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## **Pseudomonas aeruginosa expresses a functional human natriuretic peptide receptor ortholog: involvement in biofilm formation**

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1 ***Pseudomonas aeruginosa* expresses a functional**  
2 **human natriuretic peptide receptor ortholog:**  
3 **involvement in biofilm formation**  
4

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24

25 Running title: AmiC sensor acts as a bacterial hormone receptor  
26

27 **Key words:** Cystic fibrosis; Lung; Natriuretic peptides; NPR-C; AmiC; Biofilm;  
28 Bacterial adaptation; Microscale thermophoresis  
29

30 **Abstract**

31 Considerable evidence exists that bacteria detect eukaryotic communication molecules and  
32 modify their virulence accordingly. In previous studies, it has been demonstrated that the  
33 increasingly antibiotic resistant pathogen *Pseudomonas aeruginosa* can detect the human  
34 hormones Brain Natriuretic peptide (BNP) and C-type Natriuretic Peptide (CNP) at  
35 micromolar concentrations. In response, the bacterium modifies its behavior to adapt to the  
36 host physiology, increasing its overall virulence. Identifying the bacterial sensor for these  
37 hormones and interfering with this sensing mechanism offers an exciting opportunity to  
38 directly affect the infection process.

39 Here, we show that BNP and CNP strongly decrease *P. aeruginosa* biofilm formation. Isatin,  
40 an antagonist of human natriuretic peptide receptors (NPR), prevents this effect. Furthermore,  
41 the human NPR-C receptor agonist cANF<sup>4-23</sup> mimics the effects of natriuretic peptides on *P.*  
42 *aeruginosa* while sANP, the NPR-A receptor agonist, appears weakly active. We show *in*  
43 *silico* that NPR-C, a preferential CNP receptor, and the *P. aeruginosa* protein AmiC have  
44 similar 3D structures, and that both CNP and isatin bind to AmiC. We demonstrate that CNP  
45 acts as an AmiC agonist, enhancing the expression of the *ami* operon in *P. aeruginosa*.  
46 Binding of CNP and NPR-C agonists to AmiC was confirmed by microscale thermophoresis.  
47 Finally, using an *amiC* mutant strain, we demonstrate that AmiC is essential for CNP effects  
48 on biofilm formation.

49 In conclusion, the AmiC bacterial sensor possesses similar structural and pharmacological  
50 profiles to the human NPR-C receptor and appears to be a bacterial receptor for human  
51 hormones that enables *P. aeruginosa* to modulate biofilm expression.

52  
53  
54

55 **Importance**

56 *Pseudomonas aeruginosa* is a bacterium that is a highly dangerous opportunist  
57 pathogen for immunocompromised hosts, especially cystic fibrosis patients. The sites of *P.*  
58 *aeruginosa* infection are varied, with predominance in the human lung, in which bacteria are  
59 in contact with host molecular messengers such as hormones. The C-type natriuretic peptide  
60 (CNP), a hormone produced by lung cells, has been described as a bacterial virulence  
61 enhancer. In this report, we showed that the CNP hormone counteracts *P. aeruginosa* biofilm  
62 formation and we identified the bacterial protein AmiC as the sensor involved in the CNP  
63 effects. We showed that AmiC could bind specifically CNP. These results show for the first  
64 time that a human hormone could be sensed by bacteria through a specific protein which is an  
65 ortholog of the human hNPR-C. The bacterium would be able to modify its lifestyle by  
66 favoring virulence factors production whilst reducing biofilm formation.

67

68

69 **INTRODUCTION**

70

71 *Pseudomonas aeruginosa* is a well known opportunistic pathogen and a major cause of  
72 mortality among cystic fibrosis patients (1). The morbidity of this bacterium and its resistance  
73 to antibiotherapy are largely attributed to its transition in host tissues (particularly in lung, soft  
74 tissues, skin and urinary bladder) from a planktonic to a biofilm lifestyle (2, 3). The present  
75 emergence of multi-resistant strains is now critical and is prompting the need to find new  
76 therapeutic approaches (4).

77 The high adaptability of *P. aeruginosa* and its rapid response to the host environment  
78 implies that it can detect a large range of eukaryotic chemical signals (5). However, few  
79 bacterial sensors for eukaryotic molecules have been characterized to date (6). Studies on the  
80 *E. coli* quorum-sensing regulator A (QseA) led to the identification of QseC/QseB, a two-  
81 component regulatory system (7, 8). This system is not only activated by the bacterial quorum  
82 sensing signal autoinducer AI<sub>3</sub>, but also by eukaryotic neurohormones  
83 (epinephrine/norepinephrine) (9). It was suggested that QseC could be a bacterial ortholog of  
84 eukaryotic adrenergic receptors (10). Bioinformatic screening of *E. coli* QseC analogs has  
85 demonstrated the presence of proteins related to this sensor in a large number of bacterial  
86 pathogens, including *P. aeruginosa* (11), in which norepinephrine acts as a virulence inducer  
87 (12). It appears that *P. aeruginosa* is able to sense different eukaryotic communication  
88 molecules, including interferon- $\gamma$  (13), dynorphin (14), gamma aminobutyric acid (GABA)  
89 (15) and natriuretic peptides (16, 17).

