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In Vitro Activities of Dermaseptins K4S4 and K4K20S4 against *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* Planktonic Growth and Biofilm Formation

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1 *In vitro* activity of Dermaseptins K_4S_4 and $K_4K_{20}S_4$ against
2 *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas*
3 *aeruginosa* planktonic growth and biofilm formation

4

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28 Running title: antibiofilm activity of dermaseptins

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32

33 **ABSTRACT**

34

35 The rising number of infections caused by biofilm formation and the difficulties associated
36 with their treatment by conventional antimicrobial therapies have led to an intensive search
37 for novel antibiofilm agents. Dermaseptins are antimicrobial peptides with a number of
38 attractive properties that might offer alternative therapeutics against resistant microorganisms.
39 In this study, we synthesized a set of dermaseptin-derivative peptides and evaluated their
40 activity against Gram-positive and Gram-negative bacterial biofilm formation. All
41 dermaseptin-derivative peptides elicited concentration-dependant antibiofilm activity at
42 microgram concentration and their activity was dependent on the nature of the peptide, with
43 the highest levels of activity being exhibited by highly charged molecules. Fluorescent
44 binding and confocal microscopy demonstrated that dermaseptin K₄S₄, a substituted derivative
45 from the native molecule S₄, significantly decreased the viability of planktonic and surface
46 attached bacteria and stopped biofilm formation under dynamic flow conditions. Cytotoxicity
47 assays on HeLa cells showed that some of the tested peptides were less cytotoxic than current
48 antibiotics. Overall, these findings indicate that dermaseptin derivatives might constitute a
49 new lead structure for the development of potent antibiofilm agents.

50

51 *Keywords:* Biofilm, antimicrobial peptides, dermaseptins, *Pseudomonas aeruginosa*,
52 *Escherichia coli*, *Staphylococcus aureus*.

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67 INTRODUCTION

68

69 A biofilm is a microbial community of sessile microorganisms composed of cells that
70 are embedded in a matrix of extracellular polymeric substances, attached to a substratum or
71 interface. Biofilm bacteria are phenotypically and physiologically different from planktonic or
72 suspended cells (1, 2). Bacterial biofilms have been linked to a wide range of infections,
73 particularly in patients requiring indwelling medical devices, such as catheters and prostheses.
74 They have recently been associated with about 80% of all chronic human infections (3) and
75 described as important mediators of healthcare-associated infections (4).

76 Osteomyelitis, infective endocarditis, chronic wounds, and infections related to
77 indwelling devices are examples of infections that are often caused by biofilm-producing
78 strains. Staphylococci and enterobacteriaceae account for a large proportion of these
79 infections, with *Staphylococcus aureus* and *Escherichia coli* pathogens representing more
80 than 50 % of the species isolated from patients with medical device-associated infections (6,
81 7). Such infections typically exhibit increased tolerance to antimicrobial, biocidal and
82 immunological challenges, which makes their treatment with conventional chemotherapeutic
83 agents difficult, and sometimes impossible. These concerns have prompted a persistent search
84 for alternative therapies against planktonic microorganisms and biofilms. Of particular
85 interest, dermaseptins, antimicrobial peptides produced by the immune system of frogs, offer
86 a number of properties and attributes that might open promising opportunities for the
87 development of new antibiofilm agents.

88 Antimicrobial peptides are produced by both prokaryotic and eukaryotic cells and
89 have been extensively investigated in recent research because they are involved in several
90 host defense mechanisms and lend themselves to various applications as therapeutic agents.
91 (8–13). Dermaseptins are a family of eight closely related antimicrobial peptides that have
92 originally been isolated from the skin of a tree-dwelling, South American frog (*Phyllomedusa*
93 *sauvagei*). They are linear polycationic peptides composed of 28–34 amino acids that are
94 structured in amphipathic α -helices in apolar solvents (14, 15). They all have a conserved Trp
95 residue at position 3, an AG(A)KAAL(V/G)G(N/K)AV(A) consensus motif in the mid-
96 region, and a positive charge attributable to the presence of Lys residues that punctuate an
97 alternating hydrophobic and hydrophilic sequence (14). New members of the dermaseptin S
98 family (S₉–S₁₁) that do not resemble any of the so far characterized natural antimicrobial
99 peptides have recently been identified and cloned from a skin secretion-derived cDNA library
100 (16). In fact, some dermaseptins show marked abilities to inhibit microbial cells efficiently,

101 rapidly, and irreversibly without toxic effects on mammalian cells. They also display cytolytic
102 activity *in vitro* against a broad spectrum of host-free microorganisms, including bacteria
103 (Gram-positive and Gram-negative) (17–21), protozoa (22–24), yeasts and filamentous fungi
104 (19, 25) and viruses (26-28).

105 Dermaseptin S₁, from the skin secretion of *Phyllomedusa hypochondrialis* frogs, has
106 recently been reported to exhibit electroanalytical activity to dopamine (DA) oxidation. The
107 selectivity in the detection of DA is, in fact, a fundamental aspect for the development of
108 electrochemical sensors with potential applications in the biomedical and pharmaceutical
109 industries (29). Although the precise mechanism of action of antimicrobial peptides is not yet
110 fully understood, the antimicrobial action of dermaseptin is thought to be mediated by the
111 interaction of the amphipathic α -helix with the membrane phospholipids, resulting in the
112 permeation of the target cell by destabilizing the plasma membrane via either a ‘barrel-stave’
113 mechanism or a ‘non-pore carpet-like’ mechanism (30,31).

