcineurin and FKBP12/TOR complexes in a two-hybrid assay and mutations that rendered calcineurin or TOR drug-resistant prevented interaction. These observations support the hypothesis that *Streptomyces* evolved to secrete FK506, FK520 and rapamycin as toxins to inhibit the growth of competing yeast and fungi.

Keywords: FK506/FK520, rapamycin, immunosuppression, *Streptomyces*, growth inhibition

INTRODUCTION

A variety of micro-organisms produce secondary metabolites or natural products, many of which exhibit toxic activity against different cells and organisms (Chadwick & Whelan, 1992). These natural products have proved to be a valuable resource to identify drugs with specific physiological effects in animals. For example, the immunosuppressants FK506 (tacrolimus),

FK520 (ascomycin, an active FK506 derivative in which the C21 allyl group is replaced by an ethyl group) and rapamycin (sirolimus) were originally identified by screening random soil samples for biological activities and were later found to be products of *Streptomyces* sp., '*Streptomyces hygrosopicus* subsp. *ascomyceticus*' and *Streptomyces hygrosopicus*, actinomycetes that live in the soil (Baker *et al.*, 1978; Hatanaka *et al.*, 1988a, b; Kino *et al.*, 1987; Morisaki & Arai, 1992; Sehgal *et al.*, 1975; Singh *et al.*, 1979; Vezina *et al.*, 1975). FK506 is now in widespread use as an immunosuppressant to prevent and treat graft rejection in solid organ transplant

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Abbreviation: TOR, target of rapamycin.

recipients and for graft-vs. host disease in bone marrow transplant recipients. Rapamycin is in late phase III clinical trials for similar purposes (Cardenas *et al.*, 1995a).

Considerable progress has been made in delineating the biosynthetic pathways by which *Streptomyces* elaborates these complex macrolide compounds. A cluster of enzymes has been identified which mediates rapamycin biosynthesis (Schwecke *et al.*, 1995) and mutational approaches have been employed to define the pathways involved in both rapamycin (Khaw *et al.*, 1998; Lomovskaya *et al.*, 1997) and FK506 biosynthesis (Motamedi *et al.*, 1997). Finally, numerous analogues of these compounds have been produced by biotransformation, altered growth medium conditions and semisynthetic organic chemistry (Khaw *et al.*, 1998; Luengo *et al.*, 1995; Nishida *et al.*, 1995), and these analogues might have novel or improved biological activities.

FK506 and rapamycin suppress the immune system by inhibiting signal transduction cascades required for T-lymphocyte activation during tissue rejection. In mammals, the activities of FK506 and rapamycin are relatively specific for lymphocytes, with only limited effects observed in other cells and tissues. This cell type specificity of FK506 and rapamycin raises a paradox: how did soil micro-organisms evolve to produce such T-cell-specific agents when they rarely, if ever, encounter large animals? Moreover, suppressing the immune system is not a particularly effective or rapid means of doing harm to a large animal and many microbial toxins directed against mammals target the nervous system. Hence, immunosuppression by FK506 or rapamycin is not likely to be a normal role in nature. In fact, FK506, cyclosporin A and rapamycin exhibit antimicrobial activity against a variety of different bacteria, yeasts, parasites and fungi and might therefore function to inhibit the growth of competing micro-organisms (High, 1994; McCabe *et al.*, 1986; Odom *et al.*, 1997a, b; Wong *et al.*, 1998).

Recent studies reveal that the molecular mechanisms of the immunosuppressive and antimicrobial effects of FK506 and rapamycin are remarkably similar and involve inhibition of highly conserved target proteins (Abraham & Wiederrecht, 1996; Heitman *et al.*, 1992; Schreiber & Crabtree, 1992). Both drugs are hydrophobic and enter the cell by diffusion. Next they bind to a highly conserved intracellular protein, FKBP12, which is conserved from unicellular eukaryotes such as *Saccharomyces cerevisiae* to man. FKBP12 is an enzyme that catalyses *cis-trans* peptidyl prolyl isomerization, a rate-limiting step during protein refolding *in vitro*. Both FK506 and rapamycin bind to the FKBP12 active site and inhibit activity, but this is not the mechanism by which these agents are toxic. Instead, the protein/drug complexes are the active *in vivo* agents. For example, yeast mutants lacking FKBP12 are viable and resistant to FK506 and rapamycin (Breuder *et al.*, 1994; Heitman *et al.*, 1991a, b; Hemenway *et al.*, 1995; Parent *et al.*, 1993)

and increasing FKBP12 protein levels in mammalian cells by transfection results in increased drug sensitivity (Bram *et al.*, 1993).

The targets of FKBP12/drug complexes are elements of signal transduction pathways conserved from yeast to man. The target of the FKBP12/FK506 complex was first identified as calcineurin by drug affinity chromatography of mammalian cell extracts (Liu *et al.*, 1991). Subsequent studies revealed that calcineurin was the conserved target of FK506 in yeast (Breuder *et al.*, 1994; Foor *et al.*, 1992; Parent *et al.*, 1993). Calcineurin is a calcium/calmodulin activated protein phosphatase that plays a central role in signal transduction from the T-cell receptor to regulate T-cell activation and plays diverse roles in yeast involving gene regulation and cation homeostasis. The target of the FKBP12/rapamycin complex was first identified by genetic and molecular studies in yeast as the TOR (target of rapamycin) kinase homologues (Cafferkey *et al.*, 1993; Heitman *et al.*, 1991a, b; Helliwell *et al.*, 1994; Kunz *et al.*, 1993) and the mammalian target of rapamycin (mTOR, RAFT1, FRAP) shares ~ 50% sequence identity with yeast TOR proteins (Brown *et al.*, 1994; Chiu *et al.*, 1994; Sabatini *et al.*, 1994; Sabers *et al.*, 1995). TOR kinase has been functionally conserved from yeast to man and plays a central role in regulating cell cycle progression and translation in lymphocytes in response to growth factors and, in yeast, in response to nutrients (Alarcon *et al.*, 1996; Barbet *et al.*, 1996; Brunn *et al.*, 1997; di Como & Arndt, 1996).

Taken together, these observations suggest that *Streptomyces* may have originally evolved the ability to synthesize and secrete FK506 and rapamycin as antimicrobial agents to inhibit the growth of competing micro-organisms in the soil. Such a role would be analogous to that attributed to antibiotics produced by fungi. Because the proteins that FK506 and rapamycin target have been highly conserved during evolution, their original activity against yeast and fungal targets could have been maintained against the mammalian homologues. Here we have tested this hypothesis and present evidence that *Streptomyces* spp. indeed secrete potent toxins that inhibit the growth of competing cells of the ascomycetous yeast *Saccharomyces cerevisiae* and also of the very distantly related basidiomycetous pathogenic fungus *Cryptococcus neoformans*.