90 Natriuretic peptides are a family of eukaryotic hormones and neurohormones composed of  
91 three members, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type  
92 natriuretic peptide (CNP). These peptides are mainly expressed in cardiomyocytes and  
93 endothelial cells (18) explaining their major role in cardiovascular homeostasis. However,

94 ANP and CNP are also produced in significant amounts by the lung bronchial and alveolar  
95 epithelia and particularly by Clara (Club) cells (19). The circulating blood levels of natriuretic  
96 peptides are increased during community-acquired pneumonia (20), and the  
97 lipopolysaccharides (LPS) of Gram-negative bacteria induce the plasmatic release of both  
98 BNP (21) and CNP (22). In previous studies, we demonstrated that *P. aeruginosa* can detect  
99 BNP and CNP at micromolar concentrations and reacts by an overall increase of virulence  
100 (16, 17). In *P. aeruginosa*, the effect of CNP appears to be mediated by cAMP synthesis,  
101 which subsequently activates the regulatory proteins Vfr (17) and PtxR (16), leading to a rise  
102 of HCN and exotoxin A production and a reorganization of LPS structure. For these reasons,  
103 it was suggested that CNP promotes the acute infection phenotype of *P. aeruginosa* (23),  
104 favoring the switch of *P. aeruginosa* towards a planktonic lifestyle, and a decrease in biofilm  
105 formation. The effects of BNP and CNP on *P. aeruginosa* appear to be mediated by distinct  
106 mechanisms since BNP, unlike CNP, did not modify intra-bacterial cAMP levels (17) and  
107 was without effect on *P. aeruginosa* virulence towards *C. elegans* (16). In humans, each type  
108 of natriuretic peptide acts through a particular receptor subtype, suggesting the expression of  
109 specific bacterial orthologs of the human natriuretic peptide receptors in *P. aeruginosa*.

110 In the present study, we extended our knowledge of the effect of natriuretic peptides on *P.*  
111 *aeruginosa* by investigating for the first time the effect of these peptides on the biofilm  
112 formation activity of the bacterium. Using *in silico* approaches, we identified the *P.*  
113 *aeruginosa* protein AmiC as a potential CNP binding sensor site and investigated *in vitro* the  
114 binding of natriuretic peptide receptor agonists to AmiC. The pharmacological profile of  
115 AmiC as a bacterial CNP receptor was completed by studying the effect of isatin, an  
116 antagonist of human CNP receptors that is also a known bacterial metabolite and signaling  
117 compound. We further determined the affinity of AmiC for several natriuretic peptide  
118 receptor agonists. Using an *amiC* deficient mutant and its complemented derivative, we

119 validated the crucial role of *P. aeruginosa* AmiC in the effect of CNP, but not BNP, on  
120 biofilm formation. This study provides an integrated explanation of the CNP sensing system  
121 in *P. aeruginosa* and opens up the way to a new strategy to control *P. aeruginosa* biofilm  
122 formation.  
123

## 124 **RESULTS**

125

### 126 **Effects of CNP and BNP on *P. aeruginosa* biofilm formation.**

127 The influence of CNP and BNP on the biofilm formation activity of *P. aeruginosa* was  
128 investigated in dynamic and static conditions. Unless otherwise stated, peptides were used at  
129 a micromolar concentration since *i*) this corresponds to that usually used for studying the  
130 effects of peptides on eukaryotic models (24), *ii*) they were previously shown to be active at  
131 this concentration on quorum sensing molecules and toxin productions in *P. aeruginosa* (16),  
132 and *iii*) the duration of the biofilm experiment, which required a high amount of medium  
133 (continuous flow) containing the peptides, was not compatible with the realization of a whole  
134 dose response study. When *P. aeruginosa* PA14 was exposed to CNP or BNP in dynamic  
135 conditions, we observed a highly significant decrease of the biofilm volume ( $-81.0 \pm 6.6\%$   
136 and  $-80.3 \pm 4.4\%$ ,  $p < 0.001$ , respectively) and of the biofilm average thickness ( $-72.3 \pm 9.7\%$   
137 and  $-76.0 \pm 4.8\%$ ,  $p < 0.001$ , respectively) (Fig. 1A). These effects were associated with a  
138 reduction of the number and mean size of mushroom-like structures in the biofilm (Fig. 1B).  
139 Exposure of *P. aeruginosa* MPAO1 to CNP for 24 h in static conditions led to similar results  
140 with a marked decrease of biofilm formation (Fig. 2A) associated to a reduction of volume  
141 and thickness ( $-83.3 \pm 1.9\%$  and  $-91.7 \pm 2.2\%$ ,  $p < 0.001$ , respectively) (Fig. 2B).

142

### 143 **Effect of specific antagonist and agonists of natriuretic peptide receptors on *P.*** 144 ***aeruginosa* biofilm formation and virulence activity.**

145 The biofilm formation activity of *P. aeruginosa* MPAO1 was also studied in the presence of  
146 isatin, an antagonist of both A and C subtypes natriuretic peptide receptors (NPR-A and NPR-  
147 C). Administered alone for 24 h, isatin affected the organization of the biofilm which  
148 appeared more irregular with the presence of large water channels (Fig. 2A). We also noted a  
149 limited and non-significant increase of the biomass and thickness of the biofilm (Fig. 2B).



150 However, when *P. aeruginosa* MPAO1 was exposed to isatin ( $10^{-5}$  M) for 20 min prior to the  
151 administration of CNP, the inhibitory effect of CNP on the biofilm formation activity of the  
152 bacterium was abolished (Fig. 2A and 2B).

153 As we have previously shown that natriuretic peptides enhance *P. aeruginosa* cytotoxicity  
154 activity and global virulence (16), we tested the effect of sANP, a specific NPR-A agonist,  
155 and the artificial peptide cANF<sup>4-23</sup>, a specific NPR-C agonist, on bacterial cytotoxicity  
156 towards lung cells. The peptide cANF<sup>4-23</sup> (NPR-C agonist) caused a strong increase of *P.*  
157 *aeruginosa* cytotoxicity activity against A549 human lung epithelial cells ( $+ 98.3 \pm 16.9\%$ ;  $p$   
158  $< 0.001$ ) (Fig. 2C). In parallel, we observed that the sANP peptide (NPR-A agonist), also  
159 enhanced the *P. aeruginosa* cytotoxicity in the same cell model, but to a lower extent ( $+ 25.9$   
160  $\pm 12.1\%$ ;  $p < 0.05$ ) (Fig. 2C).

