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Inhibition of Bacterial Biofilm Formation and Swarming Motility by a Small Synthetic Cationic Peptide

César de la Fuente-Núñez,[a] Victoria Korolik,[b] Manjeet Bains,[a] Uyen Nguyen,[c] Elena B. M. Breidenstein,[a] Shawn Horsman,[d] Shawn Lewenza,[d] Lori Burrows,[c] and Robert E. W. Hancock[a]

Centre for Microbial Diseases and Immunity Research, Department of Microbiology and Immunology, University of British Columbia, Vancouver, Canada[a]; Institute for Glycomics, Griffith University, Gold Coast Campus, Queensland, Australia[b]; Department of Biochemistry and Biomedical Sciences McMaster University, Hamilton, Ontario, Canada[c]; and Department of Microbiology and Infectious Diseases University of Calgary, Calgary, Canada[d]

Biofilms cause up to 80% of infections and are difficult to treat due to their substantial multidrug resistance compared to their planktonic counterparts. Based on the observation that human peptide LL-37 is able to block biofilm formation at concentrations below its MIC, we screened for small peptides with antibiofilm activity and identified novel synthetic cationic peptide 1037 of only 9 amino acids in length. Peptide 1037 had very weak antimicrobial activity, but at 1/30th the MIC the peptide was able to effectively prevent biofilm formation (>50% reduction in cell biomass) by the Gram-negative pathogens *Pseudomonas aeruginosa* and *Burkholderia cenocepacia* and Gram-positive *Listeria monocytogenes*. Using a flow cell system and a widefield fluorescence microscope, 1037 was shown to significantly reduce biofilm formation and lead to cell death in biofilms. Microarray and follow-up studies showed that, in *P. aeruginosa*, 1037 directly inhibited biofilms by reducing swimming and swarming motilities, stimulating twitching motility, and suppressing the expression of a variety of genes involved in biofilm formation (e.g., PA2204). Comparison of microarray data from cells treated with peptides LL-37 and 1037 enabled the identification of 11 common *P. aeruginosa* genes that have a role in biofilm formation and are proposed to represent functional targets of these peptides. Peptide 1037 shows promise as a potential therapeutic agent against chronic, recurrent biofilm infections caused by a variety of bacteria.

Bacteria growing on surfaces often form biofilms, which represent a complex bacterial lifestyle adaptation that provides protection from environmental stresses (2, 11, 18, 30, 34, 38). Indeed, it has been estimated that biofilm cells are up to 1,000 times more resistant to most antimicrobial agents than planktonic cells (7). An estimated 80% of all bacterial infections are biofilm related (7, 11). In addition to increased recalcitrance, biofilms are able to effectively evade the host defense system, thus hindering treatment (7, 11). This explains why bacteria growing in biofilms cause a variety of infections, including chronic lung, wound, and ear infections (11). Biofilms are also very adept at colonizing medical devices (e.g., catheters, implants, etc.), resulting in increased hospital stays and adding more than 1 billion dollars per year to hospitalization costs in the United States alone (11). Despite the importance of biofilms, limited studies have focused on the identification of compounds able to specifically target and inhibit this mode of bacterial growth (1, 23, 24, 27, 33, 37, 47, 51, 59). Instead, research has traditionally been focused on the development of anti-infective agents capable of killing a wide range of multidrug-resistant, disease-causing planktonic bacteria.

Recently, cationic host defense peptides have been considered potential anti-infective agents due primarily to their antimicrobial or immunomodulatory properties (4, 6, 8, 9, 14, 17, 43, 44, 48). Natural cationic peptides are 12 to 50 amino acids in length and are amphiphilic, having 2 to 9 basic residues (R or K) and ~50% hydrophobic residues (4, 14). Their mechanism of action has been proposed to involve multiple targets, making them less prone to selecting for resistance compared to conventional antibiotics (4, 14). Thus, cationic antimicrobial peptides target the bacterial cell with low affinity through several coincident microbicidal mechanisms (4, 14). Bacterial biofilms have been found to be particularly resistant to cationic antibiotics, possibly due to the presence, in the biofilm matrix, of negatively charged polymers that bind and deactivate these antibiotics (19, 29, 32, 39). However, recently we made the breakthrough observation that the natural human cathelicidin peptide LL-37 is able to block *Pseudomonas aeruginosa* biofilm growth and accelerate disintegration of preformed biofilms (41).

Therefore, we screened our cationic peptide libraries for peptides with effective antibiofilm activity. Here, we report on the small (9-amino-acid) cationic peptide 1037, which has very weak antimicrobial activity (MIC, 304 μg/ml) and works against biofilms formed by diverse bacterial species. Comparative analysis of transcriptomic data allowed the identification of novel dysregulated genes that are involved in biofilm formation.

MATERIALS AND METHODS

Bacterial strains. *Pseudomonas aeruginosa* wild-type strains PA14 and PAO1, *Burkholderia cenocepacia* 4813, and the food-borne pathogen *Listeria monocytogenes* 568 were used. All mutants were obtained from the *P. aeruginosa* PAO1 library (21).

Peptide synthesis. All peptides used in this study, including peptide 1037 (KRFRIRVRV-NH_2), were synthesized by GenScript (Piscataway, NJ) using solid-phase 9-fluorenylmethoxy carbonyl (Fmoc) chemistry and purified to a purity of >95% using reverse-phase high-performance

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Address correspondence to Robert E. W. Hancock, bob@hancocklab.com.
C.D.L.F.-N. and V.K. contributed equally to this work.
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liquid chromatography (HPLC). Peptide mass was confirmed by mass spectrometry.

