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Synthetic Peptide Combinatorial Libraries: A Method for the Identification of Bioactive Peptides Against Phytopathogenic Fungi

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Synthetic combinatorial libraries were evaluated with an iterative process to identify a hexapeptide with broad-spectrum activity against selected phytopathogenic fungi. A D-amino acid hexapeptide (FRLKFH) and pentapeptide (FRLHF) exhibited activity against *Fusarium oxysporum* f. sp. *lycopersici, Rhizoctonia solani* (anastomosis group 1), *Ceratocystis fagacearum,* and *Pythium ultimum.* The peptides showed no hemolytic or mutagenic activity. Fluorescent microscopy studies with a membrane impermeant dye indicated that fungal cytoplasmic membranes were compromised rapidly and that the nuclear membrane was also affected.

Additional keywords: antifungal, synthetic peptide.

Plants and animals have various defense mechanisms that allow them to resist infection by pathogenic fungi and bacteria. Among such defenses are an array of naturally occurring, antimicrobial peptides produced by various organisms that elicit a potent antimicrobial response (Zasloff 1987; Bowman et al. 1991; Hansen 1993). Antimicrobial peptides constitute an important defense mechanism. Recent studies have established the composition and structure of naturally occurring peptides and have examined details of their modes of action through the use of synthetic peptides (Bessalle et al. 1990; Chikindas et al. 1993; Merrifield et al. 1994; Marcos et al. 1995). For example, Merrifield et al. (1994) employed synthetic peptide chemistry to establish the primary sequences of the cecropins and determined the steps involved in their biosynthesis. From this information, they were able to develop cecropin analogues that were more active and stable and that had a broader spectrum of activity. Marcos et al. (1995) used synthetic peptide chemistry to identify specific synthetic peptide inhibitors of plant viral infection by tobacco mosaic virus (TMV). They used synthetic peptide chemistry to develop analogues of mellitin (26-amino acid residues) that inhibited infection by TMV. Another novel way to utilize synthetic peptide chemistry has been demonstrated by Hightower et al. (1994). They transformed a synthetic cecropin gene into tobacco plants in order to produce the cecropin peptide in planta and give resistance to the leaf pathogen *Pseudomonas syringae* pv. *tabaci*. Ultimately, the peptide was produced in levels too low to provide significant disease suppression. However, the strategy of protecting plants by expression of peptide antibiotics holds much promise.

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Designed synthetic antimicrobial peptides are becoming more important as investigators search for new and innovative approaches to control disease. The use of combinatorial libraries to identify synthetic peptides that exhibit antimicrobial activity offers an efficient methodology to identify novel peptides. Blondelle and Houghten (1996) have described three categories of peptide libraries: molecular biology techniques, in which peptides are presented on the surface of filamentous-phage particles or plasmids; synthetic chemistry techniques for the generation of solid-support-bound compounds; and chemical synthesis of mixtures of soluble, free compounds. Houghten et al. (1992) have demonstrated the use of synthetic peptide combinatorial libraries (SPCLs) to identify bioactive peptides. Several studies (Houghten et al. 1993; Blondelle et al. 1994; Blondelle et al. 1995) have used the iterative process to screen SPCLs to identify peptide sequences having antimicrobial activity against pathogenic bacteria and yeast.

In the present study, we focused on a systematic evaluation of SPCLs to identify a bioactive pentapeptide (PPD1) and hexapeptide (66-10) with activity against filamentous phytopathogenic fungi. The defined peptides were compared for bioactivity to known antifungal compounds and tested for mutagenicity and hemolytic activity, and the potential target site of peptide D-FRLKFH was examined.

RESULTS

Determination of optimum inoculum concentration and incubation conditions for bioassay.

Growth studies were performed for the four fungal pathogens in order to determine the proper conditions for the bioassay. An initial inoculum concentration of 5×10^3 fungal spores, microconidia, or mycelial fragments per ml under static conditions showed balanced growth and less variability in optical density readings made over a 72-h period for *F. oxysporum* f. sp. *lycopersici* (microconidia), *R. solani* (mycelial fragments), and *P. ultimum* (oospores). Higher inoculum concentrations and/or shaking conditions resulted in more variability in the optical density readings (recorded every 24 h). The maximum optical density for each of the respective fungi was 0.950, 0.270, and 0.400 over the 72-h period. An inocu-

lum concentration of 5×10^4 spores per ml and static conditions were optimal for *C. fagacearum* (spores) based on optical density readings for 96 h (0.250 maximum). In addition to a determination of the proper inoculum concentration and incubation conditions for the respective fungal pathogens, a correlation was made of the optical density readings with the mycelial dry weights. Optical density versus dry weight plots showed a linear relationship for each of the fungal pathogens (data not shown).

Development of a defined antimicrobial hexapeptide.

To identify a hexapeptide with broad-spectrum activity, an iterative process was used (Fig. 1). The 400 D-amino acid peptide mixtures, designated the 26-series, were screened against *F. oxysporum* f. sp. *lycopersici*, *R. solani*, *C. fagacearum*, and *P. ultimum*. Peptide mixture FRXXXX (26-51) was chosen to construct the 36-series. This mixture showed IC_{50}s of 875, 557, 46, and 492 µg/ml for *F. oxysporum* f. sp. *lycopersici*, *R. solani*, *C. fagacearum*, and *P. ultimum*, respectively, with corresponding MICs of 2,030, 1083, 79, and 873 µg/ml, respectively. Screening of the 36-series showed peptide mixtures FRLXXX (36-18) and FRIXXX (36-12) to have broad-spectrum activity for the target fungi. The 36-18 mixture showed MICs of 614 ± 6, 134 ± 12, 68 ± 9, and 325 ± 3 µg/ml for *F. oxysporum* f. sp. *lycopersici*, *R. solani*, *C. fagacearum*, and *P. ultimum*, respectively (Tables 1 to 4, respectively). With the FRL sequence in positions O_1, O_2, and O_3 for the 46-series, peptide mixture FRLKXX (46-17) was found to have the best activity against the target fungi. This mixture showed MICs of 337 ± 8, 28 ± 5, 35 ± 3, and 81 ± 1 µg/ml for the respective fungal pathogens (Tables 1 to 4, respectively). This information was used to develop the 56-series designated $FRLKO_5X$, in which position O_5 consisted of one of 20 D-amino acids. The peptide mixture FRLKFX (56-3) exhibited the desired activity and its sequence was chosen to construct the 20 defined hexapeptides designated the 66-series. The 56-3 peptide mixture showed MICs of 40 ± 1, 27 ± 2, 10 ± 0, and 41 ± 1 µg/ml for the respective pathogens (Tables 1 to 4, respectively). After screening of the 66-series, hexapeptide FRLKFH (66-10) was chosen as the defined hexapeptide to undergo further investigation. Hexapeptide FRLKFH had MICs of 19 ± 1, 20 ± 3, 5 ± 0, and 36 ± 7 µg/ml for *F. oxysporum* f. sp. *lycopersici*, *R. solani*, *C. fagacearum*, and *P. ultimum*, respectively (Tables 1 to 4, respectively). With the iterative process, it was possible to obtain a 107-fold increase in activity, compared with FRXXXX (26-51), against *F. oxysporum* f. sp. *lycopersici*, a 54-fold increase against *R. solani* (AG-1), a 16-fold increase against *C. fagacearum*, and a 24-fold increase against *P. ultimum*. Testing of 66-10 against *Ophiostoma ulmi* and *R. solani* (AG-4) showed MICs of 16.5 ± 0.3 and 58 ± 5 µg/ml, respectively. In addition to the development of hexapeptide 66-10, a pentapeptide with the sequence FRLHF was developed during our studies. This peptide exhibited MICs of 66 ± 2, 66 ± 2, 14 ± 3, and 55 ± 12 µg/ml for *F. oxysporum* f. sp. *lycopersici*, *R. solani* (AG-1), *C. fagacearum*, and *P. ultimum*, respectively.

Synthesis of 26-series
↓
[a]O_1O_2XXXX [400 peptide mixtures, each containing 19^4 (130,321) hexapeptides]
↓
Screening and selection of peptide mixture exhibiting high broad spectrum activity
(FRXXXX)
↓
Synthesis of 36-series
↓
$O_1O_2O_3XXX$ [19 peptide mixtures, each containing 19^3 (6859) hexapeptides]
Position O_3 consists of 1 of 19 D-amino acids.
↓
Screening and selection of peptide mixture exhibiting high broad spectrum activity
(FRLXXX)
↓
Synthesis of 46-series
↓
$O_1O_2O_3O_4XX$ [19 peptide mixtures, each consisting of 19^2 (361) hexapeptides]
Position O_4 consists of 1 of 19 D-amino acids
↓
Screening and selection of peptide mixture exhibiting high broad spectrum activity
(FRLKXX)
↓
Synthesis of 56-series
↓
$O_1O_2O_3O_4O_5X$ (20 peptide mixtures, each consisting of 19 hexapeptides)
Position O_5 consists of 1 of 20 D-amino acids.
↓
Screening and selection of peptide mixture exhibiting high broad spectrum activity
(FRLKFX)
↓
Synthesis of 66-series
↓
$O_1O_2O_3O_4O_5O_6$ (20 individual hexapeptides)
↓
Screening and identification of a defined hexapeptide exhibiting high broad spectrum activity
(FRLKFH)

[a]Positions O_1 and O_2 represent the 20 individual D-amino acids in all possible combinations, which results in 400 peptide mixtures (i.e. $20^2 = 400$). Each X position in the sequence represents an equimolar mixture of 19 D-amino acids. Cysteine was omitted from the X position. All libraries were synthesized with D-amino acids with the carboxyl terminal aminated and the amino group unacetylated.