161  
162

163 ***In silico* study of a functional C-type natriuretic peptide receptor ortholog in *P.***  
164 ***aeruginosa*.**

165 In mammalian cells, CNP preferentially binds the NPR-C receptor and has a weak affinity for  
166 the NPR-A receptor (25), whereas BNP strongly binds NPR-A and has a weak affinity for  
167 NPR-C. The observations that the effects of CNP were blocked by the NPR-C antagonist  
168 isatin, and mimicked by the NPR-C agonist cANF<sup>4-23</sup>, suggested the presence of a bacterial  
169 ortholog of NPR-C in *P. aeruginosa*. This hypothesis was investigated using *in silico*  
170 approaches. Searching in bibliographic data, we noted a DALI structure similarity search (26)  
171 that identified a *P. aeruginosa* protein, AmiC, which shows structural homologies with the  
172 binding domain of natriuretic peptides receptors (27, 28). Using tools from the Protein Data  
173 Bank in Europe [<http://www.ebi.ac.uk/pdbe/>], we investigated in detail the structure of *P.*  
174 *aeruginosa* AmiC bound to its known ligand acetamide. We observed that it shares high  
175 structural similarity with the natriuretic peptide receptor subtype NPR-C bound to one of its

176 agonists, the atrial natriuretic peptide (ANP; *data not shown*). This supports the hypothesis  
177 that AmiC could act as a bacterial natriuretic peptide sensor.

178 The 3D structure of *P. aeruginosa* AmiC (PDB ID: 1PEA) (29) was compared to that of the  
179 human NPR-C receptor (hNPR-C) (PDB ID: 1JDP) (30). Using the STAMP software (31),  
180 we superimposed an AmiC monomer onto each protomer of the structure of an hNPR-C  
181 dimer in complex with CNP. Although the sequences of these proteins share only 19%  
182 identity and 26% similarity, these two proteins present an overall similar 3D structure in the  
183 homodimeric form (Fig. 3A), particularly at the interface between each monomer which is  
184 considered to be the binding cleft of hNPR-C. The highest similarity was found in the helix of  
185 AmiC corresponding to the region involved in the binding of CNP to hNPR-C. Next, we split  
186 the AmiC monomer into two parts, corresponding to the two lobes of the protein. The first  
187 lobe consists of amino acids 8 to 123 and 261 to 338, and the second lobe of amino acids 124  
188 to 260 and 339 to 375 of AmiC. Then, we superimposed each lobe on the 3D structure of  
189 hNPR-C (Fig. 3B). Each lobe of hNPR-C and AmiC showed a high level of secondary  
190 structure conservation with similar 3D positions, reinforcing the concept of structural  
191 homology between these two proteins. Using GASH Structural Superposition software (32)  
192 on each lobe of hNPR-C and AmiC, we observed that each of these two proteins has  
193 254 residues estimated to be involved in equivalent secondary structures, covering 61.7% of  
194 the AmiC sequence. The hinge between the two lobes was essentially formed with flexible  
195 loops. Once superimposed, we looked at the amino acids involved in the binding of CNP,  
196 supporting the hypothesis that AmiC and hNPR-C bind the peptide in a similar fashion.  
197 Supplemental material Table S1 lists AmiC amino acids that are potentially involved in CNP  
198 binding, and the corresponding amino acids involved in the binding of hNPR-C to CNP as  
199 described by He *et al.* (33). Amino acids located within 4 Å of CNP in the binding sites of  
200 both hNPR-C and AmiC are shown schematically in the supplemental material (Fig. S1). A

201 total of 24 amino acids from AmiC and 21 residues from hNPR-C with equivalent physico-  
202 chemical properties are located in the same regions and may stabilize the binding of CNP to  
203 AmiC and NPR-C equivalently.

204 We also determined the virtual capacity of *P. aeruginosa* AmiC to bind natriuretic peptides  
205 (BNP and CNP) by molecular docking using AutoDock (34). Docking revealed that CNP can  
206 bind to AmiC at the dimer interface of the bacterial protein (See Fig. S2A, B in the  
207 supplemental material). The most relevant calculated binding site of CNP on AmiC requires  
208 13 amino-acids, Arg64, Glu68, Ala90, Met92, Pro93, Glu96, Arg97, Asp99, Glu112, Tyr113,  
209 Pro115, Asn116 and Tyr366. Since these predictions were obtained by an *in silico* study, the  
210 involvement of each of these residues need to be validated by constructing and studying the  
211 13 correspondent mutant proteins, which will be addressed in a future work. In contrast, we  
212 observed *in silico* that BNP has no affinity to the AmiC protein. The NPR-C antagonist isatin  
213 also appeared capable of binding to the AmiC dimer interface (Fig. S2C, D). In this case, the  
214 AmiC amino-acids involved in the recognition of isatin are predicted to be Ile71, Arg72,  
215 Val94 and Arg97. These amino acids are located in the same region involved as the putative  
216 CNP binding site, and one residue (Arg97) could play a key role since it is potentially  
217 interacting with both CNP and isatin.