METHODS

Media. Yeast YPD medium was prepared as described by Sherman (1991). *Streptomyces* sp. and '*S. hygroscopicus* subsp. *asco-myceticus*' were maintained on yeast malt extract agar (Difco medium 0770) and *Streptomyces hygroscopicus* on sporulation agar (g l⁻¹: yeast extract, 1; beef extract, 1; tryptose, 2; glucose, 10; agar, 15).

Yeast and bacterial strains. *S. hygroscopicus* subsp. *asco-myceticus*' (ATCC 14891, hereafter referred to as '*S. asco-myceticus*') and *S. hygroscopicus* (ATCC 29253) were obtained from the American Type Culture Collection, Manassas, VA, USA. Both strains exhibited previously de-

Table 1. Yeast strains

Strain	Genotype	Reference/source
<i>Saccharomyces cerevisiae</i>		
JK9-3d α	<i>MATα trp1 his4 leu2-3,112 ura3-52 rme1 GAL⁺ HMLα</i>	Heitman <i>et al.</i> (1991b)
JHY3-3B	JK9-3d α <i>fpr1::URA3-3</i> (FK506 ^R rapa ^R)	Heitman <i>et al.</i> (1991b)
R1	JK9-3d α <i>tor1-1</i> (FK506 ^S rapa ^R)	Heitman <i>et al.</i> (1991a)
R17	JK9-3d α <i>tor2-1 HIS⁺</i> (FK506 ^S rapa ^R)	Heitman <i>et al.</i> (1991a)
IL993/5c	<i>MATα ρ° ilv5</i> (CsA ^S FK506 ^S)	Tropschug <i>et al.</i> (1989)
TB23	IL993/5c <i>ura3 leu2::hisG</i> (CsA ^S FK506 ^S)	Breuder <i>et al.</i> (1994)
6A	IL993/5c <i>ura3 leu2::hisG CMP2-1</i> (CsA ^S FK506 ^R)	Cardenas <i>et al.</i> (1995b)
TB62	IL993/5c <i>MATα ura3 ILV⁺ fpr1::URA3-3</i> (CsA ^S FK506 ^R)	Breuder <i>et al.</i> (1994)
PJ69-4A	<i>MATα trp1-901 leu2-3,112 ura3-52 his3-200 ade2 gal4Δ gal80Δ LYS2::GAL-HIS3 GAL2-ADE2 met2::GAL7-lacZ</i>	James <i>et al.</i> (1996)
SMY87-4	PJ69-4A <i>fpr1::hisG</i>	
<i>Cryptococcus neoformans</i>		
JEC21	<i>MATα</i> (FK506 ^S rapa ^S)	Moore & Edman (1993)
C21F2	<i>MATα FKR1-1</i> (FK506 ^R rapa ^S)	Odom <i>et al.</i> (1997a)
C21F3	<i>MATα frr1-3</i> (FK506 ^R rapa ^R)	Odom <i>et al.</i> (1997a)
CN111	<i>MATα cyh^R TOR1-1</i> (FK506 ^S rapa ^R)	Cruz <i>et al.</i> (1999)

scribed morphological characteristics. For example, *S. hygroscopicus* formed colonies that invaded the agar, were quite hydrophobic and formed colonies of two distinct types, either powdery whitish grey or dark black (Vezina *et al.*, 1975). The isogenic pair of rapamycin-producing (ATCC 29253) and rapamycin-deficient (WHM1651) strains of *S. hygroscopicus* were kindly provided by Dr C. Hutchinson, University of Wisconsin, Madison, WI, USA (Lomovskaya *et al.*, 1997). The isogenic pair of FK506-producing (MA6548) and FK506-reduced production strains of *Streptomyces* sp. were provided by Dr Haideh Motamedi, Merck, Rahway, NJ, USA (Motamedi *et al.*, 1997).

Saccharomyces cerevisiae and *C. neoformans* strains are listed in Table 1 and most have been described previously (Breuder *et al.*, 1994; Cardenas *et al.*, 1995b; Heitman *et al.*, 1991a, b; James *et al.*, 1996; Odom *et al.*, 1997a, b). Strain SMY87-4 is an isogenic derivative of the two-hybrid host strain PJ69-4A (James *et al.*, 1996) in which the FKBP12-encoding gene, *FPR1*, has been disrupted by *fpr1::hisG-URA3-hisG* and then selected for *fpr1::hisG* recombinants by selection for growth on 5-fluoroorotic acid (5-FOA) medium.

Growth inhibition assays. *Saccharomyces cerevisiae* or *C. neoformans* strains were cultured in YPD liquid medium and $\sim 10^7$ cells were resuspended in 3 ml top agar (0.7% Bacto agar in water), which was poured onto the surface of YPD medium (90 or 140 mm plates) and allowed to solidify. Discs containing either '*S. ascomyceticus*' or *S. hygroscopicus* were prepared by incubating a lawn of cells on sporulation agar at 24 °C. Plugs of agar with overlaying colonies, mycelium and spores were excised with the larger end of a sterile Pasteur pipette and placed (colony side down) on the surface of medium containing yeast or fungal cells. Co-cultures were incubated for 48 h at 30 or 37 °C. Growth inhibition was quantified by measuring the distance from the edge of the disc to the zone of growth (the radius of growth inhibition).

Two-hybrid interactions. Plasmids expressing the GAL4(BD)/yeast FKBP12, GAL4(AD)/yeast calcineurin A, GAL4(BD)/*C. neoformans* TOR1 FKBP12/rapamycin-binding domain and GAL4(AD)/*C. neoformans* FKBP12 have been described (Cardenas *et al.*, 1994, 1995b; Cruz *et al.*, 1999). A two-hybrid host strain, SMY87-4 (see Table 1), was co-transformed with control plasmids, with plasmids expressing GAL4(BD)/yeast FKBP12 and wild-type and mutant GAL4(AD)/yeast calcineurin fusions, and with plasmids expressing wild-type and mutant GAL4(BD)/TOR1 and GAL4(AD)/FKBP12 fusions. Cells were resuspended in top agar on the surface of synthetic medium lacking tryptophan, leucine and adenine, and were then overlaid with inverted agar discs bearing '*S. ascomyceticus*' or *S. hygroscopicus*. Control discs contained FK506 (1 μ g), rapamycin (1 μ g) or no drug. Cells were incubated for 3–5 d at 30 °C. Colony formation is indicative of GAL4-dependent expression of the GAL-ADE2 reporter gene fusion as a consequence of FKBP12/ligand/calcineurin or FKBP12/ligand/TOR interactions.