Fig. 1. Strategy used to identify hexapeptide 66-10.

Comparison of defined peptides to antifungal agents.

The defined pentapeptide (PPD1) and hexapeptide (66-10) were compared with MBC, cycloheximide, and nystatin for antifungal activity. For comparison purposes the MIC of each compound was determined with the target fungi (Table 5). Of the commercial antifungals, MBC and cycloheximide had the greatest activity against the fungal pathogens. *C. fagacearum* was the most sensitive to all the antifungal agents tested. *P. ultimum* was sensitive to cycloheximide but lacked sensitivity to MBC or nystatin. Against *F. oxysporum* f. sp. *lycopersici*, 66-10 exhibited higher activity than did cycloheximide. However, 66-10 was almost 10-fold and fourfold less active than MBC and nystatin, respectively. Hexapeptide 66-10 exhibited about 10-fold less activity than MBC and nystatin, and was 29-fold less active than cycloheximide against *R. solani* (AG-1). When compared with MBC and nystatin, 66-10 was about fivefold less active against *C. fagacearum*. Against *P. ultimum*, 66-10 was sixfold less active than cycloheximide. Overall, 66-10 was approximately threefold more active than pentapeptide PPD1.

Determination of potential target site of hexapeptide 66-10.

Fluorescent microscopy was used to determine the potential target site of hexapeptide 66-10. Two fluorescent dyes, Sytox

Green and fluorescent brightener 28, were used in the staining procedures. Figures 2A and 3A show ethanol-treated *C. fagacearum* spores and *F. oxysporum* f. sp. *lycopersici* microconidia, respectively, in the presence of Sytox. Both structures show a defined, intact nucleus where the dye has entered the spore and complexed with the nucleic acids. Figures 2B and 3B show *C. fagacearum* spores and *F. oxysporum* f. sp. *lycopersici* microconidia, respectively, observed immediately after treatment with a mixture of Sytox and hexapeptide 66-10. The photographs show that the impermeant dye has en-

Table 1. Minimum inhibitory concentrations (MICs) (μg/ml) of the peptide mixture series constructed to obtain a defined antifungal hexapeptide against *Fusarium oxysporum* f. sp. *lycopersici*

36-series		46-series		56-series		66-series	
Peptide[a]	**MIC**	**Peptide**	**MIC**	**Peptide**	**MIC**	**Peptide**	**MIC**
FRQXXX	>2,500	FRLQXX	2,435	FRLKQX	>625	FRLKFQ	40 ± 0
FRSXXX	>2,500	FRLSXX	1,889 ± 20	FRLKSX	>625	FRLKFS	39 ± 2
FRFXXX	618 ± 10[b]	FRLFXX	584 ± 12	FRLKFX	40 ± 1	FRLKFF	240 ± 4
FRAXXX	>2,500	FRLAXX	2,391 ± 19	FRLKAX	>625	FRLKFA	77 ± 3
FRNXXX	>2,500	FRLNXX	2375	FRLKNX	>625	FRLKFN	124 ± ±7
FRWXXX	1,807 ± 16	FRLWXX	>2,500	FRLKWX	70 ± 2	FRLKFW	38 ± 2
FRVXXX	534	FRLVXX	611	FRLKVX	62 ± 2	FRLKFV	39 ± 1
FRMXXX	1,164±44	FRLMXX	1,159	FRLKMX	80 ± 2	FRLKFM	76 ± 2
FRPXXX	>2,500	FRLPXX	>2,500	FRLKPX	>625	FRLKFP	532 ± 14
FRHXXX	>2,500	FRLHXX	308 ± 4	FRLKHX	>625	FRLKFH	19 ± 1
FRRXXX	>2,500	FRLRXX	1,046 ± 16	FRLKRX	>625	FRLKFR	32 ± 1
FRIXXX	318 ± 0	FRLIXX	641 ± 10	FRLKIX	42 ± 1	FRLKFI	133 ± 11
FRDXXX	>2,500	FRLDXX	>2,500	FRLKDX	>625	FRLKFD	627 ± 10
FRCXXX	*[c]	FRLCXX	*	FRLKCX	424 ± 4	FRLKFC	62 ± 1
FRGXXX	>2,500	FRLGXX	>2,500	FRLKGX	>625	FRLKFG	152 ± 5
FRTXXX	>2,500	FRLTXX	1,057 ± 1	FRLKTX	>625	FRLKFT	39 ± 1
FRKXXX	>2,500	FRLKXX	337 ± 8	FRLKKX	>625	FRLKFK	21 ± 1
FRLXXX	614 ± 6[d]	FRLLXX	631 ± 12	FRLKLX	40 ± 0	FRLKFL	72 ± 1
FREXXX	>2,500	FRLEXX	>2,500	FRLKEX	>625	FRLKFE	157 ± 1
FRYXXX	>2,500	FRLYXX	2,003	FRLKYX	160 ± 5	FRLKFY	32 ± 1

[a] All peptide mixtures contain D-amino acids with the carboxyl terminal aminated and the amino group unacetylated.
[b] (±) indicates the average of three replicate evaluations.
[C] Asterisk indicates that these mixtures were not synthesized.
[d] Boxed peptide mixture from each series was chosen to construct the suceeding series. Boxed peptide mixture of the 66-series is the selected hexapeptide 66-10.

Table 2. Minimum inhibitory concentrations (MICs) (μg/ml) of the peptide mixture series constructed to obtain a defined antifungal hexapeptide against *Rhizoctonia solani* (AG-1)

36-series		46-series		56-series		66-series	
Peptide[a]	**MIC**	**Peptide**	**MIC**	**Peptide**	**MIC**	**Peptide**	**MIC**
FRQXXX	>2,500	FRLQXX	1,724	FRLKQX	787 ± 6	FRLKFQ	41 ± 2
FRSXXX	>2,500	FRLSXX	135 ± 6	FRLKSX	>625	FRLKFS	38 ± 2
FRFXXX	135 ± 4[b]	FRLFXX	74 ± 2	FRLKFX	27 ± 2	FRLKFF	75 ± 0
FRAXXX	>2,500	FRLAXX	169 ± 13	FRLKAX	101 ± 13	FRLKFA	37 ± 4
FRNXXX	>2,500	FRLNXX	1,183	FRLKNX	697 ± 33	FRLKFN	71 ± 1
FRWXXX	236 ± 0	FRLWXX	2,225	FRLKWX	51 ± 1	FRLKFW	33 ± 1
FRVXXX	527	FRLVXX	274	FRLKVX	64 ± 29	FRLKFV	21 ± 1
FRMXXX	327 ± 14	FRLMXX	546	FRLKMX	49 ± 2	FRLKFM	38 ± 9
FRPXXX	>2,500	FRLPXX	>2,500	FRLKPX	769 ± 20	FRLKFP	137 ± 6
FRHXXX	>2,500	FRLHXX	73 ± 7	FRLKHX	379 ± 27	FRLKFH	20 ± 3
FRRXXX	>2,500	FRLRXX	69 ± 3	FRLKRX	746 ± 17	FRLKFR	24 ± 2
FRIXXX	139 ± 17	FRLIXX	160 ± 11	FRLKIX	45 ± 3	FRLKFI	25 ± 3
FRDXXX	>2,500	FRLDXX	>2,500	FRLKDX	>625	FRLKFD	148 ± 0
FRCXXX	*[c]	FRLCXX	*	FRLKCX	47 ± 6	FRLKFC	42 ± 3
FRGXXX	>2,500	FRLGXX	>2,500	FRLKGX	350 ± 11	FRLKFG	44 ± 8
FRTXXX	>2,500	FRLTXX	123 ± 0	FRLKTX	370 ± 30	FRLKFT	36 ± 4
FRKXXX	>2,500	FRLKXX	28 ± 5	FRLKKX	844 ± 49	FRLKFK	30 ± 2
FRLXXX	134 ± 12[d]	FRLLXX	138 ± 26	FRLKLX	39 ± 1	FRLKFL	22 ± 2
FREXXX	>2,500	FRLEXX	2,037	FRLKEX	>625	FRLKFE	60 ± 3
FRYXXX	>2,500	FRLYXX	>2,500	FRLKYX	98 ± 2	FRLKFY	98 ± 2

[a] All peptide mixtures contain D-amino acids with the carboxyl terminal aminated and the amino group unacetylated.
[b] (±) indicates the average of three replicate evaluations.
[C] Asterisk indicates that these mixtures were not synthesized.
[d] Boxed peptide mixture from each series was chosen to construct the suceeding series. Boxed peptide mixture of the 66-series is the selected hexapeptide 66-10.