218

### 219 **Effect of CNP on the expression of the amidase operon in *P. aeruginosa*.**

220 In *P. aeruginosa*, AmiC is considered as a sensor protein. In the absence of stimulation (basal  
221 condition), AmiC binds to the AmiR protein, an antitermination factor, which down-regulates  
222 the transcription of the *amiEBCRS* operon (35). Upon binding of acetamide AmiC releases  
223 AmiR, triggering the transcription of the whole *ami* operon (36). In order to verify that CNP  
224 could act in the same manner as acetamide on AmiC, we measured by qRT-PCR *amiB*, *amiC*,  
225 *amiE* and *amiR* mRNA levels 1 hour after exposure of the bacterium to CNP. The *amiC*,

226 *amiE*, *amiR* mRNA levels were significantly increased ( $2.97 \pm 0.2$ ,  $3.66 \pm 0.39$ , and  $2.73 \pm$   
227  $0.67$  fold,  $p < 0.001$ , respectively) after 1 hour of exposure to CNP ( $10^{-6}$  M) (Fig. 4), whereas  
228 the amount of *lecB* mRNA, encoded by a gene adjacent to the *ami* operon, was not modified.  
229 In contrast, BNP ( $10^{-6}$  M) has no significant effect on the whole *ami* operon transcriptional  
230 activity (data not shown).

231

### 232 **AmiC binding with natriuretic peptide receptor agonists.**

233 In order to confirm that the action of CNP and isatin on *P. aeruginosa* is mediated directly  
234 through AmiC, and is not an indirect effect, we investigated their binding to AmiC *in vitro*.  
235 Recombinant AmiC was expressed in *E. coli*. We first confirmed the oligomeric state of  
236 AmiC using native gel electrophoresis and analytical gel filtration (See Fig. S3 in the  
237 supplemental material). These methods showed an apparent molecular weight of  $120 \pm 30$   
238 kDa and  $136 \pm 3$  kDa, respectively. These results are most consistent with AmiC (protomer  
239 MW: 45.5 kDa) forming a dimer with an irregular shape, as predicted by the model (Fig. S3).  
240 We then tested the interaction of AmiC with natriuretic peptide receptor agonists and isatin  
241 using microscale thermophoresis (Fig. 5A). AmiC bound to CNP tightly, showing a  
242 dissociation constant ( $K_D$ ) of  $2.0 \pm 0.3$   $\mu$ M (Fig. 5). In contrast, we observed that BNP  
243 possessed no affinity for AmiC (Fig. 5A). Isatin could also bind to AmiC, although in this  
244 case the interaction was not so strong, showing a  $K_D$  of  $600 \pm 200$   $\mu$ M (Fig. 5A and 5B).  
245 Additionally, we measured the interactions of other natriuretic peptide receptor agonists.  
246 sANP, a specific NPR-A agonist, showed a weak interaction with AmiC, which had clearly  
247 not proceeded to completion at 900  $\mu$ M (Fig. 5B), the highest concentration point that we  
248 were able to assess. These data clearly predict that the  $K_D$  is greater than 600  $\mu$ M. Conversely,  
249 our initial experiments on osteocrin (a specific NPR-C agonist) showed a very strong  
250 interaction with AmiC, apparently tighter than we were able to determine using the available

251 instrument. This indicates a  $K_D$  value below 100 nM. In the same way, cANF<sup>4-23</sup>, another  
252 NPR-C specific agonist presented a  $K_D$  value of  $40 \pm 10 \mu\text{M}$  (Fig. 5).

253

#### 254 **Involvement of AmiC in CNP-regulated *P. aeruginosa* biofilm formation**

255 In dynamic conditions, the biofilm formed by the PA14 *amiC*- strain appeared reduced in  
256 comparison to wild-type strain PA14 WT (Fig. 6A). Conversely, the morphology of the  
257 biofilm formed by the PA14 AmiC+ strain (PA14 containing the wildtype *amiC* gene plus  
258 extra *amiC* plasmid-borne copies) or by the PA14 EV strain carrying the empty vector was  
259 similar to that of the wild-type (data not shown). Complementation of the *amiC*- strain with  
260 the *amiC* gene carried by a vector restored entirely the ability of the bacteria to form a biofilm  
261 (Fig. 6A and 6B). The measure of the biofilm biovolumes revealed  $64.5 \pm 12.5 \%$  reduction  
262 for the PA14 *amiC*- strain in comparison to the PA14 wild-type ( $p < 0.001$ ) whereas the  
263 biovolume of the biofilm generated by the complemented strain is similar to that of PA14  
264 wild-type ( $+13.7 \pm 14.6 \%$ ) (Fig. 6B). The biofilm formed by the strain PA14 AmiC+ was  
265 slightly, but non significantly, enhanced (Fig. 6B).

266 We observed that the ability of CNP to reduce biofilm formation is abolished in the *amiC*-  
267 strain (Fig. 6A and 6C), and is restored using the complemented strain ( $-71.9 \pm 12.7 \%$ ; ( $p <$   
268  $0.01$ ) (Fig. 6C). In contrast, BNP, which strongly reduced biofilm formation as well ( $-75.1 \pm$   
269  $9.9 \%$ ; ( $p < 0.001$ ) (Fig. 6C), continued to have the same impact on biofilm formation on  
270 *amiC*- mutant strain ( $-70.8 \pm 12.1 \%$ ; ( $p < 0.05$ ) (Fig. 6C).

271 In parallel, since it has been previously shown that CNP also modifies *P. aeruginosa*  
272 virulence, we tested the effect of AmiC on cytotoxicity towards lung cells. We observed that  
273 whereas the PA14 wild-type strain showed an increase in cytotoxic activity after CNP  
274 exposure ( $+76.6 \pm 17 \%$ ;  $p < 0.001$ ), the *amiC*- strain did not significantly modify its  
275 cytotoxicity in the presence of CNP when compared with controls (*amiC*- strain without CNP

276 exposure) ( $+14.7 \pm 6.0 \%$ ) (Fig. S4C). In accordance with these data, we observed that the  
277 increased production of HCN triggered by CNP on PA14 wild-type strain disappeared in the  
278 *amiC*<sup>-</sup> mutant strain (Figure S4D). It is interesting to note that the strain over-expressing  
279 AmiC (AmiC<sup>+</sup>) strongly lost its ability to kill lung cells (Figure S4A), confirming a role for  
280 AmiC in the regulation of *P. aeruginosa* virulence.