114 Structure-activity relationship studies performed on native dermaseptin S₄ have
115 recently led to the identification of synthetic derivatives with improved antimicrobial
116 properties (32-34). When comparing the resistance emergence rates by propagating bacteria
117 under selective antibiotic pressure, both Gram-positive and Gram-negative bacteria were
118 noted to exhibit resistance to commercial antibiotics but not to the L-or D-isomers of the
119 dermaseptin derivatives that were tested (33). Overall, the data obtained from *in vitro* and *in*
120 *vivo* experiments indicate that some dermaseptin derivatives have a variety of potential
121 medical and antimicrobial applications (32). Nevertheless, no previous work has so far been
122 performed on the antibiofilm activity of dermaseptins. Accordingly, the present study was
123 undertaken to investigate the feasibility and gain effects of using dermaseptins against
124 biofilms formed by important biofilm-forming pathogens, including *Staphylococcus aureus*,
125 *Escherichia coli*, and *Pseudomonas aeruginosa*. It particularly focused on the evaluation of
126 the antibiofilm potential of dermaseptin S₄ derivatives. A number of chemically-modified
127 peptides were synthesized and investigated for antibiofilm activity. HeLa cultures were used
128 to evaluate their safety and toxicity, and fluorescent binding and confocal microscopy assays
129 were performed to assess the speed and irreversibility of their antibiofilm activity.

130

131 MATERIALS AND METHODS

132

133 **Synthesis, purification and preparation of peptides**

134 Peptides were prepared by stepwise solid phase synthesis using Fmoc polyamide-
135 active ester chemistry on Milligen 9050 pepsynthesizer. All Fmoc-amino acids were from
136 Milligen-Waters (France). 4-(Hydroxymethyl) phenoacetic acid-linked polyamide/kieselguhr
137 resin (pepsin kA), Fmoc-aminoacid pentafluorophenyl (Pfp), and 3-hydroxy-2, 3-dehydro-4-
138 oxo-benzotriazine (Dhbt) esters were from Milligen/Bioresearch. Cleavage of peptidyl-resin
139 and side chain deprotection were carried out using 5 mg of peptidyl-resin in 1 ml mixture
140 composed of trifluoroacetic acid, para-cresol, thioanisol, water, and ethyl methyl sulfide (82.5;
141 5.5; and 2.5% v/v) for 2 h at room temperature. After filtering to remove the resin and ether
142 extraction, the crude peptides were purified by a combination of Sephadex gel filtration, ion
143 exchange chromatography, and preparative high performance liquid chromatography (HPLC).
144 The homogeneity of the synthetic peptides was assessed by analytical HPLC, amino acid
145 analysis, solid phase sequence analysis, and mass spectrometry (35). All peptides were stored
146 frozen as stock solutions at 1 mg/ml and -20°C in double-distilled water. Prior to
147 experimentation, fresh solutions were diluted in the appropriate medium. For fluorescein-
148 labeled peptides, fluorescein was introduced at the N-terminus of the peptide using fluorescein
149 N-hydroxysuccinimide ester prior to Trifluoroacetic acid (TFA) treatment.

150

151 **Bacterial strains and growth media**

152 The strains used in this study are listed in Table 1. They were maintained as follows:
153 *Pseudomonas aeruginosa* was grown in Lysogeny Broth (LB) medium, *Escherichia coli* was
154 grown in Mueller Hinton (MH) broth (for antimicrobial assays) or in M63B1 medium
155 containing 0.4% glucose medium (for biofilm assay), and *Staphylococcus aureus* was grown
156 in Tryptic Soy Broth (TSB) containing 0.25 % Glucose.

157

158 **Antimicrobial activities against planktonic cultures**

159 To investigate the antimicrobial activities of dermaseptins (Table 2), we evaluated the
160 planktonic growth of different strains in the absence or presence of each peptide at different
161 concentrations. Accordingly, an overnight culture of each bacteria grown in the appropriate
162 medium was used to inoculate 100 µl of sterile MH medium in a 96-well plate to an optical
163 density at 600 nm (OD₆₀₀) of 0.0001. The peptides were added to final concentrations ranging
164 from 0 to 100 µg/ml. Polymyxin used at 6.25 µg/ml served as a positive control. Three

165 replicates were made for each condition. The cultures were incubated at 37°C, with shaking
166 for 24 h. The minimum inhibitory concentration (MIC) was determined from the lowest
167 concentration that induced 100% inhibition.

168

169 ***In vitro* biofilm susceptibility**

170 To quantify the biofilm formation of *E. coli* in the absence and presence of peptides, a
171 microplate-based assay was performed as follows. Strains were grown in M63 media
172 containing 0.4 % glucose for 24 h at 37°C in a shaking bath and then diluted to obtain a
173 suspension with an optical density (OD) at 600 of 0.01. 100 µl of the diluted suspension was
174 added into the wells of a Polyvinyl Chloride (PVC) microtiter plate and incubated for 24 h
175 without shaking to allow biofilm formation. Planktonic cells were carefully removed by
176 pipetting, and biofilms were then treated with 100 µl of different concentrations of each
177 peptide at indicated concentrations and incubated for 24 h at 37°C. Biofilm formation was
178 measured using crystal violet (CV) staining. After treatment, planktonic cells were gently
179 removed; each well was washed three times with water and pat dried with a piece of paper
180 towel in an inverted position. To quantify biofilm formation, each well was stained with 100
181 µl of 1 % crystal violet and incubated for 15 min at room temperature. The plates were then
182 washed three times with water again to remove extra dye. After that, 125 µl of mixed 80%
183 ethanol and 20% acetone were added to each well to dissolve all the absorbed dye. After 30
184 min of incubation at room temperature, OD₅₇₀ was measured to quantify the total biomass of
185 biofilm formed in each well. The viability of biofilm cells was evaluated using the XTT
186 quantification method. After treatment, planktonic cells were removed by careful pipetting,
187 and the biofilms were washed with 125 µl of PBS 1X. Each well was supplemented with 125
188 µl of 0.05 mg/ml XTT/ 10 mM menadione and incubated at 37°C for 4 h in the dark. The
189 metabolic activity of biofilm cells reflecting their viability was assessed by determining the
190 amount of product resulting from the degradation of XTT into formazan, through OD₄₉₂
191 measurement.