MIC. The broth microdilution method with minor modifications for cationic peptides (61) was used for measuring the MIC in BM2 medium. Peptides were dissolved in water and stored in glass vials, and MIC assays were performed in sterile 96-well polypropylene microtiter plates (catalog no. 3790; COSTAR). Peptides were added to the plate at the desired concentrations, and the bacteria were inoculated to a final concentration of 5×10^5 CFU/ml per well. The plates were incubated at 37°C for 24 and 48 h. The MIC was defined as the lowest concentration of peptide at which no growth was observed.

Growth curves. All strains used in this study were grown overnight in BM2 swarming medium (62 mM potassium phosphate buffer [pH 7], 2 mM $MgSO_4$, 10 μM $FeSO_4$, 0.4% [wt/vol] glucose, 0.1% or 0.5% [wt/vol] Casamino Acids). If necessary, cultures were diluted to obtain equal optical densities. Five-microliter portions of these cultures were added to 195 μl of fresh swarming medium in 96-well microtiter plates. The growth of these cultures at 37°C under shaking conditions was monitored with a TECAN Spectrofluor Plus by determining the absorbance at 620 nm every 20 min for 24 h. Two independent experiments were performed.

Biofilm assays. Biofilm formation was initially analyzed using a static abiotic solid surface assay as described elsewhere (41, 46, 50). Dilutions (1/100) of overnight cultures were incubated in BM2 biofilm-adjusted medium [62 mM potassium phosphate buffer (pH 7), 7 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 10 μM $FeSO_4$, 0.4% (wt/vol) glucose, 0.5% (wt/vol) Casamino Acids] in polypropylene microtiter plates (Falcon, United States) in the presence of peptide 1037 for 22 h at 37°C. Planktonic cells were removed, biofilm cells adhering to the side of the tubes were stained with crystal violet, and the optical density at 595 nm (OD_{595}; 600 nm for *L. monocytogenes*) was measured using a microtiter plate reader (Bio-Tek Instruments Inc., United States). *Listeria* biofilms were grown in tryptic soy broth (TSB) medium under shaking conditions (200 rpm), with medium replacement every 24 h for a total of 72 h. Peptide 1037 was added at time zero (prior to adding the diluted, overnight cultures) in various concentrations, and the decrease in biofilm formation was recorded at 22 h for *Pseudomonas* and *Burkholderia* and at 72 h for *Listeria*.

Biofilm cultivation in flow chambers and microscopy. Biofilms were cultivated for 72 h in the presence of 20 μg/ml of 1037 at 37°C in flow chambers with channel dimensions of 1 by 4 by 40 mm, as previously described (62) but with minor modifications. Silicone tubing (VWR, 0.062 in ID by 0.125 in OD by 0.032 in wall) was autoclaved, and the system was assembled and sterilized by pumping a 0.5% hypochlorite solution through the system at 6 rpm for 1 h using a Watson Marlow 205S peristaltic pump. The system was then rinsed at 6 rpm with sterile water and medium for 30 min each. Flow chambers were inoculated by injecting 400 μl of mid-log culture diluted to an OD_{600} of 0.02 with a syringe. After inoculation, chambers were left without flow for 2 h, after which medium was pumped through the system at a constant rate of 0.75 rpm (3.6 ml/h). Microscopy was done with a Leica DMI 4000 B widefield fluorescence microscope equipped with filter sets for monitoring of green (Ex 490/20, Em 525/36) and red (Ex 555/25, Em 605/52) fluorescence, using the Quorum Angstrom Optigrid (MetaMorph) acquisition software. Images were obtained with a 63×/1.4 numerical aperture objective. Deconvolution was done with Huygens Essential (Scientific Volume Imaging B.V.), and three-dimensional (3D) reconstructions were generated using the Imaris software package (Bitplane AG).

Swarming assays. Swarming experiments were performed on BM2 swarming agar plates (62 mM potassium phosphate buffer [pH 7], 2 mM $MgSO_4$, 10 μM $FeSO_4$, 0.4% [wt/vol] glucose, 0.1% [wt/vol] Casamino Acids [0.5% Casamino Acids for PAO1], 0.5% [wt/vol] Difco agar) supplemented with different concentrations of the peptide. One-microliter aliquots of mid-log-phase (i.e., OD_{600} of 0.4 to 0.6) cultures grown in BM2 minimal medium [62 mM potassium phosphate buffer (pH 7), 7 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 10uM $FeSO_4$, 0.4% (wt/vol) glucose] were inoculated in 6-well plates. Each experiment was carried out three times with at least three replicates for each bacterial strain. All resulting dendritic colonies were analyzed by measuring the surface coverage on agar plates after 15 h of incubation at 37°C using Image J software. In the case of PAO1, due to its rounded swarming colony appearance, the swarming area was evaluated by measuring the diameter of the swarming colony.

Swimming and twitching motility assays. For swimming assays, LB medium plates with 0.3% (wt/vol) agar were used (40). One-microliter aliquots of mid-log-phase cultures grown in LB broth were inoculated onto 6-well plates containing 10 ml LB (0.3% agar) and supplemented with increasing concentrations of peptide 1037. The diameters of the swimming zones were measured after incubation for 15 h at 37°C. Twitching was assessed as described previously (41). Briefly 6-well plates containing 10 ml of LB medium supplemented with 1% (wt/vol) agar and increasing concentrations of peptide 1037 were inoculated by a toothpick stabbed through the agar to the agar-plastic interface, with 1 μl of mid-log-phase cultures grown in LB broth. Twitching motility was determined by measuring the diameters of the twitching zones after 24 h of incubation. For both swimming and twitching assays, at least three independent experiments were performed.