tered the peptide-treated spore and that the fungal structures have lost the defined nucleus and exhibit confluent staining. In companion experiments it was determined that cellular membranes were compromised and the nuclear membranes were also disrupted if the fungal structures were first incubated with 66-10 for 30 min and then stained (data not shown) or if the structures were incubated in the presence of a mixture of 66-10 and Sytox (Figs. 2B and 3B). Fungal structures were also treated with ethanol, washed, incubated with the peptide for 30 min, and then stained with Sytox without incubation, or

Table 3. Minimum inhibitory concentrations (MICs) (μg/ml) of the peptide mixture series constructed to obtain a defined antifungal hexapeptide against *Ceratocystis fagacearum*

36-series		46-series		56-series		66-series	
Peptide[a]	**MIC**	**Peptide**	**MIC**	**Peptide**	**MIC**	**Peptide**	**MIC**
FRQXXX	331	FRLQXX	161	FRLKQX	139 ± 15	FRLKFQ	10 ± 0
FRSXXX	688	FRLSXX	160 ± 4	FRLKSX	>625	FRLKFS	10 ± 1
FRFXXX	63 ± 2[b]	FRLFXX	40 ± 2	FRLKFX	10 ± 0	FRLKFF	5 ± 0
FRAXXX	593	FRLAXX	162 ± 2	FRLKAX	76 ± 2	FRLKFA	10 ± 0
FRNXXX	1,063	FRLNXX	161	FRLKNX	153 ± 3	FRLKFN	20 ± 0
FRWXXX	82 ± 2	FRLWXX	288	FRLKWX	15 ± 0	FRLKFW	9 ± 0
FRVXXX	139	FRLVXX	301	FRLKVX	10 ± 0	FRLKFV	9 ± 1
FRMXXX	154 ± 11	FRLMXX	79	FRLKMX	20 ± 0	FRLKFM	10 ± 0
FRPXXX	1,382	FRLPXX	315	FRLKPX	156 ± 3	FRLKFP	21 ± 0
FRHXXX	534	FRLHXX	65 ± 5	FRLKHX	59 ± 1	FRLKFH	5 ± 0
FRRXXX	335	FRLRXX	67 ± 4	FRLKRX	40 ± 0	FRLKFR	10 ± 0
FRIXXX	79 ± 3	FRLIXX	73 ± 4	FRLKIX	10 ± 0	FRLKFI	5 ± 0
FRDXXX	>2,500	FRLDXX	637	FRLKDX	655 ± 13	FRLKFD	40 ± 1
FRCXXX	*[c]	FRLCXX	*	FRLKCX	20 ± 0	FRLKFC	10 ± 1
FRGXXX	1,146	FRLGXX	484	FRLKGX	133 ± 9	FRLKFG	10 ± 1
FRTXXX	555	FRLTXX	120 ± 0	FRLKTX	152 ± 2	FRLKFT	8 ± 1
FRKXXX	511	FRLKXX	35 ± 3	FRLKKX	78 ± 2	FRLKFK	5 ± 1
FRLXXX	68 ± 9[d]	FRLLXX	63 ± 4	FRLKLX	15 ± 0	FRLKFL	5 ± 1
FREXXX	1,547	FRLEXX	316	FRLKEX	299 ± 10	FRLKFE	10 ± 0
FRYXXX	633	FRLYXX	>2,500	FRLKYX	32 ± 2	FRLKFY	5 ± 0

[a] All peptide mixtures contain D-amino acids with the carboxyl terminal aminated and the amino group unacetylated.
[b] (±) indicates the average of three replicate evaluations.
[C] Asterisk indicates that these mixtures were not synthesized.
[d] Boxed peptide mixture from each series was chosen to construct the suceeding series. Boxed peptide mixture of the 66-series is the selected hexapeptide 66-10.

Table 4. Minimum inhibitory concentrations (MICs) (μg/ml) of the peptide mixture series constructed to obtain a defined antifungal hexapeptide against *Pythium ultimum*

36-series		46-series		56-series		66-series	
Peptide[a]	**MIC**	**Peptide**	**MIC**	**Peptide**	**MIC**	**Peptide**	**MIC**
FRQXXX	1,043	FRLQXX	474	FRLKQX	>625	FRLKFQ	37 ± 6
FRSXXX	1,048	FRLSXX	160 ± 1	FRLKSX	>625	FRLKFS	20 ± 0
FRFXXX	320 ± 13[b]	FRLFXX	161 ± 1	FRLKFX	41 ± 1	FRLKFF	41 ± 1
FRAXXX	1,789	FRLAXX	647 ± 3	FRLKAX	>625	FRLKFA	37 ± 5
FRNXXX	2,069	FRLNXX	654	FRLKNX	>625	FRLKFN	80 ± 1
FRWXXX	659 ± 5	FRLWXX	321	FRLKWX	79 ± 2	FRLKFW	74 ± 15
FRVXXX	268	FRLVXX	32	FRLKVX	79 ± 1	FRLKFV	16 ± 2
FRMXXX	646 ± 2	FRLMXX	342	FRLKMX	81 ± 1	FRLKFM	36 ± 6
FRPXXX	>2,500	FRLPXX	1,003	FRLKPX	>625	FRLKFP	162 ± 0
FRHXXX	1,055	FRLHXX	163 ± 2	FRLKHX	>625	FRLKFH	36 ± 7
FRRXXX	1,051	FRLRXX	163 ± 3	FRLKRX	>625	FRLKFR	36 ± 6
FRIXXX	162 ± 3	FRLIXX	325 ± 4	FRLKIX	42 ± 1	FRLKFI	74 ± 11
FRDXXX	>2,500	FRLDXX	>2,500	FRLKDX	>625	FRLKFD	141 ± 29
FRCXXX	*[c]	FRLCXX	*	FRLKCX	168 ± 1	FRLKFC	37 ± 5
FRGXXX	2,155	FRLGXX	1,334	FRLKGX	>625	FRLKFG	80 ± 0
FRTXXX	2,049	FRLTXX	327 ± 2	FRLKTX	>625	FRLKFT	35 ± 7
FRKXXX	2,065	FRLKXX	81 ± 1	FRLKKX	>625	FRLKFK	17 ± 3
FRLXXX	325 ± 3[d]	FRLLXX	162 ± 3	FRLKLX	82 ± 2	FRLKFL	35 ± 7
FREXXX	>2,500	FRLEXX	700	FRLKEX	>625	FRLKFE	161 ± 1
FRYXXX	>2,500	FRLYXX	2,140	FRLKYX	161 ± 3	FRLKFY	21 ± 1

[a] All peptide mixtures contain D-amino acids with the carboxyl terminal aminated and the amino group unacetylated.
[b] (±) indicates the average of three replicate evaluations.
[C] Asterisk indicates that these mixtures were not synthesized.
[d] Boxed peptide mixture from each series was chosen to construct the suceeding series. Boxed peptide mixture of the 66-series is the selected hexapeptide 66-10.

stained with Sytox and incubated for 30 min. In each treatment the impermeant dye entered the structure through the ethanol-treated (compromised) cytoplasmic membrane and stained the nuclear structure. The microscopic results are identical to those depicted in Figures 2A and 3A. It appears that the peptide is able to disrupt intact cytoplasmic and nuclear membranes, but is unable to act on ethanol-treated membranes. Identical results were obtained by exposing the ethanol-treated structures to a mixture of peptide and Sytox without an incubation period. Untreated spores of *F. oxysporum* f. sp. *lycopersici* and *C. fagacearum* in the presence of only Sytox showed no fluorescence (data not shown).

Figures 2C, 2D, and 2E show *C. fagacearum* spores treated with fluorescent brightener 28 only, ethanol-treated spores in the presence of fluorescent brightener 28, and spores in the presence of fluorescent brightener 28 and hexapeptide 66-10, respectively. Figures 3C, 3D, and 3E show the same treatments, respectively, with *F. oxysporum* f. sp. *lycopersici* microconidia. Since fluorescent brightener 28 stains the 1,4 β-glucans of the cell wall it is evident that the cell wall is not disrupted by treatment with peptide or ethanol. Figures 2F, 3F, and 4A show ethanol-treated-washed mycelial fragments of *C. fagacearum, F. oxysporum* f. sp. *lycopersici,* and *R. solani* (AG-1), respectively, that have been stained with Sytox. As with the spores or microconidia, there are defined, intact nuclei. Figures 2G, 3G, and 4B show mycelial fragments of the respective above-mentioned fungi in the presence of hexapeptide 66-10 and Sytox. As with Figures 2B and 3B, the photographs indicate a loss of the defined, intact nucleus when the fungal structures are treated with 66-10. Figures 2H–J, 3H–J, and 4C–E show *C. fagacearum, F. oxysporum* f. sp. *lycopersici,* and *R. solani* (AG-1), respectively, treated differently and then stained with fluorescent brightener 28. Figures 2H, 3H, and 4C show mycelial fragments in the presence of fluorescent brightener 28 only. Figures 2I, 3I, and 4D show ethanol-treated-washed mycelial fragments in the presence of fluorescent brightener 28. Figures 2J, 3J, and 4E show mycelial fragments in the presence of fluorescent brightener 28 and hexapeptide 66-10. Again, all photographs depict an intact cell wall. Untreated mycelial fragments of *F. oxysporum* f. sp. *lycopersici, C. fagacearum,* and *R. solani* in the presence of Sytox only showed no fluorescence (data not shown).