RESULTS

Streptomyces inhibits *Saccharomyces cerevisiae* growth via FKBP12-dependent inhibition of calcineurin

To determine if FK506 and FK520-producing *Streptomyces* spp. secrete toxins that inhibit the growth of competing micro-organisms, we utilized a series of mutants of the yeast *Saccharomyces cerevisiae* that are sensitive or resistant to FK506 and FK520 by defined genetic mutations. Many laboratory strains of yeast are not sensitive to FK506 or FK520 because the enzyme inhibited by this drug, calcineurin, is not required for vegetative growth. A variety of mutants of *Saccharo-*

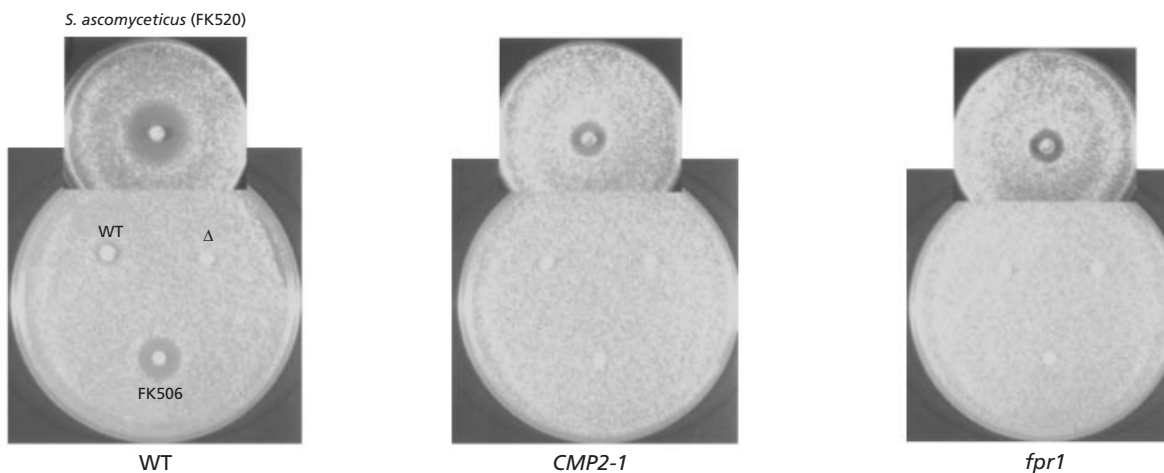


Fig. 1. *Streptomyces* sp. and '*S. ascomyceticus*' inhibit the growth of wild-type *Saccharomyces cerevisiae* but not of FK506/FK520-resistant mutant strains. Cells of isogenic yeast strains expressing wild-type FKBP12 and calcineurin proteins (WT, TB23), or expressing an FK506/FK520-resistant calcineurin mutant (*CMP2-1*, 6A), or lacking FKBP12 (*fpr1*, TB62) were resuspended in top agar on the surface of YPD medium. The medium was then overlaid with inverted agar discs bearing '*S. ascomyceticus*' (upper disc), *Streptomyces* sp. FK506-producing strain MA6548 (WT, left disc), *Streptomyces* sp. strain with reduced FK506 production (Δ , right disc) or a control disc with 1 μ g FK506 (lower disc) and incubated at 30 °C for 48 h.

myces cerevisiae have been identified in which calcineurin is essential for viability and these strains are sensitive to growth inhibition by FK506 and FK520 at a MIC of $\sim 1 \mu\text{g ml}^{-1}$ (Breuder *et al.*, 1994; Douglas *et al.*, 1994; Hemenway *et al.*, 1995; Parent *et al.*, 1993). One such calcineurin-dependent, FK506/FK520-sensitive yeast strain, TB23, has defects in ion homeostasis that render calcineurin essential (Breuder *et al.*, 1994). Isogenic FK506-resistant mutants derived from strain TB23 lack the FK506-binding protein FKBP12 (*fpr1*) or express dominant FK506-resistant forms of the FKBP12/FK506 target protein calcineurin (*CMP2-1*) that retain catalytic function but do not bind FKBP12/FK506 (Breuder *et al.*, 1994; Cardenas *et al.*, 1995b).

To establish whether '*S. ascomyceticus*' secretes the toxin FK520, the FK506- and FK520-sensitive yeast strain TB23 was cultured in the presence of '*S. ascomyceticus*'. For these experiments, yeast cells were resuspended in molten top agar and overlaid on rich YPD medium. Once the top agar had solidified, an agar disc containing '*S. ascomyceticus*' was placed on the surface. Growth of the FK520-sensitive yeast strain TB23 was potently inhibited by '*S. ascomyceticus*', resulting in a zone of inhibition with a radius of 1 cm (Fig. 1, upper discs). The major component of this toxic activity can be identified as FK520, because isogenic FK520-resistant mutant strains lacking FKBP12 (*fpr1*) or expressing a dominant FK520-resistant calcineurin mutant (*CMP2-1*) were relatively resistant to growth inhibition by the '*S. ascomyceticus*' toxin (Fig. 1). In these cases, smaller zones of growth inhibition with radii of 0.35 cm were observed. Based on these observations, we conclude that '*S. ascomyceticus*' secretes FK520 at a level sufficient to inhibit the growth of

competing yeast cells that are sensitive to FK520. '*S. ascomyceticus*' secretes an additional toxin and some residual growth inhibition was observed with mutant yeast strains that are resistant to FK506 and FK520 (Fig. 1; Breuder *et al.*, 1994; Hemenway *et al.*, 1995).

Similar results were obtained with an isogenic pair of *Streptomyces* strains, one that produces FK506 (MA6548) and the second which has a mutation in the FK506 biosynthetic machinery and produces reduced levels of FK506 (Motamedi *et al.*, 1997). As shown in Fig. 1, the wild-type FK506-producing strain inhibited the growth of the FK506-sensitive yeast strain TB23 to yield a modest zone of inhibition with a radius of 0.24 cm, whereas the mutated *Streptomyces* strain that produces a reduced level of FK506 did not inhibit growth (Fig. 1). Yeast mutants lacking FKBP12 (*fpr1*) or expressing an FK506-resistant calcineurin mutant (*CMP2-1*) were resistant to the toxic activity secreted by the wild-type *Streptomyces* strain (Fig. 1). A control disc containing FK506 inhibited the growth of the wild-type yeast strain (0.67 cm radius zone of inhibition) but not of either FK506-resistant mutant yeast strain (Fig. 1).