DNA microarray experiment. *P. aeruginosa* PAO1 was grown on glass plates in the presence (20 μg/ml) or absence of 1037. After 24 h of incubation at 37°C and shaking conditions, planktonic cells were washed off and biofilm cells were scraped from the glass surface. Once the cells were harvested, RNA isolation, cDNA synthesis, hybridization to microarray slides (The Institute for Genomic Research [TIGR], Pathogenic Functional Genomics Resource Center), and the analysis of DNA microarray slides using ArrayPipe version 1.7 were performed as previously described (41). Only genes that exhibited a change, compared to the results for the untreated control, of 2-fold or more with a P value of $\leq$0.05 were considered in this study.

Microarray data accession number. The microarray data have been deposited in ArrayExpress under accession number E-MTAB-962.

RESULTS

Synthetic peptide screen. *P. aeruginosa* is one of the three major causes of infections in hospitalized patients and is responsible for around 180,000 infections per year in North America (13, 58). This opportunistic human pathogen is also the most prevalent pathogen in patients with cystic fibrosis (CF), the most common eventually fatal recessive genetic disease in the Caucasian population (3, 36, 52, 57), and in this context commonly forms biofilms. The demonstrated ability of cationic peptide LL-37 to inhibit *P. aeruginosa* biofilms (41) encouraged the design of new, improved antibiofilm peptides. One of the main objectives was to minimize the size of the peptides, while conserving their antibiofilm activity. We argued that smaller peptides would be less expensive to produce and that a reduction in the number of amino acids would allow a more comprehensive understanding of the amino acid sequence responsible for antibiofilm activity. Therefore, we randomly selected around 50 peptides from previous libraries (based loosely on the weakly active bovine peptide Bac2A, with the same sizes and similar overall amino acid compositions) developed to investigate antimicrobial activity against planktonic bacteria (6), selecting both active and inactive peptides in this study. More than 50 derivatives were tested in 96-well plate biofilm assays, and 14 were found to have antibiofilm activity, with some peptides being able to inhibit biofilm formation by 45 to 65%. One of the best of these was HH15 (Table 1), a peptide with modest antimicrobial activity.

Around 15 other peptides were then designed and synthesized with sequence or thematic similarities to HH15. Several had antibiofilm activity, including two, 1037 and 1029, that were only 9

TABLE 1 Screening of peptide library

Peptide	Amino acid sequence[a]	MIC (μg/ml)	Biofilm inhibition at 1/2 MIC (%)
LL-37	LLGDFFRKSKEKIGKE FKRIVQRIKDFL RNLVPRTES	31	57
Bac2a	RLARIVVIRVAR	50	0
HH15	KR**FRIRVRV**IRK	12	45
1026	VQW**RIRVRV**IKK	5	54
1029	KQ**FRIRVRV**	10	40
1036	VQ**FRIRVR**IVIRK	10	43
1037	KR**FRIRVRV**	304	78
Consensus	**FRIRVRV**		
HH2	VQL**RIRV**A**V**IRA	50	0
1002	VQRWLIVWRIRK	5	0
1003	IVWKIKRWWVGR	20	15
1004	RFWKVRVKYIRF	5	15
1008	RIKWIVRFR	20	0
HH7	VRL**RIRV**A**V**RRA	12	0
1010	IRW**RIRV**W**V**RRI	>256	0
1011	RRWVVWRIVQRR	20	20
1012	IFW**R**RI**V**I**V**KKF	20	0
1013	VRL**RIRV**A	10	24
1016	LRIRWIFKR	20	30
HH8	VRL**RIRV**A**V**IRK	8	0
1020	VRL**RIR**WW**V**LRK	3	22
HH10	KR**FRIRV**A**V**RRA	0.8	0
1035	KRWRWIVRNIRR	40	15
1031	WRW**R**V**R**VW**R**	2.5	22

[a] Underlining and boldface represent consensus sequence amino acids.

TABLE 2 1037 MIC determination

Bacterial strain	MIC (μg/ml)
PAO1	304
PA14	304
B. cenocepacia 4813	>608
L. monocytogenes LM568	25

amino acids in length. A 7-amino-acid consensus sequence was apparent in the 5 best antibiofilm peptides (Table 1), although we did not rigorously explore this consensus sequence. Peptide 1026 with an F3W change in the third residue to another aromatic amino acid retained good antibiofilm activity, but peptide HH10 with an R8A change completely lost antibiofilm activity, as did HH2, HH7, and HH8 with this same change (in addition to an F3L change). Collectively, these data are consistent with the possibility that the Arg residue in position 6 of the consensus is important for the antibiofilm activity of these peptides.

Peptide 1037 that lacked only the last 3 amino acids of HH15 had a very high MIC (304 μg/ml) (Table 2) but paradoxically demonstrated the most potent decrease in biofilm mass (78% decrease) at low concentrations (1/2 MIC) (Table 1). Substitution of Arg at position 2 with Gln in peptide 1029 led to a substantial improvement in MIC but a decrease in antibiofilm activity, indicating that biofilm and antimicrobial activities were independent, as confirmed by a number of peptides, including HH10, with good antimicrobial activity but no or minimal antibiofilm activity (Table 1). Due to its small size and apparent selective potency toward biofilms, 1037 was selected for further studies.