It was also determined from time course experiments with a 66-10, Sytox, and *C. fagacearum* spore mixture that 62% of the peptide-treated spores showed compromised membranes within 5 min. By 15 min, 99% of the spores present showed confluent fluorescent staining and had lost the defined internal nuclear structure, indicating that a majority of the fungal structures had been compromised. In addition to fluorescent microscopy experiments to determine the time required for 66-10 to act, dilution plating experiments were performed to determine the kill-time of 66-10. Within 15 min, 70% of the compromised *C. fagacearum* spores were incapable of germination on PDA. By 60 min, 88% of the structures had become nonviable.

Table 5. Minimum inhibitory concentrations (MICs) (μg/ml) of peptides PPD1 and 66-10 compared with selected antifungal agents against the designated fungal pathogens[a]

Antifungal agent	*Fusarium oxysporum* f. sp. *lycopersici*	*Rhizoctoni solani*	*Ceratocystis fagacearum*	*Pythium ultimum*
PPD1	66 ± 2	66 ± 2	14 ± 3	55 ± 12
66-10	19 ± 1	20 ± 3	5 ± 0	36 ± 7
MBC[b]	2 ± 0	2.1 ± 0.2	1 ± 0	>2.5
Cycloheximide	38 ± 1	0.7 ± 0.1	0.4 ± 0	6 ± 1
Nystatin[c]	5 ± 0.4	2.1 ± 0	1 ± 0	>100

[a] MICs represent the average of three independent evaluations ± the standard deviations.
[b] Methyl-(butylcarbamoyl)-2-benzimidazole-2yl carbamate.
[c] 1 μg = 5.160 units.

Mutagenic potential of bioactive peptides.

The mutagenic potential of pentapeptide PPD1 and hexapeptide 66-10 was determined with the Ames *Salmonella* plate incorporation assay. Strains TA98 and TA100 were used to evaluate a serial dilution series (300 to 9.37 μg/ml). In two independent assays, the defined peptides PPD1 and 66-10 showed no mutagenic properties at all concentrations tested, with and without metabolic activation. Both TA98 and TA100 cells exposed to the known mutagens 2-nitrofluorene or 2-aminofluorene without activation and benzo(a)pyrene or 2-aminofluorene with activation showed the expected twofold or better increase in the reversion rate over that observed in negative controls. Under conditions of the Ames test there were no indications of a toxic effect to the test strains by either peptide, since the reversion numbers for the controls and all tested concentrations of the peptide were similar.

Hemolytic activity of the defined hexapeptide 66-10.

The hemolytic activity of hexapeptide 66-10, mellitin, and Triton X-100 (0.1%) was determined with human, bovine, and ovine red blood cells (Table 6). The anionic detergent Triton X-100 yielded 100% lysis of all the respective red blood cells. The bee venom, mellitin, a 23-amino acid peptide and a known hemolytic agent, was tested at a concentration range from 100 to 0 μg/ml. Mellitin showed lysis of the tested red blood cells at a concentration as low as 0.1 μg/ml. Hexapeptide 66-10 was tested at a range from 312 to 0 μg/ml and showed no hemolytic activity with the respective red blood cells.

Absorption studies.

To determine if hexapeptide 66-10 inserts reversibly into the fungal membrane, absorption studies were performed with *C. fagacearum* spores. Comparison of the titered control of hexapeptide 66-10 to the experimental sample containing *C. fagacearum* spores showed 7% remaining in the spore pellet. Washing of the spore pellet, however, recovered the amount that was bound. The bound peptide in combination with that found in the supernatant accounted for the total amount that was added. The controls with glass beads and latex polystyrene beads in place of the *C. fagacearum* spores showed no detectable, nonspecific binding of hexapeptide 66-10 to either hydrophobic or hydrophilic surfaces.

DISCUSSION

Evaluation of the synthetic peptide combinatorial libraries identified a biologically active hexapeptide that appears to rapidly compromise the cell membranes of *F. oxysporum* f. sp. *lycopersici, R. solani* (AG-1), *C. fagacearum,* and *P. ultimum,*

and that shows no nonspecific binding, hemolytic, or mutagenic activity. The biological activity of amphipathic α-helical peptides is thought to result from their ability to form ion channels through membrane bilayers (Lear et al. 1988; Anzai et al. 1991) and a length of approximately 20 amino acid residues is necessary to completely span the hydrocarbon portion of the lipid bilayer (Lear et al. 1988). Peptides PPD1 (FRLHF) and 66-10 (FRLKFH) are small, cationic, basic peptides, with hydrophobic backbones that are smaller than known antimicrobial peptides from natural sources (Maloy and Kari 1995; Rao 1995). However, studies show that short, cationic peptides (8 to 12 peptides in length) can form ion

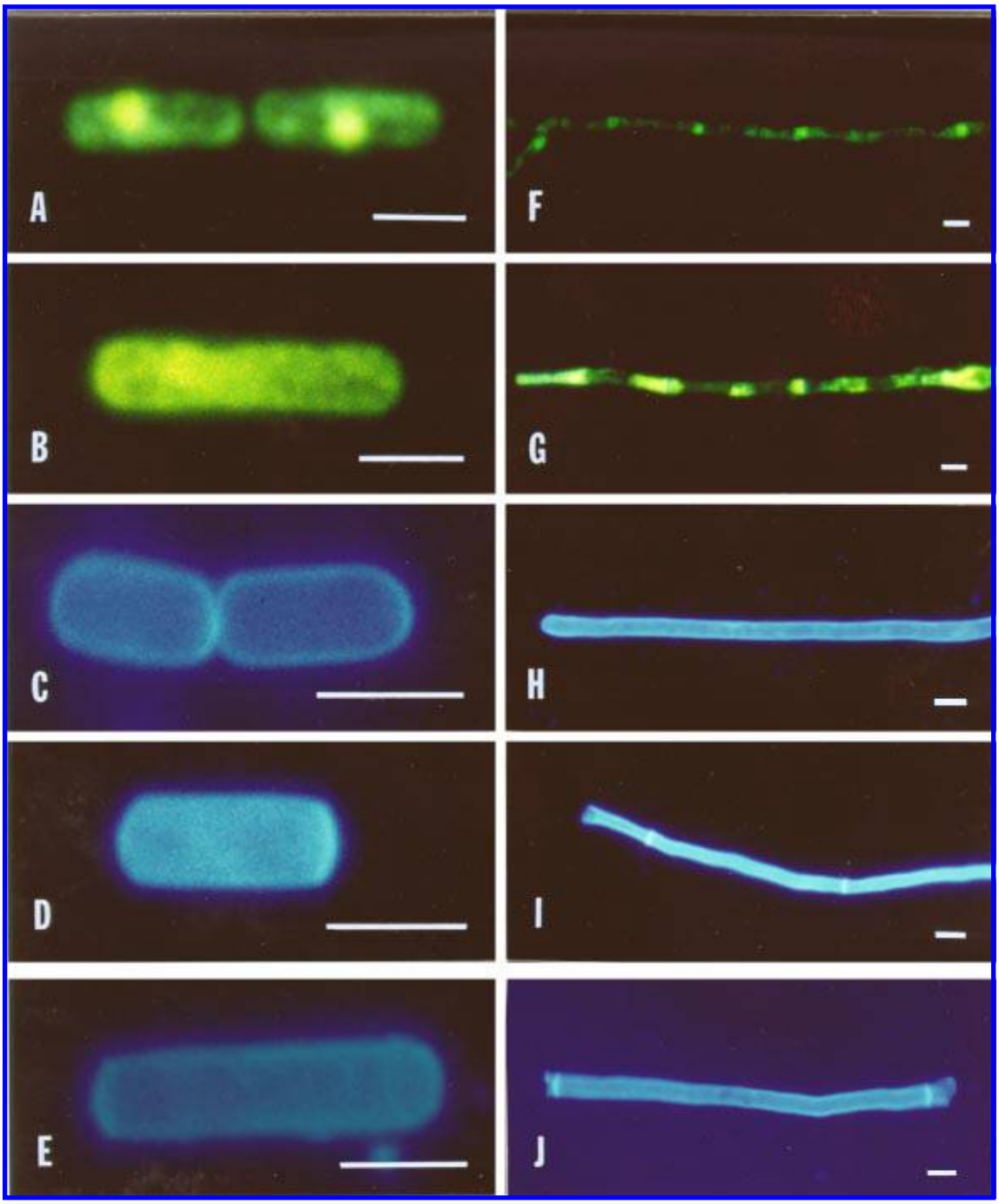

Fig. 2. Fluorescent staining of *Ceratocystis fagacearum* spores and hyphae with the membrane impermeant Sytox Green nucleic acid stain or the cell wall stain fluorescent brightener 28 in the presence or absence of hexapeptide 66-10 or ethanol treatment. **A,** Ethanol-treated spores in the presence of Sytox. **B,** Spores in the presence of Sytox and hexapeptide 66-10. **C,** Spores treated with fluorescent brightener 28 only. **D,** Ethanol-treated spores in the presence of fluorescent brightener 28. **E,** Spores in the presence of fluorescent brightener 28 and hexapeptide 66-10. **F,** Ethanol-treated mycelial fragments in the presence of Sytox. **G,** Mycelial fragments in the presence of Sytox and hexapeptide 66-10. **H,** Mycelial fragments in the presence of fluorescent brightener 28 only. **I,** Ethanol-treated mycelial fragments in the presence of fluorescent brightener 28. **J,** Mycelial fragments in the presence of fluorescent brightener 28 and hexapeptide 66-10. Bars represent 5 μm.

channels (Anzai et al. 1991). Bessalle et al. (1993) reported that short peptides with a high hydrophobic and basic nature have appreciable bioactivity. They stated that, due to their small size, short peptides cannot span the lipid bilayer, and they suggested different mechanisms of action for long-chain versus short-chain peptides. Anzai et al. (1991) and Agawa et al. (1991) postulated oligomeric bundles of head-to-tail dimers of short peptides as a potential explanation for their activity.