***S. hygroscopicus* inhibits *Saccharomyces cerevisiae* growth via FKBP12-dependent inhibition of TOR**

We performed analogous experiments to test if the rapamycin-producing actinomycete *S. hygroscopicus* also secretes toxins that inhibit yeast growth. Rapamycin potently inhibits the growth of all laboratory yeast strains that have been tested, with an MIC of $\sim 25\text{--}100 \text{ ng ml}^{-1}$ (Heitman *et al.*, 1991a). Yeast mutants that are resistant to rapamycin either lack the rapamycin drug-binding protein FKBP12 (*fpr1*) or express mutant forms of the FKBP12/rapamycin target

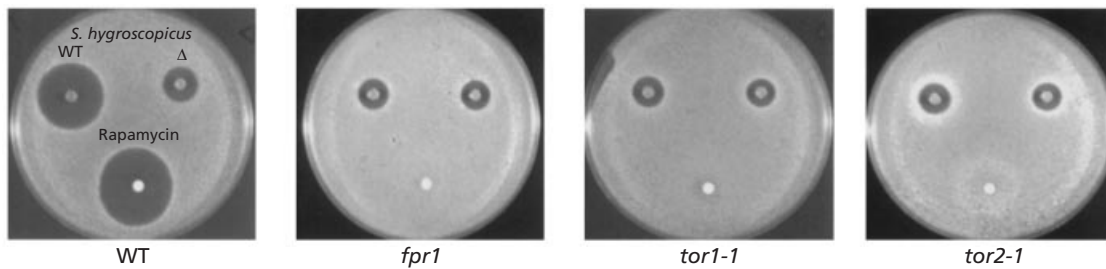


Fig. 2. *S. hygroscopicus* inhibits growth of wild-type *Saccharomyces cerevisiae* but not of rapamycin-resistant mutant strains. Cells of isogenic yeast strains expressing wild-type FKBP12 and TOR proteins (WT, JK9-3d), lacking FKBP12 (*fpr1*, JHY3-3B) or expressing rapamycin-resistant mutant forms of the TOR1 or TOR2 proteins (*tor1-1*, R1; *tor2-1*, R17) were resuspended in top agar on the surface of YPD medium, overlaid with inverted agar discs bearing *S. hygroscopicus* rapamycin-producing strain ATCC 29253 (WT, upper left disc), an isogenic *S. hygroscopicus* mutant strain that does not produce rapamycin (WHM1651; Δ , upper right disc) or control discs containing 1 μ g rapamycin (lower disc) and incubated at 30 °C for 48 h.

proteins, the TOR kinase homologues, which do not bind the FKBP12/rapamycin complex as a consequence of mutations in the FKBP12/rapamycin-binding domain on TOR (Cafferkey *et al.*, 1993; Heitman *et al.*, 1991a; Helliwell *et al.*, 1994; Lorenz & Heitman, 1995; Zheng *et al.*, 1995).

S. hygroscopicus secretes a toxin that potently inhibits the growth of the wild-type, rapamycin-sensitive yeast strain JK9-3d, resulting in a zone of inhibition with a radius of 1.56 cm (Fig. 2, upper left disc). In contrast, the isogenic rapamycin-resistant yeast mutants lacking FKBP12 (*fpr1*) or expressing rapamycin-resistant TOR mutants (*tor1-1*, *tor2-1*) were all relatively resistant to growth inhibition by the secreted *S. hygroscopicus* toxic activity, as indicated by smaller zones of inhibition with radii of 0.56, 0.53 and 0.5 cm (Fig. 2, upper left disc). Thus, *S. hygroscopicus* secretes sufficient rapamycin to inhibit the growth of competing rapamycin-sensitive yeast cells. With the isogenic *S. hygroscopicus* mutant strain that does not produce rapamycin, a smaller zone of inhibition (radius 0.5–0.63 cm) was observed that was equivalent in size in the rapamycin-sensitive and -resistant yeast strains (Fig. 2, upper right disc). In contrast, a control disc containing rapamycin potently inhibited the wild-type yeast strain (zone of inhibition radius 1.94 cm) but had no toxic effect against *fpr1*, *tor1-1* or *tor2-1* rapamycin-resistant yeast strains (Fig. 2, lower disc). Taken together, these observations indicate that *S. hygroscopicus* secretes both rapamycin and an additional toxin that inhibit growth of yeast.

***Streptomyces* sp. inhibits *C. neoformans* growth via FKBP12-dependent inhibition of calcineurin-dependent growth**

To determine if the growth-inhibiting toxins secreted by *Streptomyces* would affect another micro-organism that is quite distantly related to *Saccharomyces cerevisiae*, we employed a series of isogenic FK506/rapamycin-sensitive and -resistant strains of the pathogenic basidiomycete *C. neoformans*. As with yeast, growth of *C.*

neoformans is inhibited by both FK506 and rapamycin (Odom *et al.*, 1997a). In previous studies, we identified mutations in *C. neoformans* that confer resistance to FK506 (*FKR1-1*, mutant strain C21F2), to FK506 and rapamycin (*frr1-3*, mutant strain C21F3) or to rapamycin (*tor1-1*, mutant strain CN111) (Cruz *et al.*, 1999; Odom *et al.*, 1997a). Here we used this set of isogenic strains to further analyse the toxins secreted by *Streptomyces* sp.

As with *Saccharomyces cerevisiae*, cells of the wild-type *C. neoformans* strain JEC21 were resuspended in top agar, supported on rich YPD medium and confronted with competing cells of the wild-type FK506-producing *Streptomyces* strain MA6548 or an isogenic mutant strain that produces reduced levels of FK506. In previous studies, we found that growth of *C. neoformans* is resistant to the calcineurin inhibitor FK506 at 24 °C but markedly sensitive at 37 °C (Odom *et al.*, 1997a, b). Correspondingly, *C. neoformans* mutants lacking calcineurin are viable at 24 °C but not at 37 °C; hence, calcineurin is essential for growth at elevated temperatures in this organism (Odom *et al.*, 1997a).

