Supporting Information

Antibacterial lipo-random peptide mixtures exhibit high selectivity and synergistic interactions

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I. Experimental Procedures

Bacterial strains and growth conditions

The bacterial strains used in this study are detailed in Table S1. Unless stated otherwise, all strains were grown on nutrient agar (NA, Difco) or nutrient broth (NB, Difco), except *Pst* that was grown on Kings B agar plates[1] and NB. All strains were grown at 28 °C for 48 h. All bacterial cells used in this study were stored in 25% glycerol at -80 °C.

Synthesis of, random peptide mixtures (RPMs), lipo-RPMs and sequence-specific lipopeptides

All peptides were synthesized in SPE polypropylene single-Fritted tubes (Altech), on Rink amid resin (0.6 mmol/gr substitution, 0.1 mmol scale) using microwave irradiation (MARS: CEM, USA). Random peptide mixtures (RPMs) were synthesized according to Hayouka et al [2] using a modification of the solid phase peptide synthesis. Briefly, each coupling step was conducted with binary combinations of protected amino acids, with a freshly prepared stock solution containing the protected amino acids in the desired molar ratio between L-phenylalanine and D-lysine (2 equivalents from each amino acid, 0.2 mmol). Sequence specific peptides were synthesized according to the Fmoc solid-phase peptide synthesis method. The coupling reactions were conducted with 4 equivalents (0.4 mmol) of the needed pure protected amino acid. For generation of lipo-RPMs and sequence-specific lipopeptides, acylation was made by bounding palmitic acid to the N-terminus of the desired peptide/RPM using the same Fmoc chemistry of sequence-specific peptides, with the difference that overnight shaking at room temperature was used instead of microwave irradiation. Upon synthesis completion, RPMs/lipopeptides were cleaved from the resin [(95 % trifluoroacetic acid (TFA), 2.5 % water, 2.5 % triisopropylsilane (TIPS)], re-suspended in double distilled water (DDW), frozen and lyophilized. Evaluation of molecular weight was done using MALDI-TOF and amino acid content was quantified by amino acid analysis (Fig. S4) [2].

Purification of lipopeptides

Sequence-specific lipopeptides were dissolved in 20% acetonitrile in DDW or in DMSO and filtered before injection to reversed-phase high-performance liquid chromatography (RP-HPLC, Shimadzu) on a C18 column (Phenomenex 250 X 10.0 mm, 5 μm). Acetonitrile (Bio Lab) and 0.1% trifluoroacetic acid (Bio Lab) in DDW were used as solvents. All lipopeptides could be purified using this method except lipopeptide #32. Minimal inhibitory concentration (MIC) assays with this peptide were performed with its crude preparation. The molecular weight of the purified lipopeptides was validated by MALDI-TOF (see Table S2 for expected and observed MWs of individual lipopeptides after purification), then the lipopeptides were re-suspended in DDW, frozen and lyophilized.

Minimal inhibitory concentration (MIC) assay

MIC values were determined by broth microdilution in sterile 96-flat bottom well plates (Corning 3650) as described by Hayouka et al[2]. Briefly, *Xcc* cells were grown for 24 h in NB at 28 °C, with shaking (180 rpm) and re-suspended in fresh NB to reach an OD_{600nm} of 0.1 (~10^8 CFU/ml) that was measured using a Genesys 10uv spectrophotometer (ThermoSpectronic). One hundred microliter-aliquots of *Xcc* cells were added to 100 µl of NB medium containing peptides/lipopeptides at various concentrations. Bacterial growth was determined after incubation of 24 h at 28 °C by measurement of the optical density (OD_{595nm}), using a Tecan infinite Pro Plate reader. MIC values were determined as the lowest concentrations that showed significant growth inhibition [3]. The highest concentration tested was 200 µg/ml. Each experiment was carried out at least three times, with three replicates per strain/concentration combination in each experiment.