281

282 **DISCUSSION**

283

284 We have shown that *P. aeruginosa* is sensitive to micromolar concentrations of natriuretic  
285 peptides (i.e. BNP and CNP) and responds to these eukaryotic factors by a major increase in  
286 virulence (16, 17). Previously, the intra-bacterial cascade involving Vfr, PtxR, RhIR and LasR  
287 proteins, leading to a remodeling of the lipopolysaccharide (LPS) structure and an increase of  
288 HCN and exotoxin A production, was identified (16), but the nature of the *P. aeruginosa*  
289 natriuretic peptide sensors remained a central question.

290 *P. aeruginosa* is an opportunistic pathogen and in cystic fibrosis patients it is known to form  
291 biofilms in lung alveoli (2). As an exchange surface between air and blood, the alveolar  
292 epithelium formed by pneumocytes is particularly thin (0.2  $\mu\text{m}$ ). If bacteria are developing on  
293 this epithelium, they are certainly exposed to diffusible blood containing endocrine factors  
294 such as natriuretic peptides. These are also produced locally by endothelial cells (22), by lung  
295 airway epithelium cells and by nonciliated Clara cells (19). The growth of bacteria in biofilms  
296 is generally associated with a decrease of diffusible virulence factor synthesis (37). This  
297 assists in establishing a chronic infectious status, which progressively weakens the host  
298 leading to a potential lethal outcome (3). Conversely, an anticipated release of bacterial  
299 virulence factors, particularly when the bacterium is not protected by the biofilm, should  
300 trigger the host defence responses and the clearance of the pathogen and increase antibiotic  
301 sensitivity. Due to the effect on biofilm formation in *P. aeruginosa*, CNP and related  
302 molecules could potentially be interesting for therapy. CNP strongly decreased the biofilm  
303 formation activity of both *P. aeruginosa* PAO1 and PA14 strains confirming the hypothesis  
304 that virulence factor production and biofilm formation are inversely related. This is consistent  
305 with our previous results showing that CNP enhances cAMP production in *P. aeruginosa*  
306 (17). Indeed, cAMP and c-di-GMP synthesis appear to be antagonistically regulated in

307 bacteria. Whilst c-di-GMP promotes the growth of biofilms (23), cAMP prevents *P.*  
308 *aeruginosa* biofilm formation (38). The present results are also in agreement with the  
309 observation that CNP induces a rearrangement of the *Pseudomonas* LPS structure (17) and  
310 enhances *algC* mRNA synthesis (16), since LPS contributes to the bacterial surface properties  
311 and AlgC is involved in the synthesis of the LPS core (39).

312 In order to identify the potential natriuretic peptide sensor in *P. aeruginosa*, we started from  
313 the hypothesis that this protein should show structural homology with the binding domain of  
314 eukaryotic natriuretic peptide receptors. Van den Akker *et al.* (28) have shown that the  
315 binding domain of the eukaryotic natriuretic peptide receptor subtype A (NPR-A), a receptor  
316 which binds ANP and BNP, exhibits a type-I periplasmic binding protein fold and that the  
317 closest structural neighbour of this ANP binding domain is the AmiC protein from *P.*  
318 *aeruginosa*. Analysis of the crystal structure of the dimerized hormone binding domain of  
319 NPR-A and of the structure of the AmiC-AmiR complex showed that in NPR-A and AmiC,  
320 the same residues in helices  $\alpha 3$  and  $\alpha 7$  interact respectively with ANP and AmiR.  
321 Furthermore, the pocket forming the ANP binding site on NPR-A overlaps with the  
322 recognition site of AmiR on AmiC (27, 28). In eukaryotes, NPR-A is known as a receptor-  
323 enzyme coupled to cGMP synthesis. However, CNP does not lead to an increase of cGMP in  
324 *P. aeruginosa* whereas it stimulates cAMP synthesis (17). Then, we looked for possible  
325 similarities between AmiC and human NPR-C (hNPR-C), the sole ANP and CNP eukaryotic  
326 receptor coupled to an adenylate cyclase activity (40). We observed *in silico* that hNPR-C and  
327 *P. aeruginosa* AmiC proteins present 19% identity and 26% similarity, confirming the  
328 structural homology of eukaryotic natriuretic peptides receptors and AmiC (27). The AmiC  
329 binding domain helix  $\alpha 3$  is delineated by amino-acids 85 to 99 (41). In the present study, we  
330 noted that the binding of CNP to AmiC would occur in the same region; 6 of the 13 CNP  
331 binding amino acids (Ala90, Met92, Pro93, Glu96, Arg97 and Asp99) are in helix  $\alpha 3$ ,



332 suggesting that CNP could enter into competition with AmiR for binding on AmiC. We  
333 compared all the amino acids putatively involved in the AmiC-CNP interaction to those  
334 observed in the interaction of CNP with NPR-C in eukaryotes (33). The physico-chemical  
335 properties of amino-acids involved in these two interactions (*i.e.* CNP/AmiC in bacteria and  
336 CNP/NPR-C in eukaryotes) showed a remarkably high level of conservation (this study). We  
337 determined that AmiC (like NPR-C) (40) is functionally expressed as a dimer reinforcing the  
338 hypothesis that AmiC could have a CNP binding activity.