192

193 **Confocal microscopy: Peptides effect on a biofilm in continuous flow**

194 *Growth of biofilm*

195 The bacterial strain used for this experiment was *E. coli* MG1655 F'Tet Δ*traD*:*apra* λ
196 ATT km-mars. Biofilms were grown at 37°C in M63B1 supplemented with 0.4% glucose as a
197 carbon source, in flow chambers with individual channel dimensions of 1 by 4 by 40 mm. The

198 flow system was assembled and prepared as described previously (36). Individual flow cells
199 were inoculated with fluorescent *E. coli* (see Table 1 and legend of Fig. 2) and adjusted to an
200 optical density at 620 nm of 0.1. To allow the adherence of bacterial cells to the substratum,
201 flow cells were left without flow for 1 h after inoculation at 37°C. A laminar flow was then
202 activated with a flow rate of 2µl/sec. (7,2ml/h calculated to eliminate unattached bacteria but
203 in planktonic phase in the channel).

204

205 *Exposure of biofilm to fluorescent dermaseptins*

206 After 5 h of biofilm growth in the flow cell, the flow was stopped, and 300 µl of each
207 fluorescein-tagged peptide sample at the following concentrations (0.78 µg/ml for K₄S₄ and
208 25 µg/ml for D₄D₂₀S₄) was added into the chamber by syringe injection. After 20 min, the
209 flow was restarted with a flow rate of 0.4µl/sec per section. Normal flow rate (2µl/sec) per
210 section was set up again 20 min later. Z-stack images were obtained every 1 h for at least 6 h.
211 Flow cells were imaged under confocal microscopy Leica SP5 using the following
212 parameters: Objective 63X oil, 512x512 pixels, line average 2, 1 µm z steps, 60 µm per stack,
213 excitation Filter Wavelengths: 475-490 nanometers and 540-565 nanometers, emission
214 wavenlengths: 500-535 nanometers and 570-620 nanometers. Side-view images of the biofilm
215 were converted into 3D structures using the Software Imaris. All microscopy observations
216 and image acquisitions were performed using methods described above. For quantification of
217 biofilm development with or without peptide, images from each sample were analyzed using
218 the computer program (Imaris MeasurementPro).

219

220 **Assay for eukaryotic cell viability**

221 The potential cytotoxicity of dermaseptin S₄ and its derivatives was measured in
222 normal human cervical HeLa cells using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl
223 tetrazolium bromide)-based assay. Briefly, cells were seeded into 96-well plates at a density
224 of 2 x 10⁴ cells per well and incubated for 24 h at 37°C prior to drug exposure. On the day of
225 treatment, the culture medium was aspirated from the wells and replaced by the fresh medium
226 containing a drug concentration ranging from 0 to 500 µg/ml. Triplicate wells were used for
227 each treatment. Culture plates were then incubated for 30 min, 3 h or 6 h before the addition
228 of 10 µl of MTT solution (5 µg/ml in PBS) to each well. The wells containing only the
229 medium and MTT were used as controls for each plate. The tetrazolium/formazan reaction
230 was allowed to proceed for 4 h at 37°C, and then 100 µl of the solubilization buffer (10%

231 sodium dodecyl sulfate in 0.1% (v/v) HCL) was added to all wells and mixed thoroughly to
232 dissolve the dark-blue formazan crystals. After overnight incubation at 37°C, the optical
233 density at 540 nm was measured by a 96-well multiscanner auto-reader with the solubilization
234 buffer serving as a blank. To translate the OD₅₄₀ values into the number of live cells in each
235 well, the OD₅₄₀ values were compared with those of standard OD₅₄₀-versus-cell number
236 curves generated for each cell line. The percentage of cell survival was expressed as live cell
237 number in test group/live cell number in control group x 100.

238

239 **Statistical analysis**

240 Normality in this study was assessed using Proc Univariate in SAS software (37). All data
241 were statistically analyzed using the least-squares method with the procedure GLM for
242 General Linear Model. Effects were treatment, bacteria and the interaction treatment-bacteria.
243 The SNK multivariate test was used to compare means for bacteria and Dunnett's test to
244 compare each experimental mean of treatment (peptide) with the control mean. The following
245 equation was used for statistical calculations:

$$246 \quad Y_{ijk} = \mu + T_i + B_j + T*B_k + e_{ijk}$$

247 Where:

248 Y_{ijk} is the response variable (peptides), μ is the overall mean, T_i is the fixed effects of
249 treatment, B_j is the fixed effect of bacteria, $T*B_k$ is the effect of treatment within bacteria and
250 e_{ijk} is the residual error

251

252

253

254 RESULTS

255

256 ***In vitro* toxicity of dermaseptin and derivatives against human cells**

257 We tested the potential *in vitro* cytotoxicity of dermaseptin S₄ and its derivatives
258 (described in Table 2) in confluent monolayers of human HeLa cells using the MTT cell
259 viability assay. Cells were exposed to increasing concentrations of peptides ranging from 0 to
260 128 µg/ml. Cytotoxicity of dermaseptins was concentration-dependent (data not shown).
261 Peptides concentrations that caused 50% cytotoxicity (CC₅₀) were determined (Table 3). The
262 highest cytotoxicity rates observed for all S₄ derivatives were recorded at concentrations
263 higher than 24.12 µg/ml (CC₅₀), except for S₄(5-28) whose toxicity was slightly lower than
264 the other peptides (CC₅₀ > 100 µg/mL). Interestingly, results showed that shortening the
265 peptide in N- or C-terminal region (S₄(5-28) or K₄S₄(1-16)) and increasing its positive charge
266 by different substitutions (K₄K₂₀S₄ or K₄S₄) leads to peptides with low toxicity.