Subinhibitory concentrations of 1037 inhibited bacterial biofilm formation in a broad-spectrum manner. Antibiofilm activity was confirmed using static abiotic solid-surface assays (SSA) in which *P. aeruginosa* growing as biofilms was treated with increasing concentrations of 1037 (Fig. 1). As little as 10 μg/ml of peptide inhibited biofilm formation by ~50%. To determine whether the inhibitory effect on biofilm development was related to general growth inhibition or a change in the bacterial growth rate, growth was measured in BM2 biofilm-adjusted medium treated with increasing concentrations of 1037 under shaking conditions at 37°C. These experiments underlined the specific inhibitory effect of 1037 on biofilms, since sub-MIC levels of the peptide did not affect the planktonic growth of *P. aeruginosa* (data not shown). To further evaluate and confirm the antibiofilm properties of the peptide, a more sophisticated flow chamber biofilm model based on a flow cell system and microscopy was employed. When biofilm cells of either PAO1 or PA14 were treated with sublethal concentrations of the peptide (20 μg/ml; 1/15 MIC), biofilm formation was clearly repressed, with a strong decrease in the height of biofilms (Fig. 2). Strikingly, treated samples demonstrated a moderate increase in the number of dead biofilm cells, indicating that levels of 1037 that were more than 15-fold below the planktonic MIC were able to lead to the death of biofilm cells (Fig. 2).

To determine whether the antibiofilm action of 1037 was broad spectrum, we evaluated its activity on static biofilm cultures of *B. cenocepacia* and the Gram-positive pathogen *L. monocytogenes*. *B. cenocepacia*, a prominent CF pathogen, can cause chronic infections (in a biofilm growth mode) as well as cepacia syndrome, a fatal pneumonia accompanied by septicemia (31). It was selected, as it is notoriously resistant to the killing action of antimicrobial peptides (35). *L. monocytogenes* is a ubiquitous, intracellular pathogen that causes food-borne disease and deadly listeriosis (10), and the biofilm mode of growth is thought to be involved in its persistence in the environment and foods. Treatment with 1037 resulted in a substantial reduction in biofilm growth in both organisms (Fig. 1). In particular, *Listeria* biofilm formation was reduced by as little as 0.63 μg/ml, while 5 μg/ml caused complete inhibition of biofilm organisms, even though 20 μg/ml caused no inhibition of planktonic cells (data not shown).

Transcriptome determination. To obtain insight into the molecular mechanism(s) by which 1037 inhibits bacterial biofilms, we evaluated the effect of the peptide on gene expression of *P. aeruginosa* PAO1 biofilms. For this, we used microarray technology to analyze the global gene expression of biofilms grown in the presence and absence of 1037. A total of 398 genes (selected genes are shown in Table 3) were shown to be significantly dysregulated (P value of <0.05 by Student's t test) by at least 2-fold in the presence of 1037. Of these, 138 were downregulated and 260 were upregulated (see Table S1 in the supplemental material). These genes were analyzed for those that were likely to impact on biofilm formation.

Inhibition of swimming and swarming motilities and stimulation of twitching motility. Flagella are known to be involved in swimming motility and play a role in biofilm formation and swarming motility (3, 11, 22, 26, 30, 42). Interestingly, several genes related to flagella were downregulated by 2- to 3-fold in the presence of 1037 (Table 3). In contrast, chemotaxis genes (28) were upregulated by up to 8-fold (Table 3). Genes associated with denitrification (i.e., anaerobic respiration) (53, 64) were found to be downregulated by up to 11-fold in the treated samples.

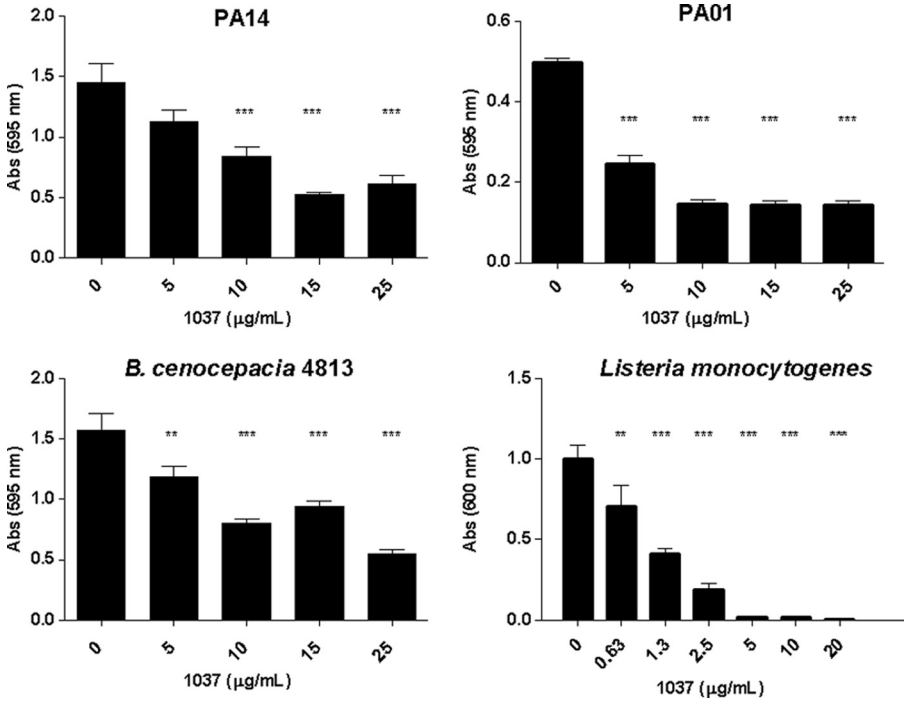

FIG 1 Dose-dependent antibiofilm effect of 1037 on Gram-negative and Gram-positive bacteria. Different bacterial strains were grown under biofilm conditions in the presence of 1037. After growth at 37°C for 22 h, biofilm growth was assessed by crystal violet staining and quantified at 595 nm. All experiments were done at least 3 times, and statistical significance was determined using one-way ANOVA (no asterisk, $P > 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