339 In *P. aeruginosa*, the *amiC* gene appears highly conserved (99.57% sequence homology and  
340 total preservation of the amino acid sequence between PAO1 and PA14 strains) suggesting  
341 that a crucial role is played by this protein in this species. The *amiC* gene is encoded in an  
342 operon including the *amiL*, *amiE*, *amiB*, *amiC*, *amiR* and *amiS* genes (36)  
343 [[www.pseudomonas.com](http://www.pseudomonas.com)]. AmiC activation by acetamide, its classical ligand in bacteria, is  
344 known to provoke the release of AmiR leading to the transcriptional activation of the whole  
345 *ami* operon (35, 41). We therefore hypothesized that, if CNP acts on AmiC in a similar  
346 fashion to acetamide, it should also induce the *ami* operon expression. This was confirmed by  
347 our observations. CNP actually induced *amiERC* transcription, and the specificity of this  
348 action was reinforced by the lack of effect of BNP on the *ami* operon transcription (data not  
349 shown). As mentioned elsewhere, *P. aeruginosa* AmiC appears functionally related to hNPR-  
350 C and it is interesting to note that, in eukaryotic cells, NPR-C preferentially binds to CNP  
351 with a higher affinity than to BNP (33). Such highly conserved specific recognition modes  
352 between eukaryotic and bacterial sensors have been previously observed in *Helicobacter*  
353 *pylori* for the human hormone somatostatin whose bacterial sensor can only recognize one  
354 agonist sub-type (42).

355 The issue of how CNP contacts AmiC in *P. aeruginosa* needs to be addressed, as  
356 AmiC is predicted to be located in the bacterial cytoplasm [[www.pseudomonas.com](http://www.pseudomonas.com)]. This

357 means that CNP must enter into the bacterium before binding to AmiC. Such an assumption is  
358 conceivable since another eukaryotic peptide, dynorphin, was shown able to cross freely the  
359 membrane of *P. aeruginosa*, inducing an increased virulence (14). This behaviour was  
360 explained by the high percentage of basic and hydrophobic amino acid residues present in  
361 dynorphin (76%) (43). Similarly, CNP contains 73% basic or hydrophobic amino acids.  
362 Moreover, natriuretic peptides have been shown to form pores in phospholipid bilayers (44)  
363 and could therefore manage their own entrance into the bacterium. However, we cannot  
364 exclude that AmiC protein could, in some conditions, reach the *P. aeruginosa* inner  
365 membrane. Indeed, AmiC belongs to the type I periplasmic binding fold protein family and  
366 the Uniprot data base claims that AmiC is located in the periplasmic space [[www.uniprot.org](http://www.uniprot.org);  
367 reference P27017]. One protein being located in multiple bacterial compartments to undertake  
368 unrelated functions is not unprecedented. Indeed, the EF-Tu protein, which acts in the  
369 bacterial cytoplasm as a ribosomal elongation factor, was shown to migrate upon stress to the  
370 plasma membrane and acquires new functions as a sensor molecule (45). In *P. aeruginosa*,  
371 EF-Tu, after migration into bacteria membrane also behaves as a plasminogen binding protein  
372 (46). To support this hypothesis, we observed, using AmiC antibodies, that whilst AmiC  
373 protein is mainly located into the bacterial cytosol, a proportion of AmiC is associated with  
374 the inner membrane compartment (See supplemental material Fig. S6). In this case, we  
375 assumed that CNP is delivered to AmiC proteins that have reached the inner membrane  
376 through protein transporters, for example. To reinforce this hypothesis we observed that a  
377 protein encoded by the gene PA14\_64270 (ortholog of PA4858 in PAO1) possesses a high  
378 structural similarity with AmiC, could virtually bind CNP, and is necessary for the CNP effect  
379 on bacterial biofilm (See supplemental material Fig. S5).

380 Although a couple of bacterial sensors for eukaryotic messengers have been identified (6),  
381 to date none of the bacterial sensors structurally identified possess a similarity with the

382 eukaryotic counterpart receptor. For example, *E. coli* QseC, the sensor protein for  
383 epinephrine, is an histidine sensor kinase that twice crosses the bacterial membrane, and  
384 which appears structurally very different from the G-coupled protein receptor (9, 47). In the  
385 same vein, interferon- $\gamma$ , which binds the eight transmembrane *P. aeruginosa* OprF protein  
386 (48), in mammals activates a single transmembrane receptor. Since our data suggest that  
387 AmiC and hNPR-C possess a homologous 3D-structure, and that CNP acts as an agonist on *P.*  
388 *aeruginosa*, we undertook to study the pharmacological profile of AmiC, as happens in  
389 eukaryotic receptor studies.

390 Results from our pharmacological studies carried out using cANF<sup>4-23</sup>, a NPR-C agonist, and  
391 isatin, an NPR-A/NPR-C antagonist (49) also support the hypothesis that AmiC acts as an  
392 ortholog of eukaryotic NPR-C. *In silico* molecular modeling revealed that isatin binding to  
393 AmiC would require only 4 amino acids but, as expected for an antagonist, these amino acids  
394 are located in the potential CNP binding pocket of AmiC. The antagonist effect of isatin was  
395 confirmed experimentally. Isatin had no intrinsic activity on the biofilm formation activity of  
396 *P. aeruginosa* but inhibited the effect of CNP. Conversely, the artificial peptide cANF<sup>4-23</sup>, a  
397 specific NPR-C agonist in eukaryotes, was able to reproduce the effects of CNP on *P.*  
398 *aeruginosa* global virulence on lung cells, as observed by Blier *et al.* (16). These data  
399 reinforced the hypothesis that AmiC acts as a sensor for natriuretic peptides, but do not  
400 exclude the possibility that these peptides bind to another protein that in turn affects AmiC. In  
401 order to validate our hypothesis, we purified *P. aeruginosa* AmiC recombinantly produced in  
402 *E. coli* and checked the interactions of CNP, BNP, isatin, osteocrin (an NPR-C agonist) (50),  
403 cANF<sup>4-23</sup> (an NPR-C agonist) and sANP (an NPR-A agonist) (51) with AmiC. As expected,  
404 AmiC bound strongly to CNP, showing a  $K_D$  of  $2.0 \pm 0.3 \mu\text{M}$ , consistent with the effective  
405 concentrations of CNP on *P. aeruginosa*. In contrast, BNP does not interact with AmiC  
406 suggesting that BNP acts on *P. aeruginosa* through another bacterial target, a hypothesis