267

268 **Antimicrobial activity of dermaseptin S₄ derivatives on planktonic bacteria**

269 We next evaluated the ability of dermaseptin S₄ and its derivatives to inhibit
270 proliferation of Gram-positive *S. aureus*, and Gram-negative *P. aeruginosa* and *E. coli*
271 planktonic cells (Table 3). All peptides under investigation inhibited antimicrobial growth.
272 Their range of activity was, however, dependent on the nature of the peptide and the highly
273 positive charged molecules. In fact, the derivatives in which positive charge was increased
274 without shortening the length of the peptide, *i.e.* K₄K₂₀S₄ and K₄S₄, were the most potent
275 inhibitors, and the derivatives in which positive charge was reduced, such as D₄D₂₀S₄, were
276 less potent. Likewise, the substituted and deleted peptide K₄S₄(1-16) and the peptide with N-
277 terminal deletions S₄(5-28) displayed a nearly homogenous potency in all assays, with a
278 progressive loss of potency as compared to K₄S₄ particularly against *S. aureus*. However, the
279 truncation of the N-terminal carboxyl S₄(5-28), which potentially resulted in a less toxic
280 peptide (33), did not affect its potency.

281

282 **Effect of dermaseptin S₄ derivatives on *in vitro* static biofilm**

283 We first assessed the activity of the dermaseptin S₄ and its derivatives against mature
284 bacterial biofilms using biofilms formed in microtiter PVC plates. While exposure of bacterial
285 biofilms to the peptides at concentrations of 2 x MIC did not dissolve biofilms as measured by
286 crystal violet staining (data not shown), survival of biofilm cells was reduced (Fig. 1). K₄S₄

287 and K₄K₂₀S₄ were the most active peptides against all the strains tested, whereas D₄D₂₀S₄ was
288 the least active one. However, K₄S₄(1-16) and S₄(5-28), which strongly affected *E. coli* and *P.*
289 *aeruginosa* biofilm, had less activity against *S. aureus* biofilm.

290

291

292 **Effect of dermaseptin S₄ derivatives on *in vitro* *E. coli* biofilm formed in continuous**
293 **flow conditions.**

294 To further investigate the inhibiting effect of dermaseptin S₄ derivatives on pre-formed
295 biofilm, we evaluated their activity on *E. coli* biofilm developed under continuous flow
296 conditions using confocal imaging to visualize both bacterial cells labeled in red (red
297 fluorescent protein, Rfp) and fluorescent dermaseptins.

298 As mentioned earlier, since K₄S₄ and K₄K₂₀S₄ exhibited a similar activity against *E. coli*
299 biofilm and due to the lack of a fluorescent K₄K₂₀S₄ peptide, this experiment was performed
300 with fluorescein tagged K₄S₄ and D₄D₂₀S₄. Confocal images (Fig 2A and B) showed that,
301 under continuous culture conditions in flow cells for 6 h in minimal media with glucose as
302 carbon source, the fluorescent peptide K₄S₄ used at 2x MIC (0.78 µg/ml, in green), targeted
303 all biofilm cells, both in the interior part and in the upper layer of the biofilm. The presence of
304 K₄S₄ appeared to completely stop the growth of the biofilm with just a little detachment,
305 suggesting that this peptide has the ability to inhibit biofilm proliferation (Fig 2A). This was
306 verified by quantitative analysis of bacterial biomass that showed that biofilm proliferation
307 was stopped right after injection of K₄S₄ (Fig. 3). Co-localization measurements indicated that
308 the peptide penetrated bacterial cells, which confirmed its bacterial cell permeation ability
309 (data not shown). The fact that dermaseptin K₄S₄ activity was also discernable in the presence
310 of the continuous flow suggests a strong effect of K₄S₄ despite the dilution factor. In contrast,
311 when the biofilm was exposed to 2x MIC of dermaseptin D₄D₂₀S₄ (25 µg/ml), the established
312 biofilm continued to grow steadily, and red cells were still visible and continued to multiply
313 (Fig 2B). Interestingly, although D₄D₂₀S₄ showed both binding cell capacity and penetration,
314 it was not as effective as K₄S₄ (co-location measures), and appeared to be unable to stop the
315 growth of *E. coli* biofilm (Fig 3).

316 Among all peptides tested, we found that K₄K₂₀S₄ and K₄S₄ were the most potent to
317 inhibit biofilm formation with non toxic concentrations comparing to the others peptides. We
318 also showed that this inhibition of growth was dependent on the nature of the peptide, and the
319 highly charged molecules were the most active.

320

321 **DISCUSSION**

322

323 Infections in which bacteria are either slow growing, dormant, or within a biofilm,
324 pose a serious clinical challenge for therapy because the cells in these states exhibit tolerance
325 to the activity of antimicrobial agents. Many of these infections, including otitis media,
326 sinusitis, cholesteatoma, and tonsillitis, are caused by biofilm-forming mucosal pathogens,
327 such as *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. Accordingly,
328 the discovery of anti-infective agents that are active against both planktonic microorganisms
329 and biofilms is of great public health significance. Antimicrobial peptides represent a
330 potential promising source for the development of new alternative agents to combat resistant
331 bacteria. These molecules are rapidly bactericidal, and since their action relates to physical
332 properties, it is all the more difficult for bacteria to develop resistance to such peptides.
333 Moreover, their structure can be engineered with relative ease, because peptide chemistry
334 allows a multitude of modifications that are relatively time and cost-effective. Nevertheless,
335 most of the agents undergoing clinical trials lack specificity and are examined only for topical
336 uses (31).

337

338 Dermaseptins are a specific class of antimicrobial peptides that have a broad range of
339 antimicrobial activity. Despite their promising properties and attributes, little work has so far
340 been performed to evaluate their effects on bacterial biofilm formation. The present study
341 aims to investigate the antimicrobial activity of five related synthetic peptides derived from
342 the natural peptide dermaseptin S₄. It evaluates the effects of dermaseptins and some of their
343 derivatives against a series of Gram-negative and Gram-positive strains, including *S. aureus*,
344 *E. coli* and *P. aeruginosa*, in planktonic and biofilm cells and describes their structure-
345 function relationship.