In accordance with these observations, a toxin secreted by *Streptomyces* strain MA6548 potently inhibited *C. neoformans* growth at 37 °C (halo radius 1.23 cm) but not at 30 °C (Fig. 3, upper left disc). Moreover, the FK506-resistant FKBP12 mutant strain (*frr1-3*, C21F3) and a dominant FK506-resistant, rapamycin-sensitive mutant strain that is likely to harbour a calcineurin mutation (*FKR1-1*, C21F2) were completely resistant to growth inhibition by *Streptomyces* sp. (Fig. 3). In comparison, the zone of inhibition of the wild-type strain was reduced to 0.68 cm with the *Streptomyces* mutant strain that produces reduced FK506 and this strain did not inhibit growth of the FK506-resistant *C. neoformans* strains (Fig. 3, upper right disc). Finally, a control disc containing FK506 potently inhibited the growth of wild-type cells at 37 °C but not at 30 °C, and did not inhibit growth of the *frr1* or *FKR1-1* FK506-resistant mutant strains (Fig. 3, lower disc). These observations support the conclusion that *Streptomyces*

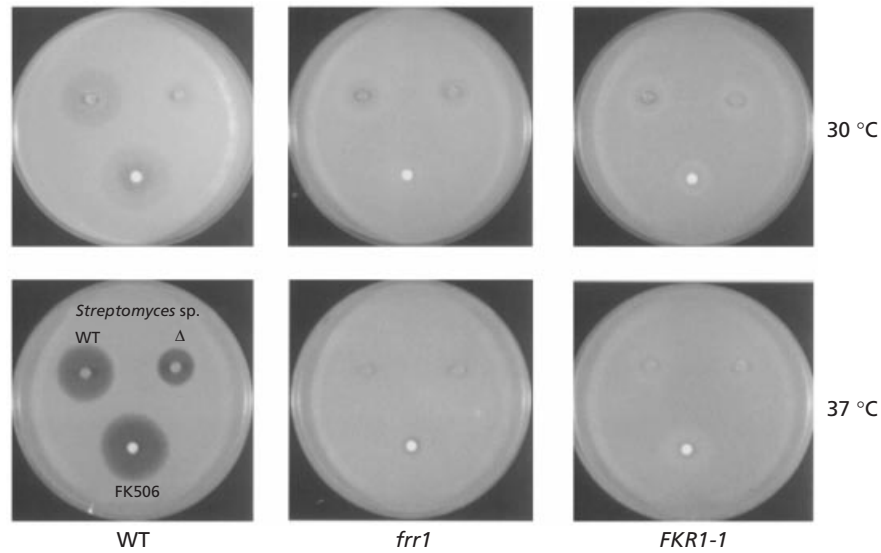


Fig. 3. *Streptomyces* sp. secretes a toxin that inhibits growth of *C. neoformans* at 37 °C but not at 30 °C and requires FKBP12 and calcineurin for action. Cells of isogenic *C. neoformans* strains expressing wild-type FKBP12 and calcineurin proteins (WT, JEC21) or FK506-resistant mutant strains that lack the FKBP12 protein (*frr1-3*, C21F3) or express the dominant FK506 resistance mutation *FKR1-1* (strain C21F2) were resuspended in top agar on the surface of YPD medium. The medium was then overlaid with inverted agar discs bearing *Streptomyces* sp. FK506-producing strain MA6548 (WT, upper left disc), *Streptomyces* sp. strain M24 with reduced FK506 production (Δ , upper right disc) or a control disc with 1 μ g FK506 (lower disc) and incubated at 30 or 37 °C for 48 h.

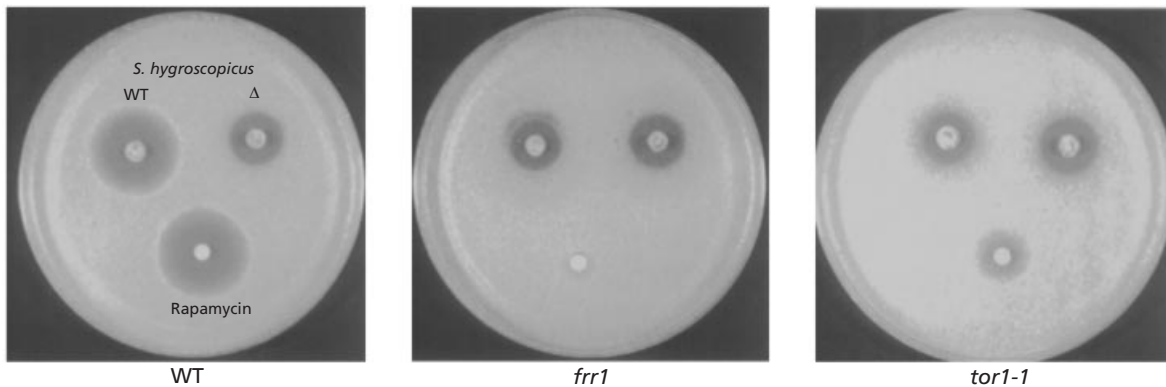


Fig. 4. Rapamycin-resistant *C. neoformans* mutants are resistant to toxins produced by *S. hygroscopicus*. Cells of isogenic *C. neoformans* strains expressing wild-type FKBP12 and calcineurin proteins (WT, JEC21), an FK506-rapamycin-resistant FKBP12 mutant (*frr1-3*, C21F3) and a rapamycin-resistant Tor mutant (*tor1-1*, CN111) were resuspended in top agar on the surface of YPD medium, overlaid with an inverted agar disc bearing a rapamycin-producing *S. hygroscopicus* strain (WT, upper left disc), the *S. hygroscopicus* mutant strain that does not produce rapamycin (Δ , upper right disc) or a control disc containing 1 μ g rapamycin (lower disc) and incubated at 37 °C for 48 h.

strain MA6548 secretes sufficient FK506 to inhibit the growth of competing *C. neoformans* cells via FKBP12-dependent inhibition of calcineurin.

***S. hygroscopicus* inhibits *C. neoformans* growth via FKBP12-dependent inhibition of TOR**

Analogous experiments were performed to examine inhibition of *C. neoformans* growth by the rapamycin-producing *S. hygroscopicus* strain ATCC 29253 and an

isogenic mutant that does not produce rapamycin (WHM1651). Rapamycin inhibits the growth of wild-type *C. neoformans* (MIC = $\sim 1 \mu\text{g ml}^{-1}$) and rapamycin-resistant mutants lacking FKBP12 or with TOR mutations have been identified (Cruz *et al.*, 1999).