Bactericidal activity assays

Xcc bacterial cells were grown for 24 h in NB at 28 °C, with shaking (180 rpm). Then the cells were washed with sterile phosphate buffered saline (PBS, 8 g/l NaCl, 0.2 g/l KCl, 0.6 g/l Na_2HPO_4, 0.2 g/l KH_2PO_4, pH 7.4) by centrifugation (3 x 2 min, 4722 g) and re-suspended in fresh PBS to OD_{600nm} of 0.1 (~10^8 CFU/ml). Bacterial cells were then incubated (28 °C, 180 rpm) with 25 µg/ml of the desired peptide/lipopeptide for different times, serially diluted in PBS and plated on NA. The plates were incubated at 28 °C for 48 h and bacterial colonies were counted to quantify the CFU/ml. The lower limit of sensitivity of colony counts was 10 CFU/ml. Each experiment was carried out three times, with two replicates per treatment in each experiment. Data were statistically analysed by non parametric comparison for all pairs using Dunn method for joint ranking using JMP Pro 12 (SAS Institute).

In planta assays

We assessed the ability of p-FdK5 to reduce disease severity using the tomato-*Xanthomonas perforans*-pathosystem (bacterial spot disease). The experiments were carried out as described [4]. Briefly, tomato (*Solanum lycopersicum*) cv. FA-144 plants were used for inoculation after they developed four fully expanded leaves (~4 weeks after transplant). The five most external leaflets of the 3 youngest fully expanded leaves were pre-treated on both sides with solutions containing p-FdK5 (200 µg/ml), Kocide® 2000 (2500 µg/ml; DuPont) or sterilized DDW (control). Twenty-four hours later each leaflet was sprayed on both sides with a bacterial suspension containing ~5x10^8 CFU/ml of *X. perforans* 97-2 (OD_{600} of 0.2). Inoculated plants were covered with a plastic bag for 24 h to promote disease. Disease

severity in each leaflet was determined every two to three days after appearance of disease symptoms [~5 to 6 days post inoculation (dpi)], using a 0-6 scale [4]. Assessment of phytotoxic effects on treated leaves was done by following appearance of symptoms in plants that were not inoculated with bacteria, but rather sprayed with sterile DDW. Three experiments were carried out. In each experiment, each treatment was applied on 5 plants, 3 leaves per plant, 5 leaflets per leaf. Data were statistically analysed by ANCOVA (dpi as covariant) using JMP Pro 15 (SAS Institute).

Cytotoxicity towards mammalian cells

HEK293T (ATCC number CRL-11268) cells were grown at 37 °C in a humidified atmosphere with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Sigma) with 2 mM L-glutamine, 1% (v/v) Penstrep and 20% (v/v) fetal bovine serum (Biological Industries). The cytotoxic potential of RPMs/lipo-RPMs was determined using the MTT assay, as described [4]. Briefly, 100 µl aliquots of $5x10^5$ viable cells/ml were transferred to wells in a 96-well microplate (Nunc, Thermo Scientific) and incubated at 37 °C. After 24 h the cells were washed, re-suspended in DMEM without phenol and incubated with tested RPMs/lipo-RPMs at two-fold serial dilutions, Tween or Triton-X (as positive controls), and PBS or DMEM (as negative controls). After incubation of 24 h, the cells were washed by removing the media by suction and replacing it with a fresh media containing 50 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) solution in culture media (0.5 mg/ml) and left for incubation for 90 min. The media were removed by suction, then 100 µl of dimethyl sulphoxide (DMSO) were added to each well. Plates were incubated in dark conditions for 10 min at 37 °C and optical density (OD_{595nm}) was measured using a Tecan Infinite Pro plate reader. The percentage of viable cells was calculated by comparison of treated cells to control cells. Experiments were carried out three times, with four replicates per RPM/lipo-RPMs concentration in each experiment. A t-test (alpha = 0.05) was performed for each treatment in the highest concentration tested as compared with the negative controls in t-tests within each independent experiment.

Synergy assessment using the checkerboard assay

The checkboard assay was used to assess possible synergistic interactions between sequence-specific lipopeptides. *Xcc* cells were grown for 24 h in NB at 28 °C with shaking (180 rpm) and re-suspended in fresh NB to reach an OD_{600nm} of 0.1 (~10^8 CFU/ml). MIC assays were conducted in sterile 96-well flat bottom well plates (Corning 3650) containing two-fold dilutions of the two tested lipopeptides combined in different concentrations starting from 4 x MIC values (columns 1-6). For each tested lipopeptide, two columns containing two-fold serial dilutions of the tested lipopeptide alone, starting at concentrations of 4 x MIC were used to evaluate the MIC (columns 7-10), while the remaining columns were used as positive control (wells containing bacteria with NB without tested lipopeptides) and negative control (wells containing NB without bacteria, columns 11 and 12, respectively, Fig. S6). The plates were incubated for 24 h at 28 °C and bacterial growth was determined by measurement of optical