To further validate the microarray results, the effect of 1037 on processes related to biofilm formation (i.e., swimming, swarming, and twitching motilities) was determined. Peptide 1037 reduced flagella-dependent swimming motility in a broad-spectrum fashion, affecting this type of motility in PA14, PAO1, and *B. cenocepacia* 4813 (Fig. 3). This is particularly interesting since flagella play a role both in biofilm formation and swarming motility. Swarming motility, which like biofilm formation is a complex adaptation dependent on flagellin and quorum sensing (but otherwise quite distinct), was significantly and nearly completely knocked down (*P* value of <0.001 by one-way analysis of variance [ANOVA]) by the action of 1037 in both *P. aeruginosa* and *Burkholderia* (Fig. 4).

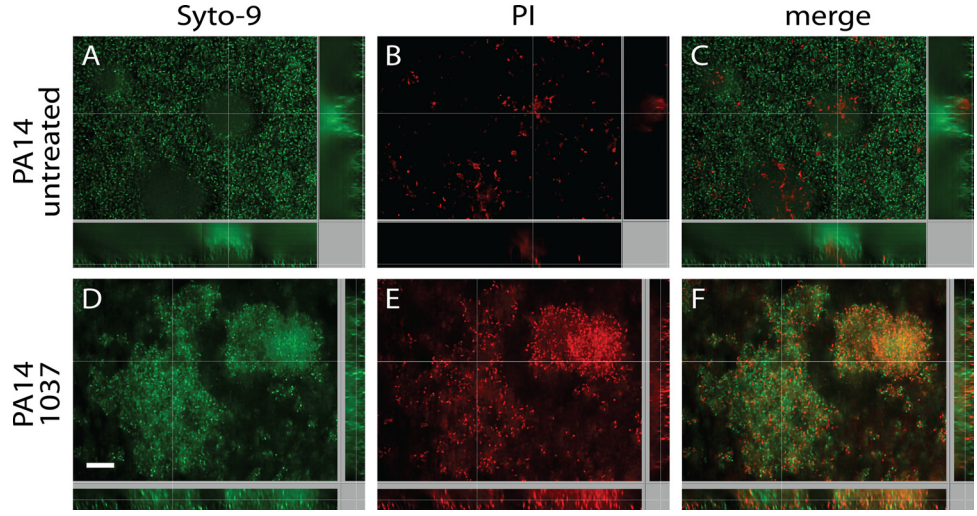

FIG 2 Flow cell analysis of *P. aeruginosa* PA14 biofilm formation in the absence and presence of 20 μg/ml 1037. *P. aeruginosa* biofilms were cultivated in minimal medium for 72 h in the presence of 20 μg/ml of 1037 peptide at 37°C in flow chambers. Biofilms were stained and visualized using SYTO-9 to stain live biofilm cells green and propidium iodide, a normally cell-impermeable stain, to stain dead cells red and examined by widefield fluorescence microscopy. The scale bar represents 15 μm in length, and each panel shows *xy*, *yz*, and *xz* dimensions. (A to C) PA14 biofilm untreated. Images correspond to PA14 biofilm stained with SYTO-9 (A), PA14 biofilm stained with propidium iodide (B), merged image (C). (D to F). PA14 biofilm treated with 20 μg/ml of 1037 peptide. Images correspond to PA14 biofilm stained with SYTO-9 (D), PA14 biofilm stained with propidium iodide (E), and merged image (F).