The initial interaction between the peptide and the target microorganism is presumably of an electrostatic nature

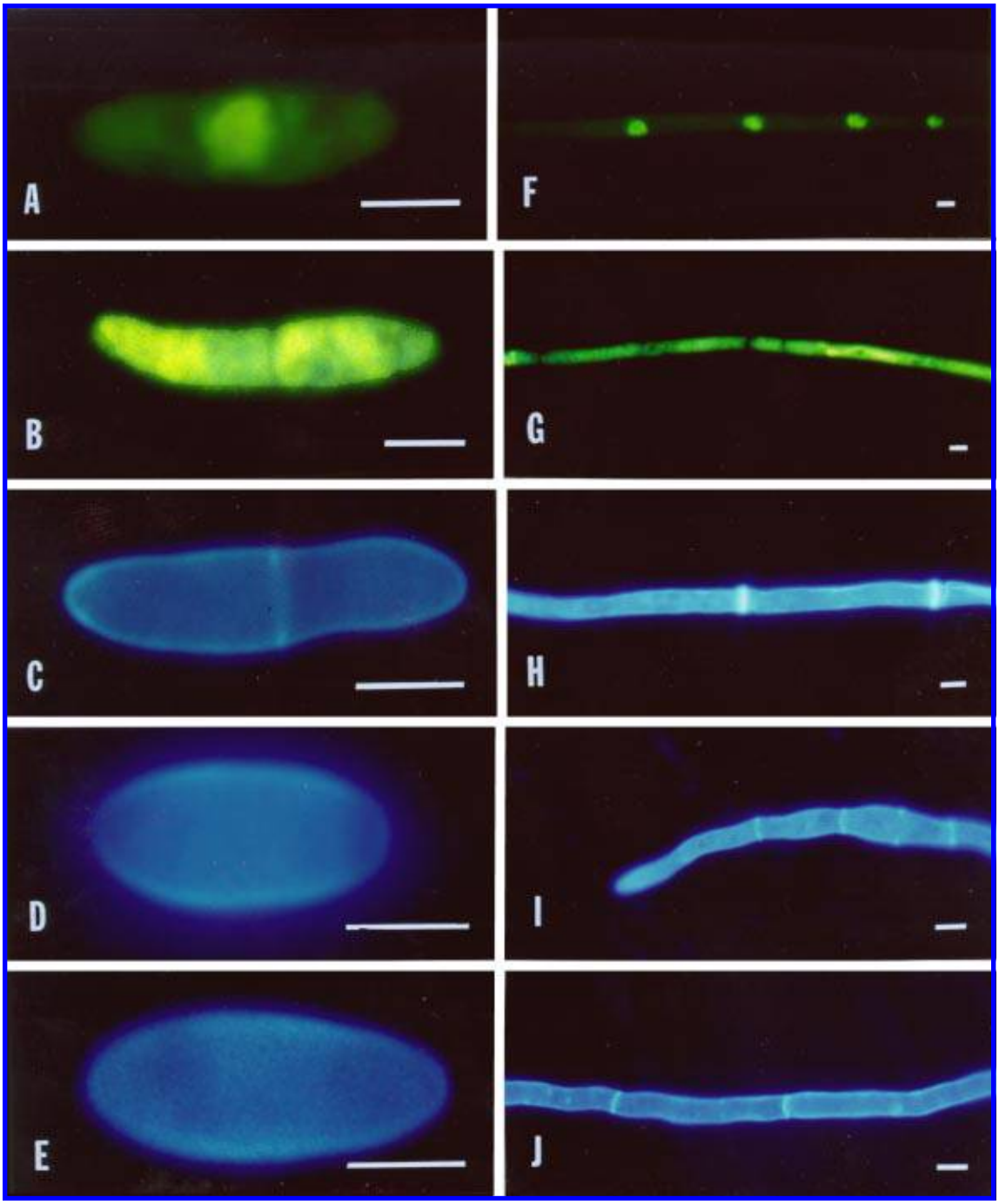

Fig. 3. Fluorescent staining of *Fusarium oxysporum* f. sp. *lycopersici* microconidia and hyphae with the membrane impermeant Sytox Green nucleic acid stain or the cell wall stain fluorescent brightener 28 in the presence or absence of hexapeptide 66-10 or ethanol treatment. **A,** Ethanol-treated microconidia in the presence of Sytox. **B,** Microconidia in the presence of Sytox and hexapeptide 66-10. **C,** Microconidia treated with fluorescent brightener 28 only. **D,** Ethanol-treated microconidia in the presence of fluorescent brightener 28. **E,** Microconidia in the presence of fluorescent brightener 28 and hexapeptide 66-10. **F,** Ethanol-treated mycelial fragments in the presence of Sytox. **G,** Mycelial fragments in the presence of Sytox and hexapeptide 66-10. **H,** Mycelial fragments in the presence of fluorescent brightener 28 only. **I,** Ethanol-treated mycelial fragments in the presence of fluorescent brightener 28. **J,** Mycelial fragments in the presence of fluorescent brightener 28 and hexapeptide 66-10. Bars represent 5 µm.

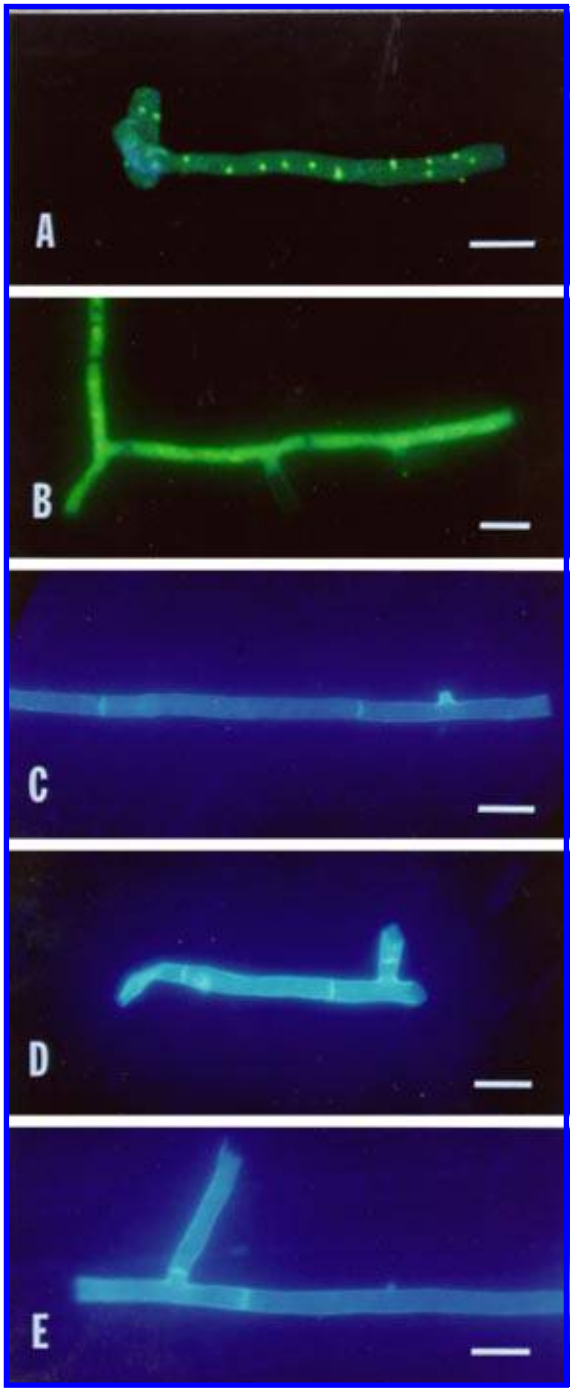

Fig. 4. Fluorescent staining of *Rhizoctonia solani* (AG-1) mycelial fragments with the membrane impermeant Sytox Green nucleic acid stain or the cell wall stain fluorescent brightener 28 in the presence or absence of hexapeptide 66-10 or ethanol treatment. **A,** Ethanol-treated mycelial fragments in the presence of Sytox. **B,** Mycelial fragments in the presence of Sytox and hexapeptide 66-10. **C,** Mycelial fragments treated with fluorescent brightener 28 only. **D,** Ethanol-treated mycelial fragments in the presence of fluorescent brightener 28. **E,** Mycelial fragments in the presence of fluorescent brightener 28 and hexapeptide 66-10. Bars represent 20 µm.