density (OD_{595nm}) using a Tecan infinite Pro plate reader. Three independent experiments were carried out for each lipopeptide combination, with two replica plates in each experiment. The interpretation of the checkerboard synergy test results was done using the Fractional Inhibitory Concertation index (FICi) according to the following equation:

$$FICi_{AB} = \frac{MIC_{A}^{Comb}}{MIC_{A}^{Alone}} + \frac{MIC_{B}^{Comb}}{MIC_{B}^{Alone}},$$

where MIC_{A}^{Comb} is the MIC of lipopeptide A when combined with lipopeptide B, and MIC_{A}^{Alone} is the MIC of lipopeptide A alone, without the presence of compound B.

Two methods were used to determine the FICi: 1) the Mean FICi, which considers the concentrations in the first non-turbid (clear) well found in each row and column along the turbidity/non-turbidity interface and then averaged; 2) the Lowest FICi, which is the lowest FICi of all non-turbid wells along the turbidity/non-turbidity interface [5]. The FICi obtained was interpreted as follows: <0.5 denoting synergy; 0.5–0.75 denoting partial synergy; 0.76–1 denoting an additive effect; 1–4 denoting indifference; and >4 denoting antagonism [6]. Experiments were carried at least three times, with two replica plates in each experiment.

Synergy assessment using the killing assay

The killing assay was used as an additional approach to assess possible synergistic interactions between sequence-specific lipopeptides. It was carried out as similar as described above in 'Bactericidal activity assays', but in these experiments *Xcc* cells were incubated (28 °C, 180 rpm) for 10 min with 1 x MIC concentration of each lipopeptide or with a combination of two lipopeptide at 1 x MIC concentration each. The Combination Index (CI) method was used to assess the nature of the interactions between the lipopeptides. CI is the standard index used to reflect the combination effect, which can be greater (CI < 1), lesser (CI > 1) or similar (CI = 1) to the expected additive effect of the two compounds being tested [7]. CI was calculated in two different ways: 1) Using the Highest Single Agent approach [8], in which the CI is determined as:

$$CI = \frac{max(E_A, E_B)}{E_{AB}},$$

where E_A is the effect observed by one of the lipopeptides, E_B is the effect observed by the second lipopeptide, and E_{AB} is the effect caused by combining the two lipopeptides; and 2) Using the response additivity approach [9] in which the CI is calculated as follows:

$$CI = \frac{E_A + E_B}{E_{AB}}$$

Experiments were carried at least three times, with two replicates per treatment in each experiment.

Statistical analysis

Statistical analysis was done using JMP Pro 15 (SAS Institute). Data from bactericidal assays (Fig. 1B), bacteriostatic activity towards *Xcc* of RPMs was statistically analysed by non-parametric comparison for all pairs using the Dunn method for joint ranking (alpha = 0.05). Data from SLMs with different Phe : D-Lys ratios (Fig. S5B) and comparisons between different p-FdK5 SLMs with individual lipopeptides from each group (Fig. 3A) were statistically analysed by non-parametric contrast comparison using the Dunn method for joint ranking (alpha = 0.05). The effect of p-FdK5 on bacterial spot disease of tomato (Fig. 1C) was statistically analysed by ANCOVA (dpi as covariant, with blocking effect, alpha = 0.05). Comparisons among 5-mer lipo-RPMs that were synthesized using varying concentrations of Phe and D-Lys (Fig. 3B) were analysed by non-parametric comparison to the p-FdK5 treatment using Dunn method for joint ranking (alpha = 0.05). Correlation between the percentage of acetonitrile at retention time of specific lipopeptides and their MIC values towards *Xcc* (Fig. S2) was statistically analysed by the Spearman's Rho correlation coefficient, alpha = 0.05).