346

347 Our findings revealed that when used at high concentrations, this natural antimicrobial
348 peptide is cytotoxic *in vitro* for host cells. This toxicity can, however, be reduced through
349 introducing a number of modifications to the native sequence without altering its activity. To
350 increase the antimicrobial activity of S₄, four deletions and substitutions were tested based on
351 previous studies performed with *Escherichia coli* and human red blood cells. Our results
352 revealed that dermaseptins K₄S₄ and K₄K₂₀S₄ displayed a hundred times higher increase in
353 antibiofilm potency than the other derivatives. The results also showed that a positive charge

354 substitution led to a lower level of cytotoxicity without altering antibiofilm activity of the
355 peptides.

356

357 Bisubstituted derived peptide K₄K₂₀S₄ exhibited the best results. This dermaseptin
358 derivative combined two substitutions, the first substitution of methionine by lysine in
359 position 4 and the second asparagine by lysine in position 20. It was previously shown that
360 increasing the number of positive charges (6 versus 4 for dermaseptin S₄) and reducing its
361 hydrophobicity index (22.7 versus dermaseptin S₄ 28.9) resulted in a reduction of hemolytic
362 activity (34). Other studies demonstrated that dermaseptin K₄K₂₀S₄ had a good antimicrobial
363 activity *in vivo* with no toxicity in mice (32). The combination of the deletion of the 12 C-
364 terminal residues with the substitution of methionine by lysine in position 4 K₄S₄(1-16), the
365 deletion of 5 N-terminal residues in dermaseptins S₄(5-28), or the reduction of positive
366 charges by substituting methionine in position 4 and 20 by aspartic acid were reported to
367 induce a marked decrease in its antimicrobial activity as compared to K₄K₂₀S₄. It can be
368 concluded from these observations that increasing the net positive charges of the peptide
369 without shortening its sequence would result in analogs that display potent antibiofilm activity
370 and low cytotoxicity. Besides, the selective activity of antimicrobial dermaseptins depends on
371 the membrane lipid composition of the microbe versus the host cell and its electrical potential
372 (33). For example, although the lipid compositions of host cell and *P. falciparum* membrane
373 are similar, the potential of the parasite membrane is higher than that of the host cell
374 membrane, leading to the discriminating effect of S₄ (34).

375

376 Furthermore, the literature shows that several other peptides isolated from different
377 sources have been investigated for their antibiofilm activity against several bacterial strains,
378 including cathelicidin (38, 39), histatin, mucins (40, 41), magainins (42, 445), pleurocidin
379 (43), and lactoferrin (44). Like dermaseptins, these peptides are cationic and unstructured and
380 fold into amphipathic α -helices upon contact with membranes. The data presented in this
381 work suggest that the antibiofilm activity of dermaseptins is significantly higher than that of
382 these peptides. For example, magainins II, which are 23-residue α -helical peptides isolated
383 from the skin of the frog *Xenopus laevis*, display antibiofilm activity against *P. aeruginosa*
384 and *E. coli* at 246.9 μ g/ml and 15.4 μ g/ml, respectively (40), whereas 0.38 μ g/ml of
385 dermaseptin K₄K₂₀S₄ was enough to achieve the same effect. In addition, a novel peptide
386 mimetic, meta-phenylene ethylene (mPE), modeled after magainin was tested for its efficacy
387 against several biofilm forming strains, including *S. aureus*, and the results showed that the

388 eradication of biofilm was observed at 10xMIC (42, 45). Used at 2xMIC, on the other hand,
389 dermaseptin was sufficient to prevent the formation of *S. aureus* biofilm. Like the archetypal
390 dermaseptins, pylospectins (PSN), a major family of phyllomedusine skin antimicrobials (45,
391 46), have recently been described to occur in some species in multiple isomeric forms. The
392 evaluation of the activity of these peptides' (PSN-1) against biofilms is, however, very
393 limited; only few strains have so far been evaluated, and no data have shown their ability to
394 inactivate bacterial strains (46). Comparatively, dermaseptins, with their broad range of
395 antibacterial activity, may certainly offer a new promising candidate for the inhibition of
396 biofilm formation. Taken together, the data indicate that the dermaseptin presented in this
397 work has a number of advantages over other antibiofilm cationic peptides tested.

398

399 Biofilms are known to constitute a diverse and complex aggregate of bacteria that
400 exhibit over 100-fold resistance to conventional antibiotics (47). Once a biofilm is
401 established, the live cells are typically buried beneath the surface or between layers of dead
402 cells and encased by a glycocalyx, an extracellular matrix of carbohydrates, proteoglycans,
403 DNA, and other cellular constituents. Not only does this complex of biological molecules
404 inhibit diffusion due to steric hindrance, but its constituents are believed to carry charges that
405 have been known to interfere with the diffusion of others antibiotics (42, 45). The confocal
406 microscopy experiments of this study showed that some dermaseptins such as K4S4 penetrate
407 well into the biofilm structure and inhibit biofilm formation.

408

409 The main question that remains is how these dermaseptins can cross a thick layer
410 established by exopolysaccharides, DNA, and proteins, to be able to reach their target cells. In
411 fact, Donlan and his coworkers showed that the matrix of exopolysaccharides also plays a
412 minor role in the antibioresistance properties of the biofilm by being directly bound to the
413 antimicrobial agents and by preventing them from penetrating within the biofilm (1). This
414 suggests that even if the matrix stops certain molecules of antibiotics, other molecules, such
415 as dermaseptins, can cross this barrier and penetrate within the biofilm. The penetration of
416 dermaseptins can stimulate exopolysaccharides production in the matrix and contribute to the
417 increase of its thickness. This increase does not seem to inhibit the peptide which remains
418 active against all the tested biofilm even at low concentrations, suggesting that the matrix may
419 not hold these peptides which are positively charged and that they can reach the target cells.
420 Once in contact with these cells, dermaseptins establish electrostatic interactions with the

421 bacterial membranes, leading to the formation of ionic channels in the lipid bilayers inducing
422 the death of the cell.