The *S. hygroscopicus* wild-type strain produces a toxin that potently inhibits the growth of the wild-type *C. neoformans* strain JEC21 (zone of inhibition radius 1.45 cm) (Fig. 4, upper left disc). In comparison, the

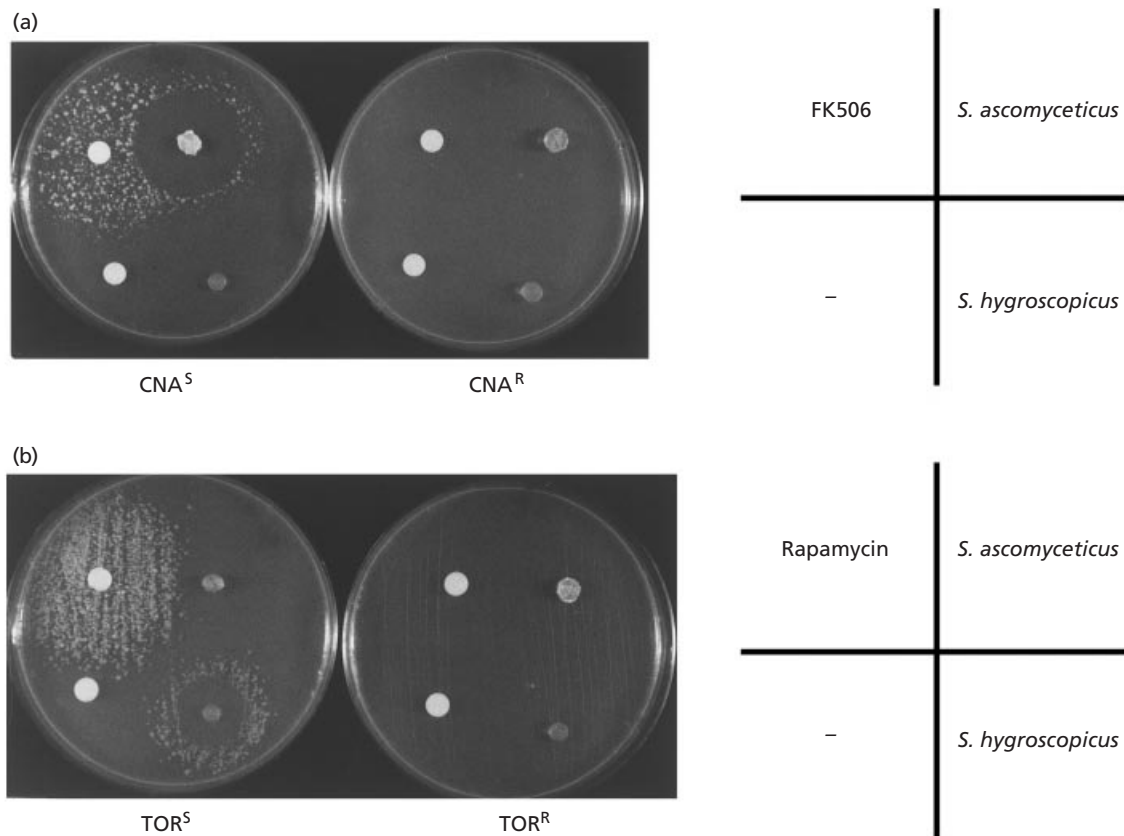


Fig. 5. *Streptomyces* sp. secretes ligands that promote FKBP12/calcineurin and FKBP12/TOR interactions in the yeast two-hybrid assay. (a) Cells of the yeast two-hybrid host strain SMY87-4 (*fpr1::hisG*) co-expressing the GAL4 DNA-binding domain fused to FKBP12 [GAL4(BD)/FKBP12] and the GAL4 activation domain fused to wild-type calcineurin A (CNA^S) or an FK520-resistant mutant form of calcineurin A (CNA^R) were resuspended in top agar and grown on the surface of medium lacking adenine. Discs bearing FK506 or no drug (–), or agar plugs bearing ‘*S. ascomyceticus*’ or *S. hygroscopicus*, were placed on the surface of the agar. Growth indicates GAL4-dependent expression of the *GAL–ADE2* reporter gene and results from formation of a GAL4/FKBP12/FK506/FK520/calcineurin/GAL4 complex. (b) Cells of the yeast two-hybrid host strain SMY87-4 (*fpr1::hisG*) co-expressing the GAL4 activation domain fused to FKBP12 [GAL4(AD)/FKBP12] and the GAL4 DNA-binding domain fused to wild-type TOR (TOR^S) or a rapamycin-resistant mutant form of TOR (TOR^R) were resuspended in top agar and grown on the surface of medium lacking adenine. Discs bearing rapamycin or no drug (–), or agar plugs bearing ‘*S. ascomyceticus*’ or *S. hygroscopicus*, were placed on the surface of the agar. Growth indicates GAL4-dependent expression of the *GAL–ADE2* reporter gene and results from formation of a GAL4/FKBP12/rapamycin/TOR/GAL4 complex.

rapamycin-resistant FKBP12 mutant strain C21F3 (*frr1-3*) was relatively resistant to the *S. hygroscopicus* toxin, with a smaller zone of inhibition (radius 0.75 cm) compared to the isogenic wild-type strain JEC21 (Fig. 4). Similarly, an isogenic mutant strain expressing a TOR1 mutant protein that no longer binds FKBP12/rapamycin (Cruz *et al.*, 1999) was also relatively resistant to the *S. hygroscopicus* toxin, with a zone of inhibition with a radius of 0.75 cm (Fig. 4). In accordance with these findings, the zone of growth inhibition with the *Streptomyces* WHM1651 mutant strain that does not produce rapamycin was reduced to 0.75 cm in both the rapamycin-sensitive wild-type strain JEC21 and the rapamycin-resistant *frr1* and *tor1-1* mutant strains (Fig. 4, upper right disc). Finally, a control disc containing rapamycin potently inhibited the growth of

the wild-type strain (zone of inhibition radius 1.45 cm), whereas the *frr1* mutant strain was completely resistant to rapamycin and the *tor1-1* mutant strain was largely resistant to rapamycin (Fig. 4, lower disc). Taken together, these observations indicate that *S. hygroscopicus* produces both rapamycin and a second toxin with a different mechanism of action (Fig. 4).

‘*S. ascomyceticus*’ toxin mediates FKBP12/calcineurin interaction

To provide another means to determine if FK520 produced by ‘*S. ascomyceticus*’ promotes an interaction between FKBP12 and calcineurin *in vivo*, we employed the yeast two-hybrid system. Protein–protein and protein–ligand–protein interactions can be monitored in

the two-hybrid assay by reconstitution of the GAL4 transcriptional activator (Fields & Song, 1989).