II. Supplementary Figures and Tables

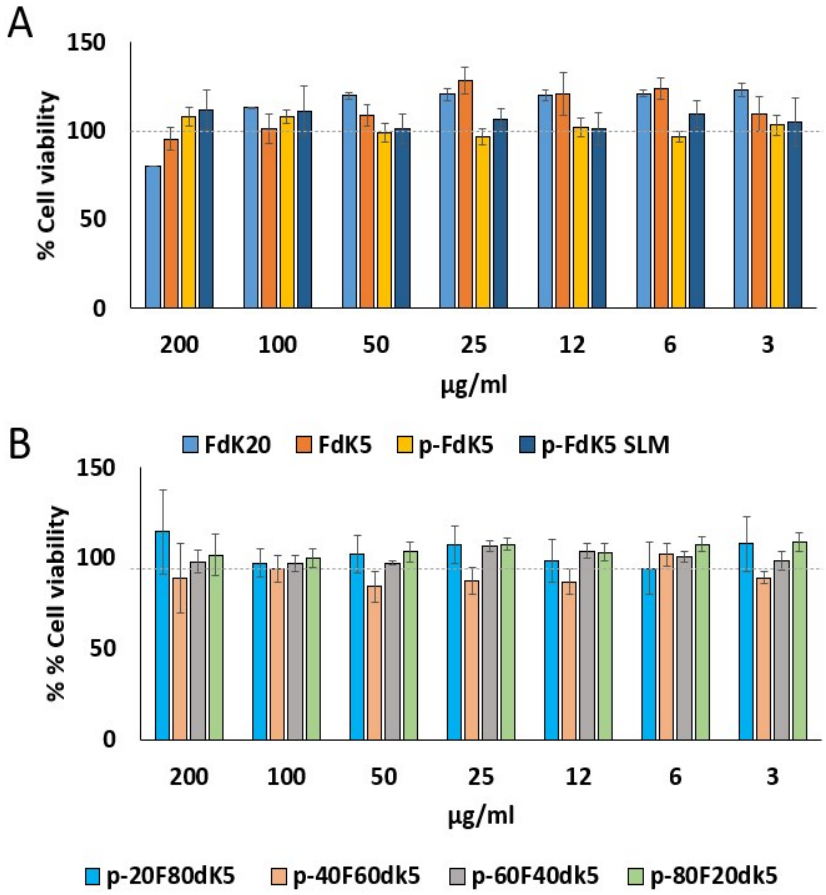

Figure S1. Assessment of cytotoxicity of lipo-RPMs in HEK293T-2 MTT assays. A. Percentage of viable cells in response to different concentrations of the lipo-RPM p-FdK5 and the p-FdK5 specific lipopeptide mixture (SLM, a mixture of equal amounts of the 32 purified lipopeptides that compose p-FdK5) in comparison with the RPMs FdK5 and FdK20. RPMs were synthesized with a 1:1 ratio of L-F and D-K. B. Percentage of viable cells in response to p-FdK5 lipo-RPMs that were synthesized using different ratios of L-F and D-K. The cytotoxic effects were determined on exponentially growing HEK293T mammalian cells using the MTT assay. After 24 h incubation, cells were stained and the percentage of viable cells was determined using a plate reader. Data represent averages and standard errors (SE) of at least 3 independent experiments with four replicates for each lipo-RPM/RPM concentration. No significant differences were found for any treatment in the highest concentration tested as compared with the negative controls in t-tests within each independent experiment (not shown).