407 strongly reinforced by the fact that BNP retains an effect on biofilm formation in the *amiC*-  
408 mutant strain (this study). Isatin also showed a specific interaction, although at a significantly  
409 higher concentration ( $600 \pm 200 \mu\text{M}$ ). As normal blood isatin concentrations are usually of  
410 the order of 0.3-1.3  $\mu\text{M}$  (52), this implies that *in vivo*, isatin is unlikely to be at a sufficiently  
411 high concentration to antagonise the effects of CNP on AmiC. Finally, we attempted to test  
412 specific agonists of NPR-A and NPR-C, to confirm that AmiC reflects NPR-C specificity.  
413 sANP showed some interaction with AmiC, but this interaction was weak, and indeed was not  
414 complete at the highest ligand concentration available, indicating a  $K_D$  (likely considerably) in  
415 excess of 600  $\mu\text{M}$ , explaining probably the weak effect of this peptide on bacteria cytotoxicity  
416 (this study). In contrast, initial experiments on osteocrin showed that it bound sufficiently  
417 strongly that it was beyond the limit of detection of our experiment (100 nM), and cANF<sup>4-23</sup>  
418 possesses a  $K_D$  in the same range as CNP. These observations suggest that AmiC does indeed  
419 show the same specificity as NPR-C, and is selective against NPR-A agonists. Finally, using  
420 an *amiC* deficient mutant strain and its complemented derivative, we physiologically  
421 validated the crucial role of AmiC sensor in CNP effects on *P. aeruginosa* virulence  
422 expression and biofilm formation.

423 The last puzzling question is the reason why a bacterium such as *P. aeruginosa* should  
424 express a sensor system for peptides which are naturally labile molecules only present in the  
425 inner medium of vertebrates. The fact that the CNP sensor should also be a binding site for  
426 isatin provides some clues on a possible explanation. Isatin, or indole-2,3-dione, is an  
427 interspecies signal molecule produced in large amounts by bacteria such as *Escherichia coli*  
428 (53). Until now, Pseudomonads were not thought to synthesize isatin or isatin related  
429 compounds but as shown by Lee *et al.* (54) and in the present study, *P. aeruginosa* can detect  
430 this molecule. Isatin is also produced in large amounts by vegetals where it is a precursor in  
431 auxin biosynthesis (55) and by mammalian tissues including lungs (56, 57). Therefore, in the

432 host, *P. aeruginosa* is regularly exposed to significant amounts of isatin. As is the case for  
433 GABA (15), it will gain an advantage by adapting its behavior in response to the host  
434 physiology. Nevertheless, even if this explanation is correct, the fact that AmiC showed  
435 common properties with the eukaryotic NPR-C, including CNP recognition, remains to be  
436 explained.

437 In conclusion, the present work provides the first demonstration that bacteria are not only  
438 sensitive to eukaryotic messengers but could express eukaryotic receptor-like structures to  
439 detect human hormones. The AmiC sensor protein acts as an ortholog of the eukaryotic  
440 receptor NPR-C, acting as a CNP/Isatin sensor in *P. aeruginosa* that modulates bacterial  
441 biofilm formation. Since the CNP hormone is produced in the lung, the effects of CNP on the  
442 biofilm formation activity of *P. aeruginosa* could represent a natural defense mechanism  
443 against lung colonization. Furthermore, a natriuretic peptide receptor agonist has been used as  
444 bronchodilator drug (58). This could on the one hand pose a risk of enhancing *P. aeruginosa*  
445 virulence; and on the other it may prevent biofilm formation. This opens up the tantalising  
446 prospect that compounds in development for other indications could be re-targeted for use  
447 against biofilms.

448

449 **MATERIALS AND METHODS**

450 **Reagents and test substances.**

451 The CNP peptide was obtained from Polypeptide (Strasbourg, France). Isatin and hBNP were  
452 purchased from Sigma-Aldrich (St Quentin Fallavier, France). Osteocrin, sANP and cANF<sup>4-23</sup>  
453 were synthesized by Dr J. Leprince (PRIMACEN Platform, University of Rouen, France).  
454 DMSO was purchased from Acros Organics (Geel, Belgium).

455

456 **Bacterial strains and bacterial cultures.**

457 *Pseudomonas aeruginosa* PAO1 was obtained from an international collection (17). *P.*  
458 *aeruginosa* MPAO1 was originally from Iglewski lab. A transposon library was constructed  
459 from the MPAO1 strain at the University of Washington (59). The *P. aeruginosa* PA14 was  
460 from Harvard Medical School (Boston, MA) (60) and kindly provided by the Biomerit  
461 Research Center (Univ. Cork, Ireland). All strains used in this work are listed in Supplemental  
462 material Table S2. All strains were grown at 37°C in Luria Bertani medium (LB). For  
463 treatment with CNP, an overnight culture was diluted to OD<sub>600</sub> = 0.08 using a  
464 ThermoSpectronics (Cambridge, UK) spectrophotometer. CNP was added two hours after the  
465 onset of the culture, a time corresponding to the middle of the exponential growth phase. For  
466 all studies performed using isatin, because of its hydrophobicity this molecule was initially  
467 dissolved in a solution of dimethyl sulfoxide (DMSO) in water (5/95). The final concentration  
468 of DMSO in bacterial culture medium was kept under 0.2%. In this case, control experiments  
469 were conducted in the presence of the same final concentration of DMSO. The final bacterial  
470 density and the absence of contamination were controlled by plating.