(Bessalle et al. 1993). The pentapeptide PPD1 contains two terminal Phe residues at positions 1 and 5, a Leu residue at position 3, and Arg and His residues at positions 2 and 4, respectively. Similarly, hexapeptide 66-10 also contains Phe residues at positions 1 and 5, a Leu residue at position 3, and Arg, Lys, and His residues at positions 2, 4, and 6, respectively. The developed peptides exhibit a cationic, hydrophobic character that is prevalent in other antimicrobial peptides (Agawa et al. 1991; Bessalle et al. 1993; Blondelle and Houghten 1992; Evans and Harmon 1995). The cationic character of the naturally occurring peptides appears to allow for a strong interaction between the peptide and the negatively charged phospholipids of cell membranes (Agawa et al. 1991), which might also be the operational mechanism for the peptides described herein. Cationicity is conferred to the peptides by the presence of positively charged amino acids arginine, lysine, and/or histidine. Also, the hydrophobic residues Phe and Leu that separate the positively charged residues of the peptides enhance the capabilities of the charged residues to interact with the hydrophobic chains of the phospholipids.

Cecropins B and D, isolated from the pupae of the Chinese oak silk moth (*Antheraea pernyi*) contain 46 and 24%, respectively, of the aforementioned amino acids in their primary sequences (Qu et al. 1982). Forty-eight percent of the primary sequence of magainins I and II contain either Phe, Ile, Leu, His, or Lys, and contribute to their antimicrobial properties (Maloy and Kari 1995). Approximately 50% of the primary sequence of various defensins also consist of these same hydrophobic or cationic amino acids (Ganz and Lehrer 1995). It becomes apparent that a cationic, hydrophobic character is important for expression of significant antimicrobial activity. At the pH used for our in vitro assay (pH 6.0) the His, Lys (only 66-10), and Arg residues are fully protonated (pKa = 6.7, 10, and 12, respectively), thus giving the peptides a positive charge that appears to be associated with the potency of peptides. Powell et al. (1995) reported MICs of 2.5, 15, and 20 µM for *F. oxysporum* f. sp. *lycopersici* with peptides ESF1 (20 residues), magainin II (23 residues), and cecropin B (35 residues), respectively. Although we did not use the same isolate, we observed in replicate experiments an MIC of 22 µM for *F. oxysporum* f. sp. *lycopersici* with peptide 66-10. On a molar basis the activity of the hexapeptide compares well with that of cecropin B, but not with that of ESF1 or magainin II.

Activity of the peptides was directly compared with MBC, cycloheximide, or nystatin, with all antifungals having different modes of action (Jawetz et al. 1982; McGinnis and Rinaldi

Table 6. Hemolytic activity of hexapeptide 66-10, mellitin, and Triton X-100

	Red blood cells		
	Human	**Bovine**	**Ovine**
Compound	**Hemolysis (%)**		
66-10			
312 to 0 µg/ml	0	0	0
Mellitin			
100 to 10 µg/ml	100	100	100
5 µg/ml	98	100	86
1 µg/ml	95	3	7
0.1 µg/ml	11	2	4
0 µg/ml	0	0	0
Triton X-100 (0.1%)	100	100	100

1991; Pinto et al. 1993). *F. oxysporum* f. sp. *lycopersici* and *R. solani* (AG-1) were sensitive to each of the antimicrobials tested. *P. ultimum*, however, was insensitive to MBC and nystatin, as previously documented (Williams and Ayanaba 1975; Kerwin and Duddles 1989; Hancock 1993). Hexapeptide 66-10 was approximately 3 to 10 times lower in activity than the commercial antifungals, with the exception of cycloheximide against *F. oxysporum* f. sp. *lycopersici*. Cycloheximide exhibited 2× lower activity than 66-10 against *F. oxysporum* f. sp. *lycopersici*. Compared with 66-10, PPD1 showed less activity against the test fungi. This may be explained by the fact that 66-10 contains 6 amino acids, whereas PPD1 contains 5 amino acids. Of the test fungi, *C. fagacearum* was the most sensitive to all the antifungals tested, including the synthetic peptides 66-10 and PPD1. This was observed throughout the development of the peptides, and may indicate that *C. fagacearum* may have unique features in the structure of its membrane, making it more susceptible to peptide action.

During the development of new antimicrobials it is important to determine if a compound may possess mutagenic or hemolytic activity. The *Salmonella* plate incorporation assay (Ames et al. 1973), with and without microsomal activation, demonstrated that neither PPD1 nor 66-10 possessed mutagenic properties at a concentration as high as 300 μg/ml based on the twofold rule of Chu et al. (1981). In a study to determine the potential for the formation of ion channels through cell membranes by an amphipathic peptide, Blondelle and Houghten (1992) evaluated the peptide AC-LKLLKKLLKKL KKLLKKL-NH_2 through the preparation of analogs that varied in length from 8 to 22 residues while maintaining amphipathicity. They found that hemolytic activity was more sequence dependent than lysis of bacterial cells and suggested that two different mechanisms of action were involved in the lysis of bacteria and red blood cells. Stronger hydrophobic interactions than that necessary for antimicrobial activity were shown to be necessary for the lysis of red blood cells. Additionally, they found that as they reduced the peptide length while maintaining amphipathicity, hemolytic activity was reduced and antimicrobial activity varied with the bacterial isolate. They observed no hemolytic activity with peptides smaller than 12 residues. This was consistent with the lack of hemolytic activity observed with 66-10 at concentrations up to 312 μg/ml. These two tests are critical first steps in the development of a chemical that could be introduced into the environment.

The fluorescent microscopy experiments with the membrane impermeant dye Sytox strongly suggest that 66-10 compromises the integrity of the cell membrane and, in addition, affects the nuclear membrane (Figures 2B and G, 3B and G, and 4B). It is also evident that the ethanol-treated structures are infiltrated by Sytox, but that the nuclear structure is not disrupted (Figures 2A and F, 3A and F, and 4A). This indicates that an intact or biologically active membrane may be necessary for the peptide to act. The ethanol treatment acts to compromise the membrane in a manner (dehydration) that makes it no longer susceptible to peptide action. The highly cationic, hydrophobic peptide, 66-10, is certainly not long enough to span the membrane. However, its structure might allow for a limited α-helical formation. Although we can only speculate on the exact mode of action for the peptide, we know that the interaction takes place in a manner that results in 62 and 99% of treated *C. fagacearum* spores having a compromised membrane in 5 and 15 min, respectively. This, along with a 70% kill rate within 15 min of exposure of spores to the peptide, illustrates the rapid manner in which the peptide acts. The rapid effect observed is consistent with an interaction of the peptide and the membrane phospholipids, as discussed above.

Combinatorial chemistry offers a systematic method for the development of antimicrobial peptides that show high broad-spectrum activity against an array of microorganisms. The observed high broad-spectrum activity of the identified small peptides warrants further investigation to elucidate possible mechanisms of action and alternate expression systems.

MATERIALS AND METHODS

Fungal strains and media used in the study.

The fungal strains used in this study were *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *lycopersici* (Sacc.) W. C. Snyder & H. N. Hans., race 1, strain RM1, obtained from R. D. Martyn, Texas A&M University (TAMU) (FOLRM-1); *Rhizoctonia solani* Kühn strain TM-101, anastomosis group 1, obtained from M. A. Marchetti, USDA-Beaumont, TX (RS101); *Rhizoctonia solani*, anastomosis group 4, obtained from J. Johnk, TAMU Research Center, Dallas, TX; *Pythium ultimum* Trow strain LB-1, isolated from cotton, obtained from L. Barnes, TAMU (PULB-1); *Ceratocystis fagacearum* (T. W. Bretz) J. Hunt strain BAN 102 (CFBAN-102), and *Ophiostoma ulmi* (Buisman) Nannf. (OU-1) obtained from D. N. Appel, TAMU. All cultures were routinely transferred to potato dextrose agar (PDA) (Difco, Detroit, MI) plates. Potato dextrose broth (PDB) (Difco) was used in all microtiter plate studies. Clarified V8-cholesterol broth, as described by Ayers and Lumsden (1975), was used for oospore production by *P. ultimum*. A mineral salts medium (FLC), as described by Esposito and Fletcher (1961), was used for microconidia production by *F. oxysporum* f. sp. *lycopersici.*

Growth and storage conditions for stock cultures and production of inoculum for bioassays.

All fungal cultures were grown at 25°C and routinely maintained on PDA plates. The plates were stored at 4°C. *R. solani* and *P. ultimum* were maintained as mycelial cultures on PDA. *F. oxysporum* f. sp. *lycopersici* was stored as microconidia that were produced in FLC medium. Microconidia were produced as follows with the FLC medium. A 4-mm square of an actively growing culture was taken from PDA to 50 ml of FLC broth in a sterile, 250-ml, screw-cap flask. The culture was incubated with shaking (200 rpm; model 4710, Queue Systems, Parkersburg, WV) at 25°C. After 96 h, the slurry of mycelia and microconidia was filtered twice through 3 layers of sterile cheesecloth to obtain the microconidial suspension. The microconidial suspension was adjusted to a concentration of 1 to 5 × 10^7 microconidia/ml with a Brightline hemacytometer (Hausser Scientific, Horsham, PA) and was frozen in 1-ml aliquots in 20% sterile glycerol at –80°C. *C. fagacearum* spores were harvested from PDA plates that were incubated at 25°C for 8 days. The spores were harvested in sterile PDB, filtered, adjusted to a concentration of 1 to 5 × 10^7 spores per ml, and stored as described above.