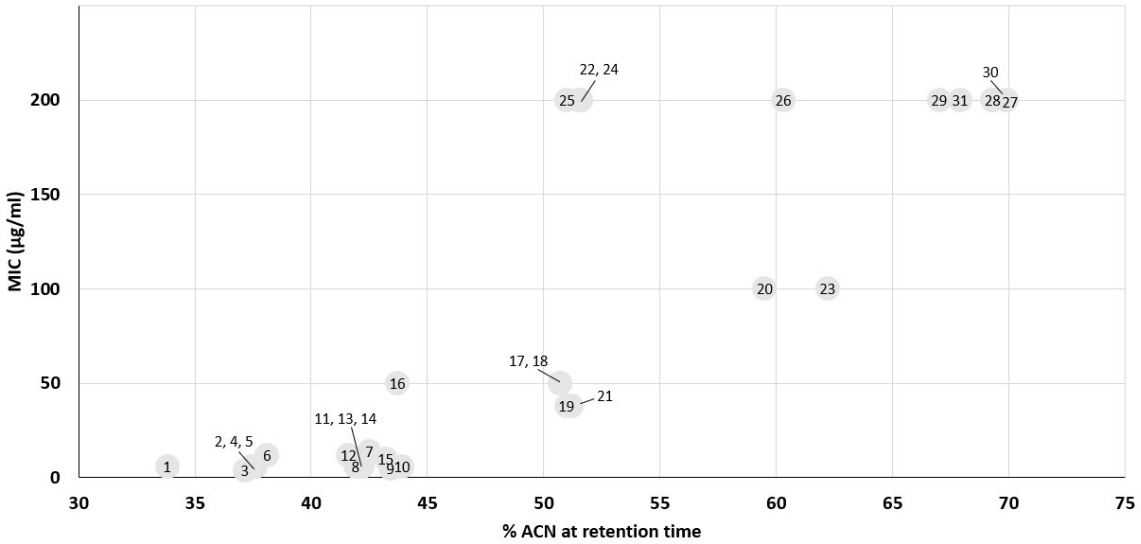

Figure S2. Correlation between bacteriostatic ability and level of hydrophobicity of lipopeptides. The 32 different lipopeptides that compose the p-FdK5 mixture were synthesized individually, purified using RP-HPLC (acetonitrile and 0.1% trifluoroacetic acid in DDW were used as solvents), and tested in MIC assays with *Xcc*. Each number represents a specific lipopeptide (see Table S2 for details). Spearman's Rho correlation coefficient = 0.8704, $p<0.001$.

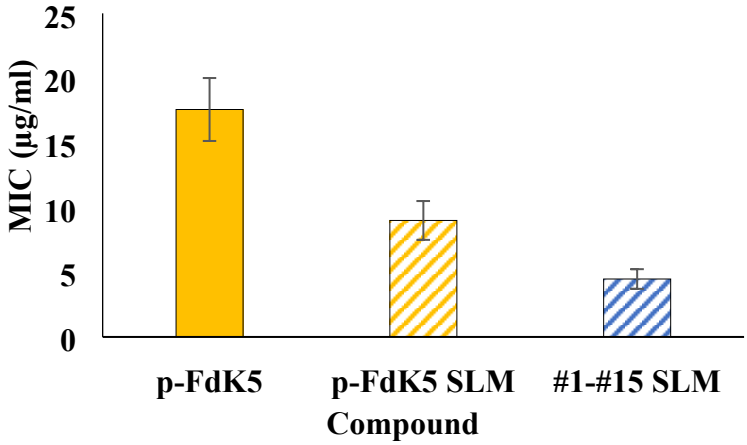

Figure S3. Bacteriostatic effects of lipo-RPMs towards *Xcc* as determined by MIC values. p-FdK5 is the original, randomly synthesized mix. p-FdK5 specific lipopeptide mixture (SLM) is a mixture of the 32 individually synthesized and purified lipopeptides that compose p-FdK5 at equal amounts. #1-15 is a mix of lipopeptides #1 to #15 that possess the lowest MIC values (<15 µg/ml), at equal amounts. MIC values were determined after 24 h incubation at 28 °C. Data represent averages and SE of at least 3 independent experiments with three replicates for each compound.

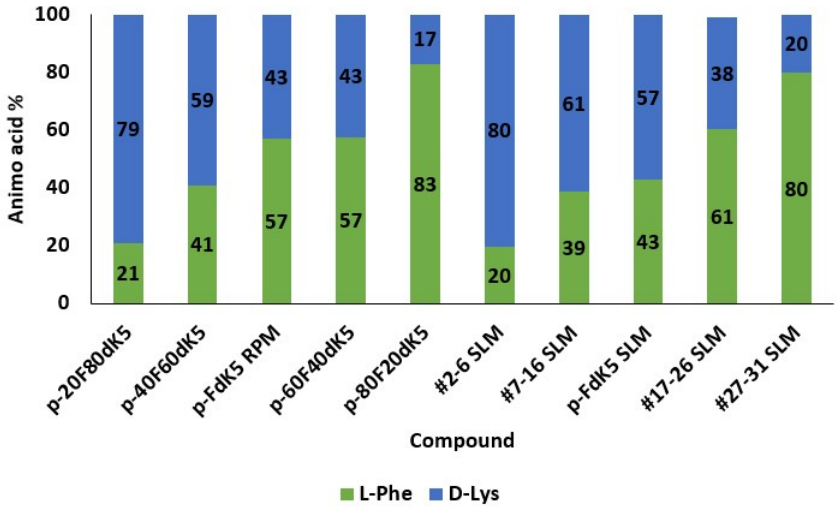

Figure S4. Amino acid analysis of RPMs and SLMs composed of different ratios of L-phenylalanine and D-lysine. Samples of 20 µg each were lyophilized and sent to the Mass Spectrometry and Chemical Analysis Laboratory, Department of Chemical Research Support at the Weizmann Institute of Science (Rehovot, Israel) for amino acid analysis. Data represent average percentage of two independent experiments with two replicates per peptide mixture.