423

424 Finally, dermaseptin S₄ derivatives present a new lead structure for potent antibiofilm
425 agents. Nevertheless, new studies are needed to correlate the activity of S₄ in the *in vivo*
426 situation. The *in vitro* biofilm assays employed in this study are essentially preliminary
427 screens. They provide evidence that dermaseptins have a strong effect against biofilm strains,
428 and further studies on their mechanisms of action and activity against other biofilm sources
429 are warranted.

430

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435

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- 584
- 585

Table 1. Different stains used in this study.

Strains	Relevant characteristics	Reference
<i>E. coli</i>		
MG1655_λatt_Km_mars_F'tetΔtraD	Red fluorescent biofilm forming <i>E. coli</i> strain producing the F pilus, Km ^R , Tet ^R	Laboratory collection
<i>P. aeruginosa</i>		
PAO1	Wild type, prototroph, <i>chl-2</i>	B. Holloway
<i>S. aureus</i>		
15981	Biofilm forming strain	(48)

Table 2 Dermaseptin S₄ derivatives used in this study

Dermaseptins	Sequence
K ₄ S ₄	ALW K TLLKKVLKAAAKAALNAVLVGANA
K ₄ K ₂₀ S ₄	ALW K TLLKKVLKAAAKAAL K AVLVGANA
D ₄ D ₂₀ S ₄	ALW D TLLKKVLKAAAKAALDAVLVGANA
K ₄ S ₄ (1-16)	ALW K TLLKKVLKAAAK-----
S ₄ (5-28)	TLLKKVLKAAAKAALNAVLVGANA

Table 3. Antimicrobial activity and dose-dependent effect of different derivatives of dermaseptins

Peptides	CC ₅₀ ^a	MIC ^b		
		<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>E. coli</i>
K ₄ S ₄	28.11 ^{**}	12.5 ^{**}	12.5 ^{***}	0.39 [*]
K ₄ K ₂₀ S ₄	27.92 ^{**}	0.19 [*]	0.39 [*]	0.19 [*]
D ₄ D ₂₀ S ₄	24.12 ^{**}	25 ^{***}	12.5 ^{***}	12.5 ^{***}
K ₄ S ₄ (1-16)	28.51 ^{**}	25 ^{***}	12.5 ^{***}	3.125 ^{**}
S ₄ (5-28)	103.11 ^{***}	25 ^{***}	12.5 ^{***}	6.25 ^{***}
Polymyxin	ND	25 ^{***}	6.25 ^{**}	6.25 ^{***}

^a Peptide concentration (µg/ml) that causes 50% cytotoxicity of dermaseptin S₄ and derivatives on HeLa cells,

^b Minimal inhibitory concentration (MIC). Values *in vitro* expressed in µg/ml for all strains tested. ND: not determined. According to SNK multivariate test, we noticed a significantly difference (p <0.001) between peptides, with the best activity, especially against *E. coli*, was reported in K₄K₂₀S₄ and K₄S₄ (0.39 µg/ml and 0.19 µg/ml respectively) compared to other peptides with a means activity (12.5 µg/ml, 6.25 µg/ml and 3.12 µg/ml), respectively for D₄D₂₀S₄, S₄(5-28) and K₄S₄ (1-16).