Frozen glycerol stocks of *F. oxysporum* f. sp. *lycopersici* microconidia and *C. fagacearum* spores were used for the bioassays. Microconidia or spores were thawed on ice, washed 2× in PDB, and diluted in PDB to obtain the appropriate inoculum concentration for the assay. Inoculum of *R. solani* consisted of mycelial fragments that were prepared from an actively growing broth culture. To obtain these fragments, a single sclerotium from an actively growing culture was inoculated to 50 ml of sterile PDB in a 250-ml, sterile, screw-cap, Erlenmeyer flask and incubated with shaking (125 rpm; model 4710, Queue Systems, Parkersburg, WV) at 25°C. After 4 days, the mycelial mass had grown to approximately 2 cm in diameter and was divided in the flask by a sterile glass pipette. One half was removed and placed into a sterile tissue grinder (#7725-19, Corning Glass Works, Scientific Products Division, Corning, NY). The mycelial mass was macerated in PDB (5 ml) on ice to obtain fragments with an average size of 52 µm (average of 100 mycelial fragments). The mycelial fragment suspension was calibrated with a hemacytometer and adjusted to 3 to 5×10^4 mycelial fragments per ml of PDB for the bioassay experiments. Inoculum of *P. ultimum* consisted of oospores produced from an actively growing culture. A PDA plug of an actively growing culture of *P. ultimum* was inoculated to 20 ml of sterile V8-cholesterol broth (Ayers and Lumsden 1975) in sterile, glass, petri dishes and incubated at 21°C. After 10 days, the mycelial mat was washed 2 times in sterile, deionized water. The mycelial mat was divided in half and each half was macerated with a hand-held homogenizer as described for *R. solani*. After maceration, the suspension of oospores was filtered through 3 layers of sterile cheesecloth. The oospore suspension was calibrated with a hemacytometer and diluted to the proper inoculum concentration with sterile PDB. An aliquot of the prepared microconidial, spore, mycelial fragment, or oospore suspension was streaked to a tryptone nutrient agar (Olsen and Hansen 1976) plate. The plates were incubated at 25°C for 24 h to verify that the suspensions were bacteria-free.

Determination of optimum inoculum concentration and incubation conditions for bioassay.

All of the fungal isolates were inoculated to sterile, disposable, flat-bottom, polystyrene, 96-well (with lid), microtiter plates (Corning Glass Works) for growth studies in order to determine the optimum conditions for the bioassay. Parameters such as optimal inoculum concentration, temperature, length, and method of incubation were determined to obtain optical density readings over the growth period that was reflective of balanced growth conditions. Balanced growth conditions were defined as those conditions that allowed for a short lag phase, an exponential phase, and a stationary phase of growth.

F. oxysporum f. sp. *lycopersici* microconidia and spores from *C. fagacearum* were inoculated to 96-well microtiter plates. Frozen stocks of the microconidia or spores were removed from the –80°C freezer and allowed to thaw on ice. Each suspension was washed 2× in PDB and then diluted to obtain an inoculum concentration in the range of 1 to 7×10^5 microconidia or spores per ml. The inoculum was stored on ice until needed. The microtiter plates were aliquoted with 50 µl of PDB per well and 50 µl of the microconidia or spore inoculum per well. Wells A1 to A3 (media control wells) received 100 µl of PDB. All of the microtiter plates were incubated both with and without shaking, in the dark, at 25°C, for up to 96 h. To follow growth in the microtiter plates, an Emax Precision Microplate Reader (Molecular Devices, Menlo Park, CA) attached to a Think-Jet printer (Hewlett-Packard, Vancouver, WA) was used to obtain the absorbance (at 595 nm) readings, which were recorded at 24-h intervals.

A mycelial fragment suspension of *R. solani* was prepared as previously described. PDB was aliquoted into microtiter wells, as described above, that were individually inoculated with 50 µl of the respective mycelial suspensions of 3×10^4 or 3×10^5 mycelial fragments per ml. All microtiter plates were incubated with and without shaking, at 25°C, in the dark, for 96 h. Absorbance readings were recorded on a 24-h regime.

For *P. ultimum* growth studies, an oospore suspension was obtained as described. The stocks were diluted in PDB to obtain a final concentration of 6.6×10^4 or 3.4×10^5 oospores per ml. Microtiter plates were aliquoted with 50 µl of PDB and then inoculated with 50 µl per well of the respective oospore suspensions. All microtiter plates were incubated with and without shaking, in the dark, at 25°C, for 72 h. Absorbance readings were recorded.

Once growth conditions were determined, absorbance versus dry weight correlations were resolved for each of the fungal isolates. Briefly, all microtiter plates were inoculated as described earlier and incubated without shaking. At each time interval, the contents of 22 wells were collected and combined to form one sample. The sample was vacuum filtered through a pre-weighed, 0.45-µm, glass filter (Millipore, Bedford, CT). The filter was dried at 15 in. Hg (1 in. = 2.54 cm), in a 70°C Thelco vacuum oven (Precision Scientific, Chicago, IL) to constant weight. The dried filters were allowed to come to room temperature in a vacuum desiccator. The tared weight of the filter was subtracted from the sample-filter weight to determine mycelial mass dry weight. At each time interval, the dry weight was calculated as mg per well. Absorbance readings, for each of the 22 wells collected at each time interval, were averaged.

Development of a defined antimicrobial hexapeptide.

The starting peptide mixture library, designated 26-series, was synthesized by Houghten Laboratories (Torrey Pines Institute for Molecular Studies, San Diego, CA), using a process described by Houghten et. al. (1992). The 26-series peptide library was prepared with methylbenzhydrylamine polystyrene resin and standard t-Boc chemistry in combination with simultaneous multiple peptide synthesis. Each mixture in the 26-series library consists of six positions. The first two positions each consist of individual D-amino acids, while the remaining four positions each consist of an equimolar mixture of 19 D-amino acids. Cysteine was omitted from the mixture position because a reducing agent is needed and it is difficult to define disulfide aggregates. The peptide mixture libraries employed in the iterative steps, following screening of the 26-series, were synthesized in a similar manner by Quality Controlled Biochemicals (Hopkinton, MA). The designated amino acids for each series were based on the bioassay results of the previous series.

The antimicrobial activity of the initial 400 peptide mixture library (26-series) and those libraries involved in the iterative steps was determined with a 96-well microtiter plate bioassay

under established conditions. *F. oxysporum* f. sp. *lycopersici* and *R. solani* were used to screen the entire 26-series. All four fungal isolates were used to retest selected 26-series peptide mixtures and peptide mixtures involved in the iterative steps. Fifty microliters of 1× PDB was added to all wells of a 96-well microtiter plate, except A1 to A3 and B1 to H1. Wells A1 to A3 (media control) received 100 µl of 1× PDB to function as media control wells. Wells B1 to H1 received 50 µl of 2× PDB. Wells A4 to A12 were designated growth control and received no test compound. The individual aqueous peptides or peptide mixtures were added to each of wells B1 to H1 in 50-µl aliquots and a twofold serial dilution was carried out across each row with a starting concentration of either 2,500, 1,250, or 625 µg/ml and an ending concentration of either 1.25, 0.625, or 0.313 µg/ml, respectively. Once the individual peptide mixtures had been diluted across each row, the microtiter plate (excluding wells A1 to A3) was overlaid with 50 µl of the selected fungal inoculum. The inoculum was prepared as described earlier. Absorbance readings (at 595 nm) were taken at 0, 24, 48, and 72 h for *F. oxysporum* f. sp. *lycopersici*, *R. solani*, and *P. ultimum*, with an additional reading at 96 h for *C. fagacearum*.

After the 26-series peptide mixture library, tested in a single dilution series, was screened for antimicrobial activity, an iterative process was employed to develop the defined antimicrobial hexapeptide. The starting library contained individual mixtures with a sequence of O_1O_2XXXX. Positions O_1 and O_2 were specifically defined amino acids and each X position represented an equimolar mixture of 19 amino acids (cysteine omitted). The 400 mixtures resulted from all possible combinations involving positions O_1 and O_2 (i.e., $20^2 = 400$). The 26-series peptide mixture that exhibited the highest broad-spectrum activity for the microorganisms tested was chosen to construct the 36-series consisting of positions $O_1O_2O_3XXX$. Positions O_1 and O_2 were those determined in the 26-series peptide mixture and position O_3 consisted of one of 19 D-amino acids (cysteine omitted). Each X position represented an equimolar mixture of 19 amino acids (cysteine omitted). Following screening of the 36-series, the peptide mixture that exhibited the highest broad-spectrum activity was chosen to construct the 46-series consisting of positions $O_1O_2O_3O_4XX$. The 56-series and 66-series with positions $O_1O_2O_3O_4O_5X$ and $O_1O_2O_3O_4O_5O_6$ defined were synthesized by following the same strategy.

Analysis of data.