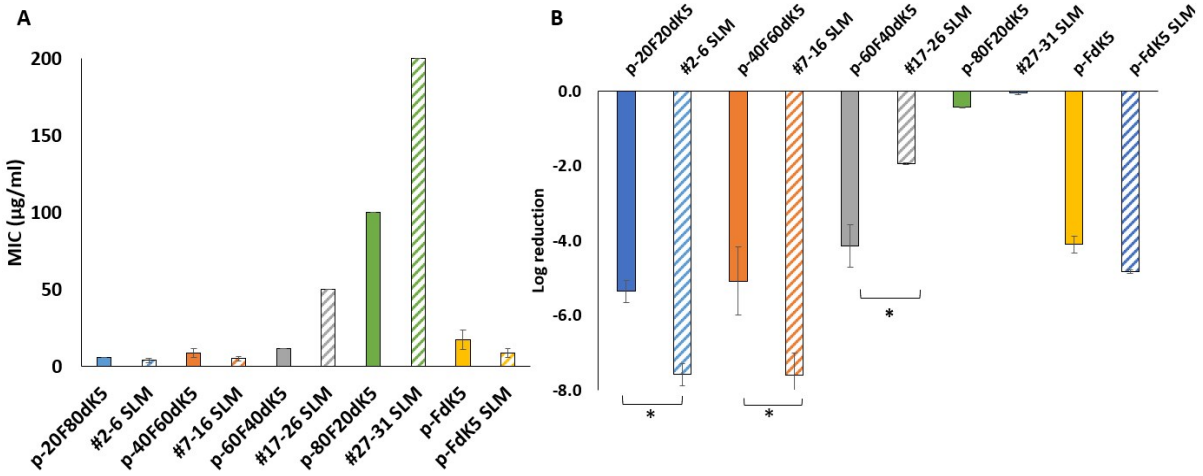

Figure S5. Antimicrobial activity towards *Xcc* of RPMs and SLMs with different F:dK ratios. A. Bacteriostatic effect (MIC values) was determined after 24 h incubation at 28 °C. The highest concentration tested was 200 µg/ml, therefore values ~200 µg/ml mean that no effect was detected under tested conditions. Data represent averages and SE of at least 3 independent experiments with three replicates per treatment. B. The bactericidal effect was measured after 10 min incubation at 28 °C of 10^8 CFU/ml *Xcc* cells with the different lipopeptides [25µg/ml]. Asterisks indicate significant differences between groups ($p<0.05$). Data represent averages and SE of at least 3 independent experiments with two replicates per treatment in each experiment.