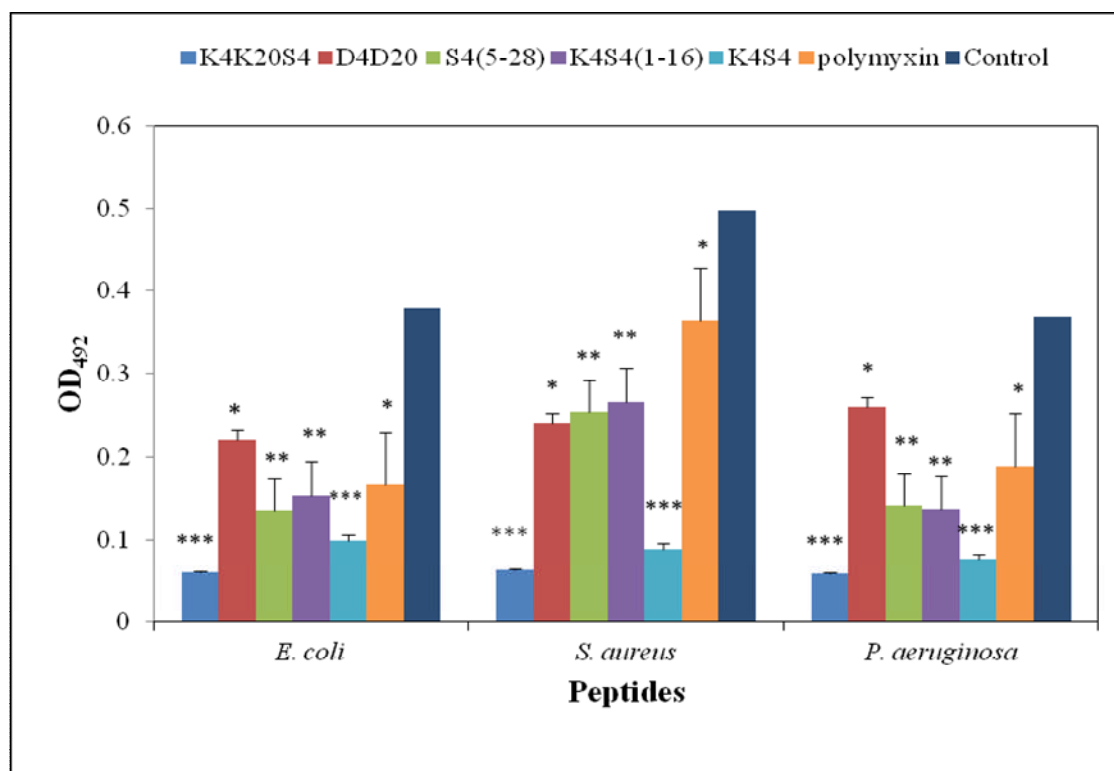


Figure 1. Effects of dermaseptins S₄ derivatives on biofilm formation. The viability of biofilm cells was evaluated using the XTT quantification method: Biofilm-forming *S. aureus*, *P. aeruginosa* and *E. coli* strains were grown in 96-well polystyrene plates and biofilms formed exposed to 2 x MIC concentrations of peptides during 24h. Control (negative control wells containing an appropriate medium for each strain only), Polymyxin (positive control wells). Statistical analysis was performed by using SNK multivariate test and Dunnett's test. K₄K₂₀S₄ and K₄S₄ were significantly different from control p<0.001, S₄(5-28) and K₄S₄(1-16) were different from control (p<0.01), and polymyxin is different from control (p< 0.05) (** p<0.001; * p<0.01; * p<0.05)

A

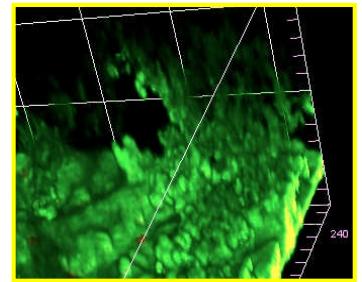
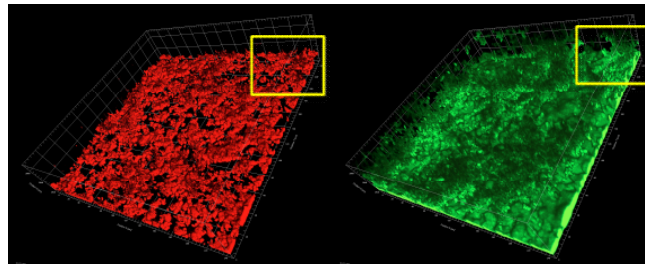
Time
post-Injection

Bacterial Biofilm

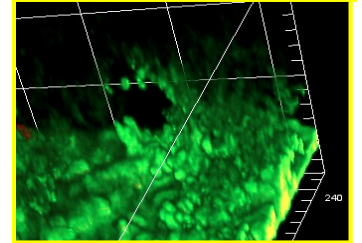
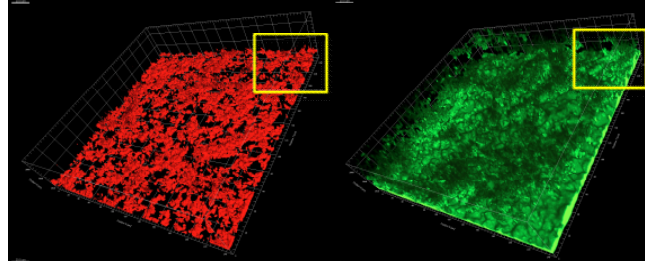
K_4S_4

MERGE X20

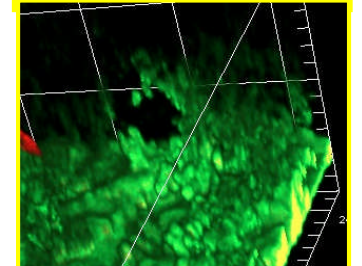
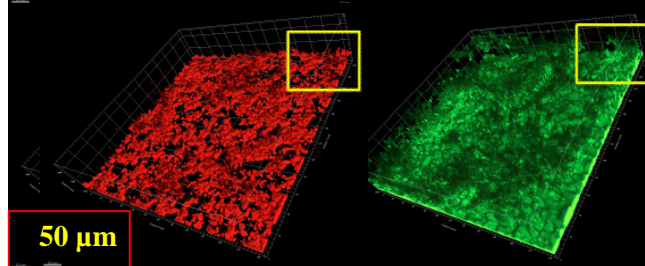
2h



4h



6h



B

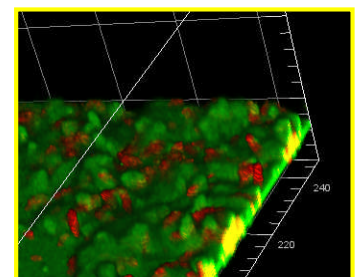
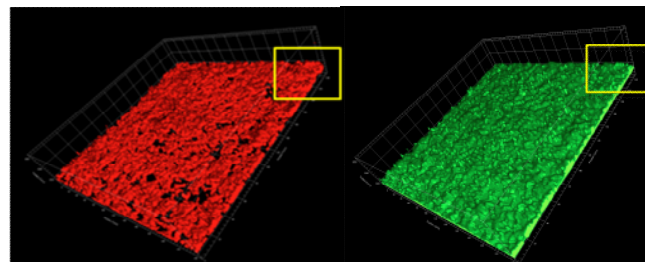
Time
post-Injection

Bacterial Biofilm

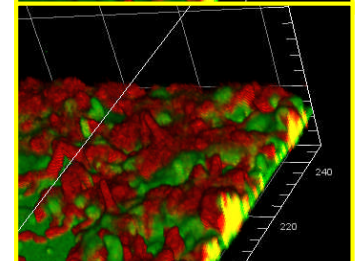
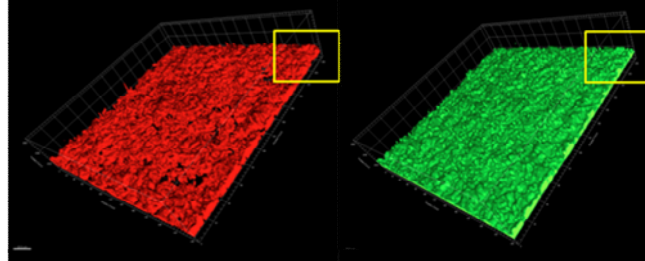
$D_4D_{20}S_4$