TABLE 3 Selected *P. aeruginosa* genes dysregulated by 1037 in biofilms

Probe ID by type	Gene	Protein	Fold change	*P* value
Flagella				
PA1077	*flgB*	Flagellar basal-body rod protein FlgB	−3.84	5E−08
PA1078	*flgC*	Flagellar basal-body rod protein FlgC	−2.19	0.0002
PA1079	*flgD*	Flagellar basal-body rod modification protein	−2.52	0.0002
PA1081	*flgF*	Flagellar basal-body rod protein FlgF	−2.27	0.01
Chemotaxis				
PA4953	*motB*	Chemotaxis protein MotB	6.06	0.003
PA0176	*aer2*	Aerotaxis transducer Aer2	3.64	0.03
PA1608	PA1608	Probable chemotaxis transducer	8.05	0.0005
PA2788	PA2788	Probable chemotaxis transducer	2.28	0.05
PA3704	*wspE*	Probable chemotaxis sensor/effector fusion	4.85	0.008
Anaerobic growth				
PA0519	*nirS*	Nitrite reductase precursor	−3.56	0.004
PA0523	*norC*	Nitric-oxide reductase subunit C	−11.51	5E−08
PA3392	*nosZ*	Nitrous-oxide reductase precursor	−4.71	0.01
Others				
PA4959	*fimX*	Type IV pilus assembly	5.49	0.004
PA3361	*lecB*	Fucose-binding lectin PA-IIL	−4.79	0.007
PA4479	*mreD*	Rod-shape-determining protein MreD	4.43	0.007
PA5053	*hslV*	Heat shock protein HslV	11.84	4E−05
PA3478	*rhlB*	Rhamnosyltransferase chain B	−3.45	0.005
PA4230	*pchB*	Salicylate biosynthesis protein PchB	−2.64	0.03
PA4228	*pchD*	Pyochelin biosynthesis protein PchD	−2.88	0.001
PA4226	*pchE*	Dihydroaeruginoic acid synthetase	−2.70	0.03
PA1202	PA1202	Probable hydrolase	−2.41	0.026
PA2145	PA2145	Hypothetical protein	−3.28	0.047
PA2204	PA2204	Probable binding protein of ABC transporter	−3.58	0.0023
PA2330	PA2330	Hypothetical protein	−3.93	0.035
PA2781	PA2781	Hypothetical protein	−2.17	0.038
PA3369	PA3369	Hypothetical protein	−7.93	2.5E−08
PA4739	PA4739	Hypothetical protein	−5.31	2E−07
PA0267	PA0267	Hypothetical protein	3.57	0.033
PA3234	*actP*	Probable sodium-solute symporter	2.73	0.0164
PA3903	*prfC*	Peptide chain release factor 3	8.57	0.0003
PA4454	PA4454	Hypothetical protein	4.77	0.009

Pilus-mediated twitching motility involves the movement of *Pseudomonas* on solid surfaces. Twitching motility has been shown to be involved in the disassembly of biofilm structures (45, 56). Array results demonstrated that a gene required for twitching motility (*fimX*) (25) was upregulated by more than 5-fold. Consistent with this result, sub-MIC concentrations of 1037 significantly (P value of <0.05 by one-way ANOVA) enhanced twitching motility by about 45% (Fig. 5).

Screening of genes dysregulated by the action of cationic peptides LL-37 and 1037. Since 1037, like LL-37, is a cationic amphipathic peptide with antibiofilm activity, we wondered whether these peptides inhibited biofilms using similar mechanisms. We therefore compared microarrays evaluating the effects on *P. aeruginosa* biofilms of LL-37 (41) and 1037 (see Table S1 in the supplemental material). A common set of 14 genes, out of more than 400 dysregulated genes, was found to be dysregulated in biofilms treated with either peptide, including 10 downregulated genes and 4 upregulated genes (Fig. 6). To assess the involvement of these genes in biofilm formation, transposon mutants in each gene were grown in static biofilm cultures. Among the downregulated genes, mutants in all but one (PA2781) exhibited various deficiencies in biofilm formation by 13 to 83% (Fig. 6A). Prominent biofilm deficiency phenotypes were found for mutants in the nitrogen metabolism gene PA0519, the flagella gene *flgB* (PA1077), and particularly in a gene predicted to be a probable ABC transporter binding protein (PA2204). Mutants corresponding to the upregulated genes were also grown in static cultures to determine their biofilm phenotypes. Two mutants demonstrated significantly increased biomasses (PA3234 and PA4454) compared to that of the wild-type strain (Fig. 6B).

DISCUSSION

The objective of the present study was to identify a very short peptide with full antibiofilm capability. Here, we identified a novel 9-amino-acid peptide, 1037, capable of knocking down bacterial biofilms. By comparing the primary structures of a series of peptides that exhibited reasonable antibiofilm activity (Table 1), a consensus amphipathic sequence (FRIRVRV) was identified, with 3 cationic residues (i.e., R) and 4 hydrophobic amino acids, analogous to host defense peptides.