A two-hybrid host strain whose growth is resistant to FK520 (SMY87-4, see Table 1) was transformed with plasmids that express yeast FKBP12 fused to the GAL4 DNA-binding domain [GAL4(BD)/FKBP12] and yeast calcineurin A fused to the GAL4 activation domain [GAL4(AD)/CNA]. Cells were plated in top agar on synthetic medium lacking leucine and tryptophan (to select for the plasmids) and also lacking adenine (as a measure of expression of the *GAL-ADE2* reporter gene). The cells were then overlaid with inverted agar discs bearing '*S. ascomyceticus*' or a disc containing FK506. A halo of colonies was observed surrounding both the disc bearing '*S. ascomyceticus*' and the control disc containing FK506, but not around a control disc lacking FK506 or an agar disc bearing *S. hygroscopicus*, which secretes rapamycin but not FK520 (Fig. 5a). The presence of growth indicates the formation of a GAL4/FKBP12/ligand/calcineurin/GAL4 complex that then activates transcription of the *GAL-ADE2* reporter gene to support growth in medium lacking adenine. In contrast, no growth was observed when a mutation (W388C, CMP2-1) that prevents FKBP12/FK506 binding was introduced into the GAL4/calcineurin A fusion protein (Fig. 5a, CNA^R). Finally, the finding that a zone of inhibition surrounded the disc of '*S. ascomyceticus*', but not the control disc with FK506 alone, indicates that '*S. ascomyceticus*' secretes FK520 and an additional toxin. In summary, these observations support the conclusion that '*S. ascomyceticus*' secretes sufficient FK520 to form FKBP12/FK520/calcineurin complexes in competing yeast cells.

S. hygroscopicus toxin mediates cryptococcal FKBP12/TOR interaction

Analogous experiments were performed using the yeast two-hybrid system to test if *S. hygroscopicus* secretes rapamycin and promotes formation of an FKBP12/rapamycin/TOR complex. In this case, the yeast two-hybrid strain was co-transformed with plasmids that express GAL4(BD)/TOR and GAL4(AD)/FKBP12 fusion proteins. In addition, to render growth of this strain resistant to rapamycin, a third plasmid was introduced that expresses a dominant, rapamycin-resistant allele of the yeast TOR1 protein. Again, growth was readily apparent surrounding the agar disc bearing *S. hygroscopicus* and around a disc bearing rapamycin, but not around a control disc or an agar disc bearing '*S. ascomyceticus*' (Fig. 5b). In this assay, growth indicates formation of an FKBP12/rapamycin/TOR complex and GAL4-dependent expression of the *GAL-ADE2* reporter gene. In contrast, no growth was observed when a mutation that prevents FKBP12/rapamycin binding to TOR was introduced into the GAL4(BD)/TOR fusion protein (Fig. 5B, TOR^R). In control experiments, strain WHM1651, which does not produce rapamycin, did not promote interaction of FKBP12 and TOR in the two-hybrid assay (data not shown). These findings support

the conclusion that *S. hygroscopicus* secretes rapamycin and promotes the formation of an FKBP12/rapamycin/TOR complex in adjacent yeast cells. The observation that a zone of growth inhibition is found surrounding the disc of *Streptomyces* cells, and not around the control disc containing rapamycin alone, again indicates that *S. hygroscopicus* secretes an additional toxin.

DISCUSSION

Streptomyces and a variety of other micro-organisms produce a vast array of natural products, many of which are antibiotics, toxins and important pharmaceuticals. Here we present evidence that *Streptomyces* spp. produce the immunosuppressants FK506, FK520 and rapamycin as antimicrobial agents to inhibit the growth of competing micro-organisms (Fig. 6).

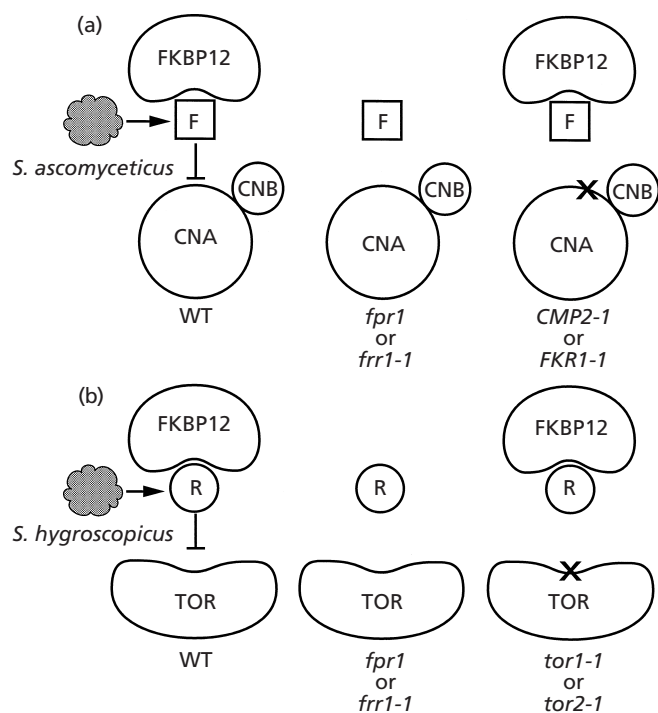


Fig. 6. The antifungal actions of FK506, FK520 and rapamycin are mediated by interactions with the conserved FKBP12, calcineurin and TOR proteins. (a) *Streptomyces* sp. produces a toxin that inhibits the growth of yeast and fungi. A major component of this toxic activity is FK506 or FK520 (F) which, in a complex with the FKBP12 protein, inhibits the protein phosphatase calcineurin, here indicated as a heterodimer of the catalytic CNA and regulatory CNB subunits. Growth of wild-type yeast and fungi is inhibited by FK506, whereas mutants lacking FKBP12 (*fpr1*, *frr1-1* or *-3*) or expressing FK506-resistant calcineurin mutants (*CMP2-1*, *FKR1-1*) are drug-resistant. (b) *S. hygroscopicus* produces toxins that inhibit the growth of yeasts and fungi. One major component of this activity is rapamycin (R) which, in complex with the FKBP12 protein, inhibits the TOR kinase homologues. Growth of wild-type yeasts and fungi is inhibited by rapamycin, whereas mutants lacking FKBP12 (*fpr1*, *frr1-1* or *-3*) or expressing rapamycin-resistant TOR mutants (*tor1-1*, *tor2-1*) are rapamycin-resistant.