For each peptide mixture, the data was expressed as a minimum inhibitory concentration (MIC). The MIC was defined as the lowest concentration of the test peptide at which there was no growth. Some data were expressed as a 50% inhibitory concentration (IC_{50}). The IC_{50} was defined as that concentration of the test peptide or peptide mixture that inhibited 50% of growth. To obtain the MIC and IC_{50}, absorbance readings from the final time interval were used to calculate the percent inhibition of growth. For all microtiter plates, absorbance values of nine control wells (A4 to A12) were averaged. The average absorbance value obtained for wells A4 to A12 was individually divided into the absorbance values obtained for the dilution series of a particular peptide or peptide mixture. Then, each value was subtracted from 100 to acquire the percent inhibition. By using percent inhibition as *x* values and peptide concentration as *y* values, linear regression analysis was performed on each peptide mixture. The *y* intercept, *n*, correlation coefficient, MIC, and IC_{50} were calculated for each peptide or peptide mixture. The MIC or IC_{50} data were expressed as the mean of three replicates ± the standard deviation, where replicates were performed.

Comparison of defined hexapeptide to antifungal agents.

The defined hexapeptide, designated 66-10, and pentapeptide, designated PPD1, were compared with cycloheximide (Sigma Chemical, St. Louis, MO), nystatin (Sigma), and MBC (methyl-(butylcarbamoyl)-2-benzimidazole-2yl carbamate; 99.6% active; Dupont De Nemours, Wilmington, DE) for antifungal activity. These studies were performed, in triplicate, for all fungal isolates, using the protocol as described for the peptide assays. All fungal isolates were exposed to MBC at a concentration range of 2.5 to 0.0012 µg/ml. *F. oxysporum* f. sp. *lycopersici*, *R. solani*, and *C. fagacearum* were tested for growth inhibition by nystatin at a concentration range of 50 to 0.025 µg/ml, whereas *P. ultimum* was tested at a concentration range of 100 to 0.050 µg/ml. *F. oxysporum* f. sp. *lycopersici*, *R. solani*, and *P. ultimum* were tested against cycloheximide at a concentration range of 50 to 0.006 µg/ml, and *C. fagacearum* was tested at a concentration range of 250 to 0.061 µg/ml. MICs were calculated as previously described.

Determination of potential target site of hexapeptide 66-10.

Spores and mycelial fragments from *F. oxysporum* f. sp. *lycopersici* and *C. fagacearum*, and mycelial fragments from *R. solani*, were used to determine the potential target of 66-10. The fluorescent dyes Sytox Green, a membrane impermeant nucleic acid stain (Molecular Probes, Eugene, OR), or fluorescent brightener 28 (Sigma), which stains the β-1,4 glucans of the cell wall (Springer and Yanofsky 1989), were used in the study at final concentrations of 1 nM and 4 µg/ml, respectively. With non-ethanol-treated fungal structures (spores, microconidia, or mycelial fragments), 66-10 (final concentration of 50 µg/ml) and Sytox were mixed with the respective fungal structures and incubated at 25°C for 30 min. In a different regime, 66-10 was first incubated with the fungal structures for 30 min and then Sytox dye was added without an incubation period. The fungal structures were also incubated with 66-10 in the presence of fluorescent brightener 28 for 30 min to determine what effect 66-10 would have on cell wall integrity. To obtain compromised membranes under control conditions, the fungal structures were incubated in 70% ethanol for 10 min at 25°C (Springer and Yanofsky 1989) and then washed 2× in PDB. The fungal structures were then stained with Sytox or exposed to a mixture of Sytox and 66-10 without incubation. The structures were also incubated with 66-10 for 30 min and then stained with Sytox and observed immediately or incubated with 66-10 for 30 min and stained with Sytox for 30 min before observation. The ethanol-treated fungal structures were also incubated for 30 min in the presence of fluorescent brightener 28 to observe any effects on cell wall integrity. Other controls consisted of a mixture of each fluorescent dye with each fungal structure from the respective fungal isolates without 66-10 or ethanol treatment. The stained structures were examined with an Olympus microscope (model BH-2) equipped with an Olympus fluorescent microscopy attachment

(model BH2-RFC) and an Olympus automatic photomicrographic system (model PM-10ADS) with an Olympus 35 mm camera (model C-35AD-4) (Olympus, Lake Success, NY). Kodak Ektachrome P1600 film (EPH 135-36) was used to capture the images. A time course study with *C. fagacearum* spores, 66-10, and Sytox was used to resolve the exposure time necessary for the peptide to compromise fungal membranes. A series of microfuge tubes (1.5 ml) were set up to contain 500 µl of a 1×10^6 spores per ml suspension and Sytox (1nM). The peptide was then added to individual tubes, mixed, and allowed to incubate for time intervals of 0, 5, 10, 15, and 30 min before being observed by fluorescent microscopy. Each time interval was performed in duplicate in three independent experiments. The ratio of fluorescent spores to nonfluorescent spores was used to determine the percentage of compromised spores present in the observed field. To determine the kill-time for *C. fagacearum* spores with 66-10, a time course study was performed. Spores (5×10^6) were incubated with 66-10 (10 µg/ml) in a shaking water bath (25°C) for time intervals of 0, 5, 10, 15, 30, 45, and 60 min. After the respective time intervals had expired, the tubes were removed from the water bath and a dilution plating was performed on PDA. After 4 days at 25°C, the number of CFU was obtained. Each time interval exposure and plating was performed in triplicate. The percent difference between the number of CFU for the peptide-treated spore platings and for the control platings was used to calculate the percent kill at each time interval.

Mutagenic potential of bioactive peptides.

The mutagenic potential of the defined hexapeptide and pentapeptide was evaluated by a *Salmonella* plate incorporation assay as described by Ames et al. (Ames et al. 1973). Hexapeptide 66-10 and pentapeptide PPD1 were tested at concentrations of 300 to 9.37 µg/ml in a serial dilution series. Two *Salmonella typhimurium* tester strains (Ames et al. 1973) were used in the assay, TA98 (hisD3052, rfa, ΔuvrB, pKM101) or TA100 (hisG46, rfa, ΔuvrB, pKM101). This assay was completed with and without metabolic activation. Metabolic activation was accomplished with Aroclor-1254–induced S9 rat liver microsomes (Microbiological Associates, Rockville, MD). Two independent assays were completed for each bacterial test strain. All assays were performed in triplicate at the designated peptide concentrations. Both negative and positive controls were included in all the assays. Dimethyl sulfoxide (DMSO) (Sigma) served as the negative solvent control in all assays. The compounds benzo(a)pyrene (0.2 mg/ml in DMSO) and 2-nitrofluorene (0.5 mg/ml in DMSO) served as positive controls with and without metabolic activation, respectively, for peptide PPD1 testing. The compound 2-aminofluorene (0.5 mg/ml in DMSO) (Sigma) served as the positive control with and without metabolic activation for 66-10 testing. A test was considered positive if the bacterial counts were increased twofold over the negative control at two consecutive doses (Chu et al. 1981).

Hemolytic activity of the defined hexapeptide 66-10.

The potential hemolytic activity of the defined hexapeptide 66-10 was established with human, bovine, and ovine red blood cells. Each of the respective red blood cells was isolated by centrifugation at 3,024 × *g* for 5 min at 5°C. Following isolation, the cells were washed 3× in phosphate buffered saline (PBS) (10 mM Na_2PO_4, 0.9% NaCl, pH = 7.4) and diluted to a final optical density of 1.200 at 600 nm. The hexapeptide 66-10 (suspended in sterile, distilled, deionized H_2O) was diluted in 2× PBS to obtain a stock suspension of 624 µg/ml in 1× PDB. Peptide concentrations tested ranged from 312 to 0 µg/ml in repeated twofold dilutions with 0.5-ml, sterile, microfuge tubes (final volume of 100 µl per tube). Following the dilution series, 100 µl of each of the respective red blood cells was added to each tube and the tubes were incubated in a 37°C water bath. After 1 h, the tubes were centrifuged (13,000 × *g*) for 5 min. The supernatant was removed from the tubes and absorbance readings (at 414 nm) were recorded on a Beckman DU-64 spectrophotometer (Beckman Instruments, Fullerton, CA). A 0.1% solution of the anionic detergent Triton X-100 (Sigma) or a concentration range of mellitin (Sigma) served as the positive (hemolytic) control. The negative (nonhemolytic) control consisted of only PBS and red blood cells. Each of the experiments was performed in duplicate.

Absorption studies.

To determine if hexapeptide 66-10 inserted reversibly into the fungal membrane, absorption studies were performed with *C. fagacearum* spores. *C. fagacearum* spores (1×10^6 spores per ml) were incubated in the presence of 66-10 (100 µg/ml) at 25°C. After 1 h, the spores were centrifuged (13,000 × *g*) and the supernatant (50 µl) was titered against *C. fagacearum* with the protocol for peptide testing as described. The pellet was washed 2× with 25 µl of PDB and the combined supernatant was titered against *C. fagacearum.* Inert, acid-washed glass beads (1×10^4 beads per ml) (Sigma; mean diameter 106 µm) having a hydrophilic surface, or latex polystyrene beads (6×10^6 beads per ml) (Sigma; mean diameter 3 µm) having a hydrophobic surface, were also incorporated in place of *C. fagacearum* spores to determine nonspecific binding. Supernatants were assayed as above for activity.

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