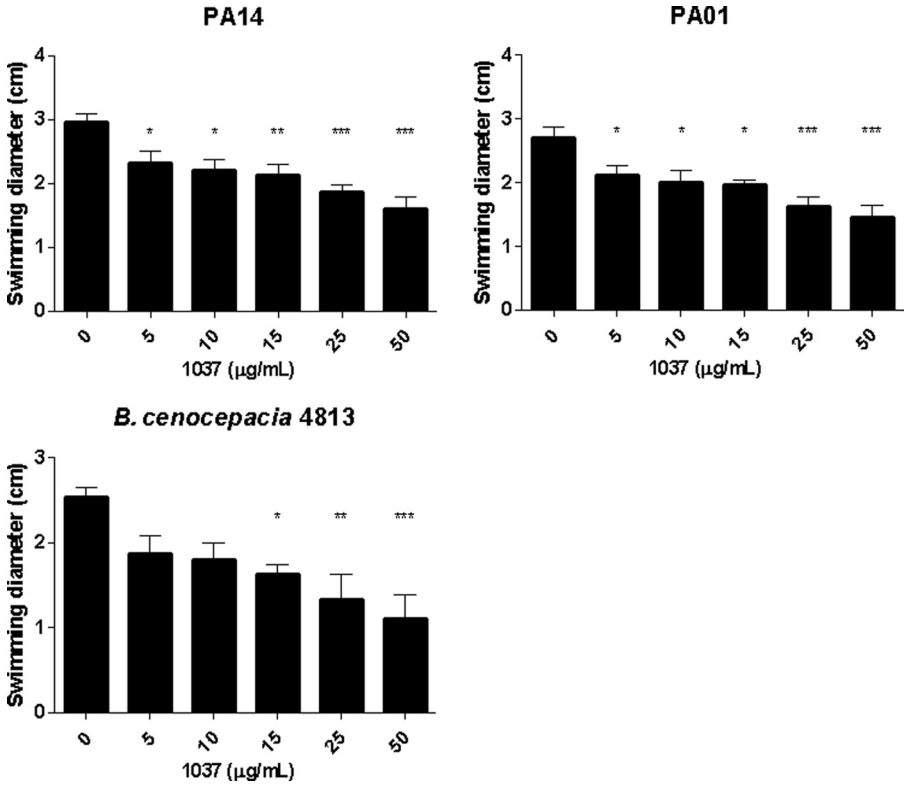

FIG 3 Swimming motility in the presence of 1037. Swimming motility was evaluated on LB plates containing 0.3% (wt/vol) agar and different concentrations of 1037. The diameters (in cm) of the swim zones were measured after incubation for 20 h at 37°C. All experiments were done at least 3 times, and statistical significance was determined using one-way ANOVA (no asterisk, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Interestingly, 1037, one of the smallest peptides that we designed, was found to have the most potent antibiofilm activity (Table 1). Indeed, levels of 1037 more than 30-fold lower than the MIC for planktonic cells were able to significantly reduce biofilm formation in both Gram-negative (*P. aeruginosa*, *B. cenocepacia*) and Gram-positive (*L. monocytogenes*) bacteria (Table 2; Fig. 1).

Intriguingly, 1037, as well as altering the thickness and morphology of biofilms, led to a decreased number of biofilm cells of *P. aeruginosa* (Fig. 2), even though, paradoxically, it failed to show significant direct antimicrobial activity against planktonic cells

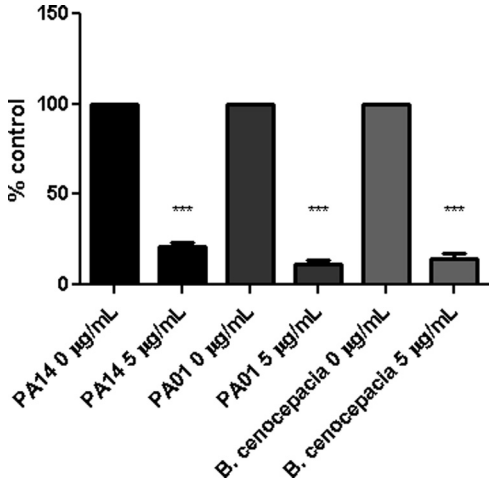

FIG 4 Bacterial swarming in the presence of 1037. Swarming was examined on BM2-swarm plates containing 0.5% (wt/vol) agar (Difco) after incubation for 20 h at 37°C. Swarming colonies were quantified as described in Materials and Methods. All experiments were done at least 3 times, and statistical significance was determined using one-way ANOVA (***, $P < 0.001$).

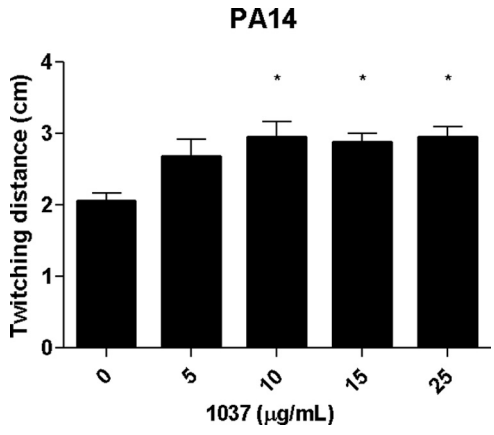

FIG 5 Twitching motility of *P. aeruginosa* PA14 in the presence of 1037. *P. aeruginosa* cells were spot inoculated on LB plates with 1% (wt/vol) agar and increasing concentrations of 1037. Twitching motility was determined by measuring the diameter of the twitching zones after 24 h of incubation at 37°C. Four independent experiments were performed, and statistical significance was determined using one-way ANOVA (*, $P < 0.05$).

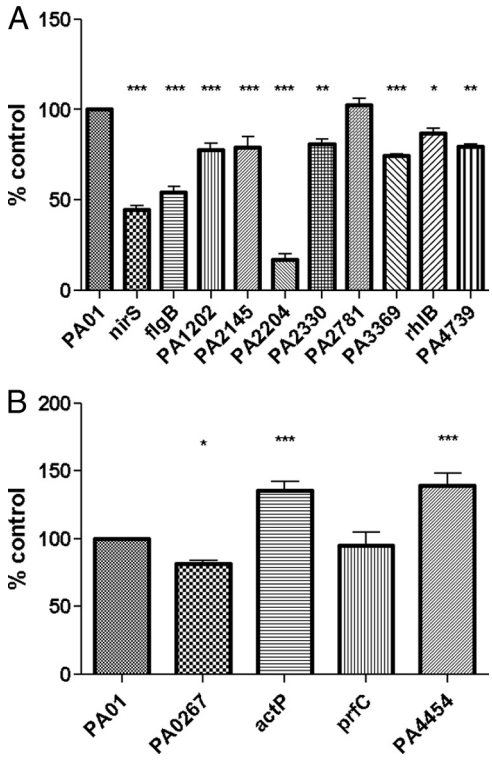

FIG 6 Mechanism of action of antibiofilm activity. Comparison of the LL-37 and 1037 microarrays. Biofilm formation by mutants of genes downregulated by both LL-37 and 1037 (A) and upregulated by both peptides (B). Transposon mutants corresponding to genes dysregulated in biofilm cells by the action of both LL-37 and 1037 were grown in polypropylene microtiter plates at 37°C for 22 h, and residual biofilm formation was assessed by crystal violet staining All experiments were done at least 3 times, and statistical significance was determined using one-way ANOVA (no asterisk, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