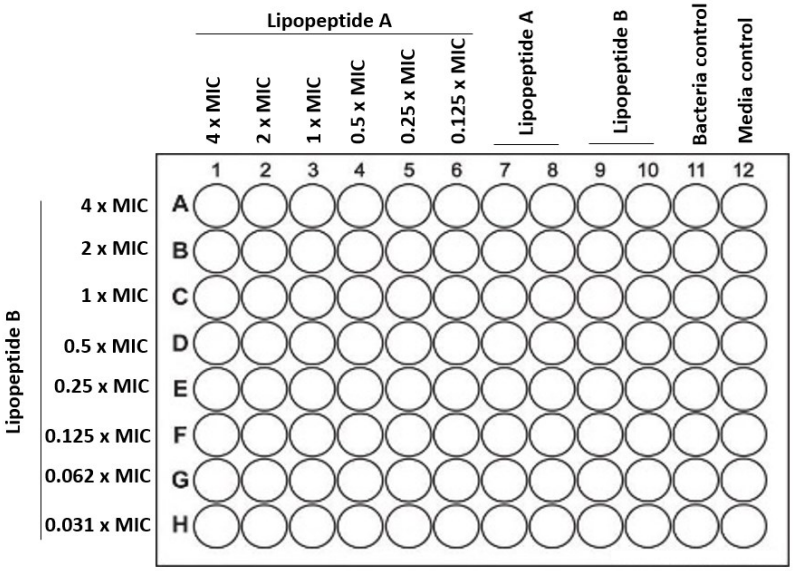

Figure S6. Schematic representation of the checkerboard assay plate used to assess synergistic effects between lipopeptides. Assays were conducted in 96-well plates containing two-fold dilutions of the two tested lipopeptides combined in different concentrations, and starting from 4 x MIC values (columns 1-6). For each tested lipopeptide, two sets of replicates (corresponding to columns 7-8 for lipopeptide A and columns 9-10 for lipopeptide B) were included that contained each lipopeptide alone, at different concentrations (under two-fold serial dilutions, starting at concentrations of 4 x MIC). Wells corresponding to column 11 contained Xcc at a concentration of ~108 CFU/ml without tested lipopeptides, and column 12 were negative controls (wells containing NB without bacteria).

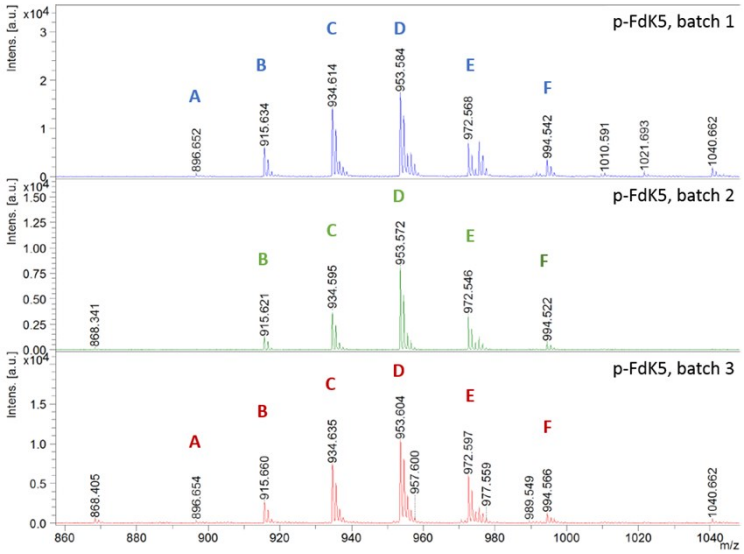

Figure S7. Mass Spectrometry spectra of p-FdK5 lipo-RPMs from three different batches. Mass spectra of the different lipopeptides that compose the mixtures are marked as (A) 896 g/mol for lipopeptides predicted as p-kkkkk; (B) 915 g/mol for lipopeptides containing 4 D-lysine and 1 phenylalanine; (C) 934 g/mol for lipopeptides containing 3 D-lysine and 2 phenylalanine; (D) 953 g/mol for lipopeptides containing 2 D-lysine and 3 phenylalanine; (E) 972 g/mol for lipopeptides containing 1 D-lysine and 4 phenylalanine; and (F) 994 g/mol for lipopeptides predicted as p-FFFFF.

Table S1. Bacterial strains used in this study.

Pathogen	Strain	Gram	Disease	Abbreviation	Source
Acidovorax citrulli	M6, AAC00-1	(-)	Bacterial fruit blotch of cucurbits	*Ac* M6, *Ac* AAC00-1	[10,11]
Xanthomonas campestris pv. *campestris*	ATCC 33913	(-)	Black rot disease of crucifer plants	*Xcc*	[12]
Xanthomonas perforans	97-2	(-)	Bacterial spot disease of tomato and pepper	*Xp*	[13]
Pseudomonas syringae pv. *tomato*	DC3000	(-)	Bacterial spot disease of tomato	*Pst*	[14]
Clavibacter michiganensis subsp. *michiganensis*	NCPPB 382	(+)	Bacterial canker and wilt of tomato	*Cmm*	[15]
Streptomyces scabies	Av	(+)	Potato common scab	*Ssc*	*Burdman lab collection*

Table S2. Lipopeptides, RPMs and lipo-RPMs used in this study.