MERGE X20

2h



4h



6h

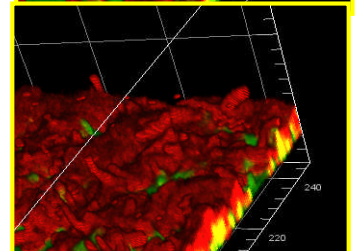
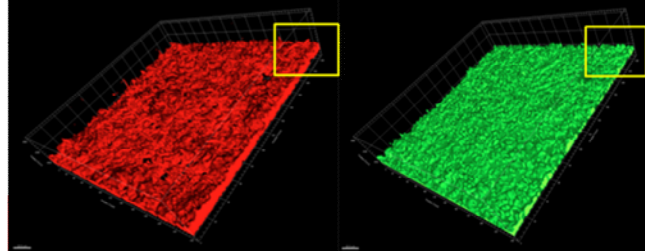


Figure 2 Effect of K₄S₄ on biofilms. To allow attachment of the bacterial cell to the substratum, flow cells were left without flow for 1h after inoculation at 37°C. Afterwards, a laminar flow with a flowrate of 2µl/sec was activated. After 5h of biofilm growth, the flow was stopped and 300 µl (0.78 µg/ml for K₄S₄ and 25 µg/ml for D₄D₂₀S₄) of each peptide sample was added. Confocal images acquisition showed that the fluorescent peptide (**A**): K₄S₄ (green) has an instant effect on biofilm and stops its formation under dynamic flow conditions. Whereas, D₄D₂₀S₄, at 25µg/ml, (**B**), shows a less activity, biofilm cells exhibit high fluorescent signals (red) and survived the dermaseptin treatment.

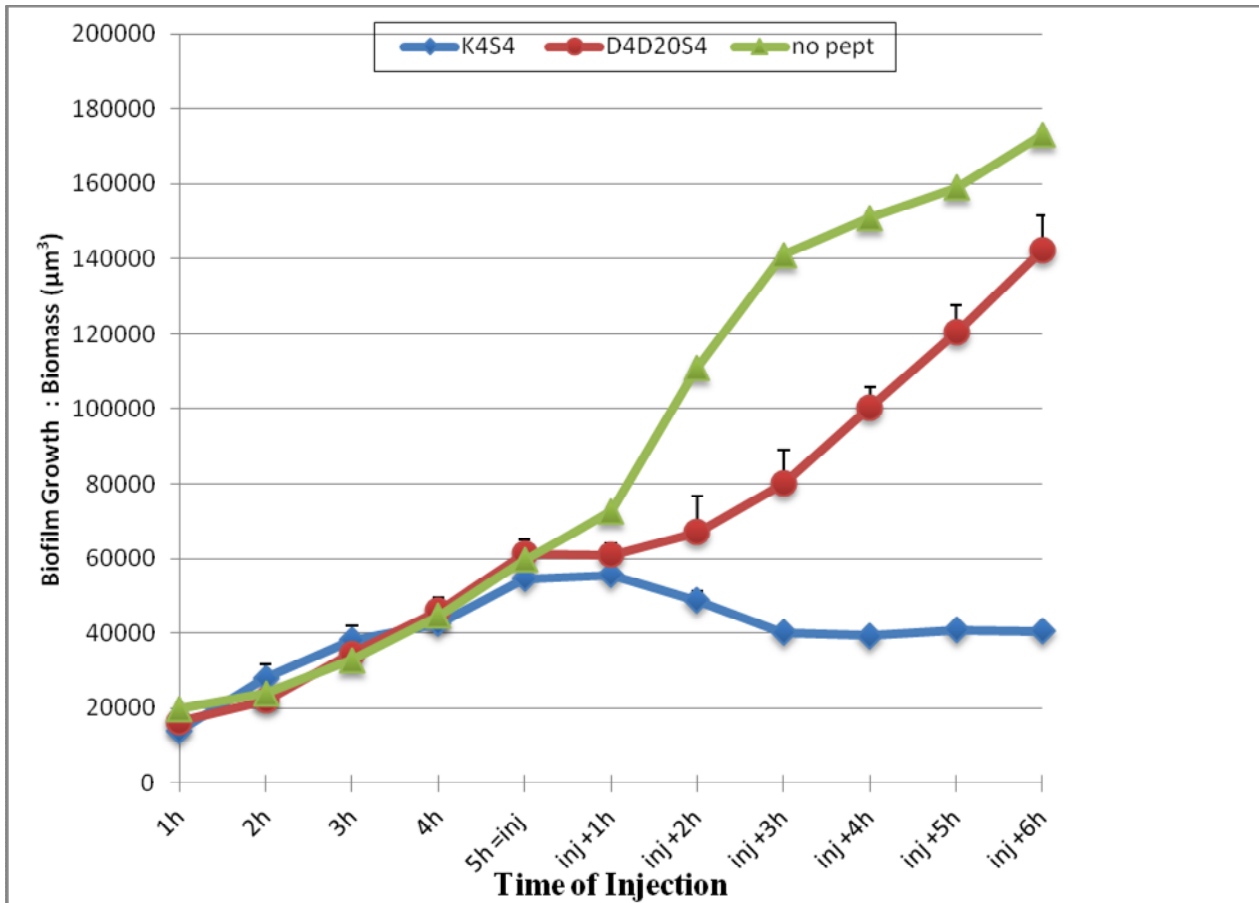


Figure 3. Confocal assays were confirmed by the quantification of biofilms. Differences in bacterial survival in the biofilms were quantified using the computer program Imaris MeasurementPro. Statistical analysis was performed by using SNK multivariate test and Dunnett's test. K₄S₄ was significantly different from control (p<0.001).