(Table 2). This indicates that the peptide is able to trigger uptake of a normally impermeable stain, propidium iodide (usually interpreted as cell death), or release of DNA to which it binds by acting on a target that is either selectively expressed in biofilm cells or is underexpressed and thus more susceptible to inhibition. Table S1 in the supplemental material and Fig. 6 present a large number of candidate genes, but given the penchant of the antimicrobial peptides to demonstrate multiple targets, including cell membranes, cell wall biosynthesis, RNA, protein and DNA synthesis, cell division, autolytic enzymes, and inhibition of particular enzymes (14), it is likely that such a mechanism will be quite complex. The lack of large amounts of propidium iodide staining DNA outside the biofilm cells suggested that the peptide might not be inducing cell lysis but rather might be compromising the cytoplasmic membrane, as indicated by the presence of propidium iodide inside a subset of the cells (Fig. 2).

Cationic peptides are known to be able to freely translocate into cells (15, 49), bind to DNA in a sequence-specific manner (16, 63), and directly alter gene expression (this and LL-37 papers). Using microarray technology and *in vitro* assays, it was demonstrated here that very low concentrations of 1037 affected the development of biofilms in a variety of ways. First, flagellum-dependent swimming motility was reduced in a concentration-dependent manner (Fig. 3). Inhibition of swimming motility might limit the number of bacterial cells reaching the surface, therefore decreasing biofilm formation (3, 26, 30). Second, 1037 potently inhibited bacterial swarming (Fig. 4), which is known (in a nutritionally conditional fashion) to impact on (55) and share (3, 42) regulatory relationships with biofilm formation. As swarming cells are thought to be relevant to growth on mucosal surfaces and demonstrate increased resistance to antimicrobial agents and overproduction of virulence factors (5, 40), the antiswarming effect of 1037 might contribute to its potential therapeutic value. Importantly, the antiswimming and antiswarming properties of 1037 were not observed with LL-37 (C. de la Fuente-Núñez and R. E. W. Hancock, unpublished observations). Third, 1037 was found to stimulate twitching motility (Fig. 5), a type of surface motility that promotes the disassembly of biofilm structures (45, 56).

As expected, flagellar genes were downregulated, as were genes (*nirS*, *norC*, and *nosZ*) known to play a role in anaerobic biofilm developmental process by encoding proteins involved in anaerobic respiration (53, 64). Other downregulated genes involved in biofilm formation included the quorum-sensing-regulated gene *rhlB*, which is involved in rhamnolipid production (40, 54), and the fucose-binding lectin gene *lecB*, which is required for biofilm formation (23).

Since LL-37 served as a general model for the design of peptides culminating in 1037, we questioned whether these peptides inhibited biofilms in a similar manner. To answer this question, the impact of 1037 on bacterial global gene expression was compared with the LL-37 results previously reported by our lab (41). LL-37 causes upregulation of 311 genes and downregulation of 475 genes, while 1037 was found to induce the expression of 260 genes and repress 138. However, only 10 genes were found to be downregulated by both peptides (Fig. 6A). Transposon mutants corresponding to each gene were utilized to evaluate the potential impact of decreased expression of these 10 genes on biofilm formation. All but one of these mutants led to significant reductions in biofilm formation (Fig. 6A). Three mutants led to more substantial biofilm deficiencies: an *nirS* mutant, consistent with a role for anaerobic respiration in biofilm development (53, 64), the *flgB* mutant in the flagellar basal body, and a gene encoding the unknown ABC periplasmic transporter gene (PA2204).

On the other hand, 4 genes were upregulated by both LL-37 and 1037 (Fig. 6B). In this case, two mutants (PA3234 and PA4454) grew significantly more biofilm than the control, consistent with a role for these proteins in suppression of biofilm formation. Indeed, a previous study showed that PA3234, a probable sodium-solute symporter, was repressed in biofilms (60). Moreover, expression of the ABC superfamily gene *yrbD* (PA4454) is known to gradually increase during biofilm formation (20). Interestingly, PA4454 was also found to be upregulated in a biofilm-deficient *phoQ* mutant (12). Taken together, our results indicate that LL-37 and 1037 induce the dysregulation of relatively few common genes, and this might imply that these dysregulated genes likely play an active role in biofilm development that is antagonized by their dysregulation by 1037.

In conclusion, we have demonstrated a small peptide, 1037, that improves on the previously described antibiofilm activity of its predecessor (LL-37) and additionally is able to inhibit another complex adaptation, swarming motility. Despite its conceptual similarity to antimicrobial peptides, results with 1037, confirmed by results for other peptides, clearly demonstrated that direct an-

timicrobial activity and antibiofilm activity are separately determined, since 1037 had very weak activity against planktonic bacteria and inhibited biofilm production even in *Burkholderia* that is completely resistant to polymyxin B and other cationic peptides. In the process of library screening, we identified a consensus sequence (FRIRVRV) present in several peptides with antibiofilm activity that will serve as a basis for iterative design of improved peptides. Small cationic peptides that simultaneously target biofilms and swarming while retaining either direct antimicrobial or immunomodulatory activities might provide the basis for a new generation of anti-infective agents. Alternatively, the combination of 1037 plus a second agent with antimicrobial properties could also provide a good therapeutic strategy.

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