#[1]	Peptide[2]	Predicted MW[3]	Observed MW[3]	% Purity[4]	%ACN at retention time	Average MIC
1	p-kkkkk	896.31	896.40	>95	34	6
2	p-kkkkF	915.32	915.31	>95	37	6
3	p-kkkFk	915.32	915.02	>95	37	4
4	p-kkFkk	915.32	915.56	>95	38	6
5	p-kFkkk	915.32	915.61	>95	37	6
6	p-Fkkkk	915.32	915.61	>95	38	12
7	p-kkkFF	934.31	934.63	>95	43	14
8	p-kkFkF	934.31	934.23	>95	42	6
9	p-kFkkF	934.31	934.30	>95	43	5
10	p-FkkkF	934.31	934.31	>95	44	6
11	p-kkFFk	934.31	934.29	>95	42	6
12	p-kFkFk	934.31	934.46	>95	42	12
13	p-FkkFk	934.31	934.29	>95	42	6

14	p-kFFkk	934.31	934.27	>95	42	8
15	p-FkFkk	934.31	934.29	>95	43	10
16	p-FFkkk	934.31	934.22	>95	44	50
17	p-kkFFF	953.32	953.04	>95	51	50
18	p-kFkFF	953.32	953.04	>95	51	50
19	p-FkkFF	953.32	953.17	>95	51	38
20	p-kFFkF	953.32	955.19	>95	60	100
21	p-FkFkF	953.32	953.56	>95	51	38
22	p-FFkkF	953.32	953.42	>95	52	ND[5]
23	p-kFFFk	953.32	955.17	>95	62	100
24	p-FkFFk	953.32	953.48	>95	52	ND
25	p-FFkFk	953.32	953.52	>95	51	ND
26	p-FFFkk	953.32	953.75	>95	60	ND
27	p-FFFFk	972.32	973.78	>95	70	ND
28	p-FFFkF	972.32	973.90	>95	69	ND
29	p-FFkFF	972.32	974.13	>95	67	ND
30	p-FkFFF	972.32	972.90	>95	69	ND
31	p-kFFFF	972.32	972.25	>95	68	ND
32	p-FFFFF	991.32	1015.32		-	ND
-	FdK5	657.88 - 752.89	696-734		-	ND
-	p-FdK5	896.31-991.31	916-954		-	18
-	FdK20	2580.49-2960.53	2829		-	25
-	p-20F80dk5	896.31-991.31	915-934		-	6
-	p-40F60dk5	896.31-991.31	915-953		-	9
-	p-60F60dk5	896.31-991.31	915-953		-	12
-	p-80F20dk5	896.31-991.31	934-953		-	125

[1] #, serial number.
[2] Sequence from N to C-terminus; p, palmitic acid.
[3] Predicted and observed MW in Da. All lipopeptides (except #32, which could not be successfully purified under tested conditions) were purified using HPLC and all lipopeptides/RPMs/lipo-RPMs were analyzed using MALDI-TOF. For RPMs and lipo-RPMs, the range of predicted MW and the observed main peaks MW are presented.
[4] Percentage of purity was estimated by analytical HPLC (5-65% ACN, 1% ACN per minute gradient).
[5] ND, non-detected at tested concentrations (up to 200 µg/ml).

Table S3. Interactions between sequence-specific lipopeptides.

	Checkerboard assay		Killing assay	
# lipopeptide	**Lowest FICi**	**Average FICi**	**Highest Single Agent**	**Response Additivity**
2+5	partial synergy (0.75)	Indifference (1.11)	Synergy (0.53)	Synergy (0.92)
8+12	partial synergy (0.67)	Additivity (0.92)	Synergy (0.83)	Indifference (1.46)
18+23	partial synergy (0.71)	Indifference (1.36)	Synergy (0.76)	Indifference (1.21)
2+8	partial synergy (0.58)	Additivity (0.82)	Synergy (0.45)	Synergy (0.69)
2+18	Additivity (0.87)	Indifference (1.12)	Synergy (0.46)	Synergy (0.60)
8+18	partial synergy (0.61)	Additivity (0.94)	Synergy (0.77)	Synergy (0.77)

The interactions between sequence-specific lipopeptides were assessed using a checkerboard microdilution assay, followed by calculation of fractional inhibitory concentration indexes (lowest FICi and average FICi), as well as by a killing assay followed by calculation of the highest single agent and the response additivity indexes. See experimental procedures for details. Data represent averages of at least 3 independent experiments with two replicates per treatment in each experiment.

III. References for Supporting Information

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IV. Author Contributions

STR synthesized the RPMs/lipo-RPMs, carried out most of the experiments alone. EM assisted STR in some of the experiments. Data analyses were done by STR. STR, ZH and SB conceived all experiments and wrote the manuscript. ZH and SB supervised the work of STR and were responsible for funding acquisition.