Using a series of *Saccharomyces cerevisiae* and *C. neoformans* mutant strains with defined mutations that confer sensitivity or resistance to FK506/FK520 and rapamycin, we have demonstrated that the principle components of these toxic activities are indeed FK506, FK520 and rapamycin. These observations are summarized in Fig. 6. First, mutants lacking the FK506/rapamycin-binding protein FKBP12 are viable and resistant to a toxin produced by '*S. ascomyceticus*' and *S. hygroscopicus*. Correspondingly, yeast and *C. neoformans* mutants expressing mutant, drug-resistant forms of the FKBP12/FK506 target protein calcineurin that do not bind FKBP12/FK506 (Cardenas *et al.*, 1995b) are resistant to FK506 produced by *Streptomyces*. Similarly, yeast and fungal mutants expressing mutant drug-resistant forms of the FKBP12/rapamycin target proteins TOR1 and TOR2 in yeast (Lorenz & Heitman, 1995), or the TOR1 homologue in *C. neoformans* (Cruz *et al.*, 1999), are resistant to rapamycin produced by *S. hygroscopicus*. Finally, using the yeast two-hybrid system, we have demonstrated that '*S. ascomyceticus*' secretes a ligand that promotes FKBP12 binding to wild-type calcineurin, but not to a calcineurin mutant that fails to bind FKBP12/FK520, and that *S. hygroscopicus* secretes a ligand that promotes FKBP12 binding to wild-type TOR, but not to a mutant TOR that fails to bind FKBP12/rapamycin. We conclude that *Streptomyces* secrete sufficient FK506, FK520 and rapamycin to inhibit the growth of competing, drug-sensitive yeast and fungal cells (Fig. 6).

The mechanisms by which *Streptomyces* produce these antifungal agents provides further evidence that these compounds may have evolved early and for a specific purpose. FK506 and rapamycin are members of the macrolide family of compounds, which are synthesized by polyketide synthases. In the case of rapamycin, a large array of related biosynthetic enzymes have been characterized that catalyse the sequential steps in the synthesis of the macrolide ring (Aparicio *et al.*, 1996; Molnár *et al.*, 1996; Schwecke *et al.*, 1995). A related series of enzymes is involved in the biosynthesis of FK506 and FK520 (Motamedi *et al.*, 1996; Nielsen *et al.*, 1991). In both cases, a large, complicated series of genes is required to synthesize the natural product. Other macrolides, such as the antibiotic erythromycin, are synthesized by similar, related enzyme clusters (Cortes *et al.*, 1990; Donadio *et al.*, 1991; Katz, 1997). The diversity of compounds produced by related means suggests that these enzymes may have evolved quite early, perhaps prior to the evolution of multicellular eukaryotes and consistent with a role as antimicrobial agents. Many other members of the macrolide family, including erythromycin and streptomycin, are well-known antibiotics, again suggesting that the role of other members of this family of natural products could serve to inhibit the growth of competing micro-organisms. The great complexity of the biosynthetic machinery for these natural products requires that the producing organism invests a great deal of energy in their synthesis, which would again be consistent with a

role in growth competition. An alternative hypothesis is that the natural products FK506 and rapamycin might play a signalling role as pheromones, but no evidence to support such a role has as yet been adduced.

A general question that applies to all micro-organisms that make toxins is how the producing organism protects itself from potential suicide. In some cases, such as colicin production in bacteria, the host cell produces an immunity protein that counteracts the lethal action of the toxin. In the case of *Streptomyces* that produce FK506 and rapamycin, these organisms have been found to express an FKBP12 homologue that binds FK520 and rapamycin with high affinity (Pahl & Keller, 1992). On the other hand, both *S. hygroscopicus* and '*S. ascomyceticus*' were found to be resistant to both FK520 and rapamycin (Pahl & Keller, 1992). One plausible hypothesis is that these bacteria lack the calcineurin and TOR protein targets of FKBP12/drug complexes. Alternatively, calcineurin and TOR homologues might be present but not required for vegetative growth, as is the case with calcineurin in wild-type *Saccharomyces cerevisiae* strains and may be the case for TOR in *Schizosaccharomyces pombe* in which vegetative growth is not sensitive to rapamycin (Weisman *et al.*, 1997). Finally, *Streptomyces* spp. could have evolved mutations in FKBP12, calcineurin or TOR that prevent inhibition by FK506, FK520 and rapamycin.

Why might natural products that target fungal and yeast cells have any activity against mammalian cells, let alone such a specific immunosuppressive action in mammals? The targets of these agents, the FKBP12 prolyl isomerase, the phosphatase calcineurin and the TOR kinase homologues, are highly conserved from yeast to man with ~ 50% sequence identity despite ~ 1 billion years of evolution. In the case of calcineurin and TOR, one hypothesis is that these proteins play important signal transduction roles involving other conserved signalling molecules and that this, in part, has constrained their evolution. The case of FKBP12 is much more mysterious given that its stable interactions with calcineurin and TOR occur only when FK506 or rapamycin are present. Our previous findings have suggested that these proteins might normally interact under some physiological conditions (see Cardenas *et al.*, 1994). In summary, natural products that evolved to target yeast and fungal growth can have quite specific effects in multicellular eukaryotes by targeting conserved target proteins.

Our studies underscore the highly conserved nature of life and the power of studies on natural product toxins, both as probes of cell function and as a source of valuable pharmaceuticals. These studies may also enable mutagenic approaches to study FK506- and rapamycin-producing organisms and enzymes, and their specificity. For example, our approaches could be readily adapted to screen for mutant bacteria that make toxins that act via FKBP12 but not on calcineurin or TOR. Alterations in culture conditions and biotransformation approaches have been found to result in the production of drug analogues (Khaw *et al.*, 1998; Nishida *et al.*, 1995) and these could be readily screened for activity against novel

targets using the approaches described here. Finally, novel FKBP12-binding ligands may already exist in nature and in fact two classes of novel FKBP12-binding ligands, meridamycin and the antascomycins, have recently been described (Fehr *et al.*, 1996; Salituro *et al.*, 1995). Although the biological properties of these novel agents remain to be explored, our studies suggest that one role might be as novel antimicrobial agents.

ACKNOWLEDGEMENTS

We thank Drs C. Richard Hutchinson and Haideh Motamedi for supplying strains of *Streptomyces* and Ronald Germain for stimulating discussions. Rapamycin was provided by the Drug Discovery branch of the NCI and FK506 by Fujisawa Pharmaceuticals. This research was supported by K01 career development award CA77075 from the NCI (to M.E.C.) and NIAID grants R01 AI39115, AI41937, a supplement to AI41937 (to M.C.C.), and P01 award AI44975 to the Duke University Mycology Research Unit. Joseph Heitman is an associate investigator of the Howard Hughes Medical Institute and a Burroughs Wellcome Scholar in Molecular Pathogenic Mycology.

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Received 12 April 1999; accepted 23 April 1999.