471

472 **Biofilm formation in dynamic conditions.**

473 After 3 h of pre-culture in LB at 37°C, *P. aeruginosa* PA14 was inoculated at OD<sub>600</sub> = 0.08 in  
474 LB medium and sub-cultured for 2 h. CNP (1 µM final concentration) was added and bacteria  
475 were grown for additional 3 h. Bacteria were then washed and adjusted to OD<sub>600</sub> = 0.1 in 0.9%  
476 NaCl supplemented with CNP (1 µM). The bacterial suspensions were then used to study  
477 biofilm formation under dynamic conditions at 37°C in a three-channel flow cell as described  
478 by Bazire *et al.* (61). Briefly, each bacterial suspension was injected into a flow cell channel  
479 and bacteria were allowed to attach onto the glass coverslip for 2 h. A flow (2.5 ml/h) of LB  
480 medium containing CNP (1 µM) was then applied for 24 h. At the end of the experiments, the  
481 biofilms were stained with 5 µM Syto 61 Red dye or 5 µM SytoX (Molecular probes).  
482 Observations were made using a confocal laser scanning microscope (LSM 710 confocal  
483 laser-scanning microscope, Zeiss). The biofilm thicknesses and corresponding biovolumes  
484 were estimated by measuring field samples from at least 3 independent experiments using the  
485 COMSTAT software (62).

486

#### 487 **Biofilm formation in static conditions.**

488 The static bacterial biofilm formation activity was determined after 24 h of development. Pre-  
489 cultures of *P. aeruginosa*, containing the pSMC2.1 plasmid harbouring the GFP gene, were  
490 grown overnight at 37°C. An aliquot of pre-culture (100 µl) was then added to 5 ml of LB  
491 medium supplemented with 400 µg/mL kanamycin. Tested molecules or the corresponding  
492 excipient were added after 2 hours of sub-culture. Three hours later, bacteria were collected  
493 by centrifugation (7,000 x g, 10 min). The supernatant was then removed and the pellets were  
494 resuspended in 25 ml of physiological solution (0.9% NaCl) and adjusted to OD<sub>600</sub> = 0.1.  
495 Sterile glass slides, previously cleaned with 4% TFD4 in water (Dutscher, Brumath, France),  
496 were layered in Petri dishes and covered by the bacterial suspension. The slides were then  
497 incubated for 2 h at 37°C in static condition. At the end of the attachment period the

498 physiological solution was replaced by LB medium and incubated for 24 h at 37°C in static  
499 condition. The slides were then rinsed twice in 25 ml sterile phosphate buffered saline (PBS  
500 0.1 M, pH=7.4) and bacteria were heat fixed. Biofilms were observed using a confocal laser  
501 scanning microscope (LSM 710, Zeiss). Images were treated using a 3 median filter and  
502 segmented using Zen® 2009 software (Zeiss). The volumes and thicknesses of the biofilms  
503 were estimated as described elsewhere (62). All 2D images are representative from 15  
504 observations.

505

#### 506 ***In silico* studies.**

507 The STAMP software (31) was used within VMD (63) on a single computer. The GASH  
508 Structural Superposition software (32) was used on the web server ([http://sysimm.ifrec.osaka-  
509 u.ac.jp/gash/](http://sysimm.ifrec.osaka-u.ac.jp/gash/)). Potential ligand/protein interactions were investigated *in silico* by the  
510 molecular docking technique using the amidase sensor protein of *P. aeruginosa* AmiC  
511 (Protein Data Bank 1QO0). Essential hydrogen atoms, Kollman united atom type charges and  
512 solvation parameters were added with the aid of AutoDock tools (34). Affinity (grid) maps of  
513 20X20X20 Å grid points and 0.375 Å spacing were generated using the Autogrid program  
514 (34). AutoDock parameter set- and distance-dependent dielectric functions were used in the  
515 calculation of the van der Waals and electrostatic terms, respectively. Docking simulations  
516 were made using the Lamarckian genetic algorithm (LGA). Initial position, orientation and  
517 torsions of the ligand molecules were set randomly. Each docking experiment was derived  
518 from three different runs that were set to terminate after a maximum of 1,000,000 energy  
519 evaluations. The population size was set to 100.

520

#### 521 **Quantitative reverse transcription-PCR (qRT-PCR) mRNA assays.**



522 RNA extraction and quantification were realized as previously described (61) using bacteria  
523 grown for 3 h in LB. The primers employed are presented in supplemental material Table S3.  
524 The transcription level of 16S rRNA was used as the endogenous control. PCR reactions were  
525 performed in triplicate and the standard deviations were lower than 0.15 CT. Relative  
526 quantification of mRNA was obtained according to Bazire *et al.* (64) using the comparative  
527 CT ( $2^{-\Delta\Delta C_T}$ ) method (65).

528

#### 529 **Lung epithelial cells cytotoxicity tests.**

530

531 The human A549 lung epithelial cell type II line (ATCC-CCL185TM) (American Type  
532 Culture Collection, Manassas, VA) was grown at 37°C in 5% CO<sub>2</sub> atmosphere in Dulbecco's  
533 modified Eagle's medium (DMEM, Lonza) supplemented with 10% fetal calf serum (Lonza)  
534 and 1% of penicillin and streptomycin (Penistrep, Lonza). For cytotoxicity assays, cells were  
535 seeded in 24 well plates at a final density of  $3 \times 10^5$  cells per well, and grown for 48 h before  
536 use. A minimum of 24 h before infection assays, cells were deprived of antibiotics and fetal  
537 calf serum by addition of a fresh serum- and antibiotics-free medium. For the assays, A549  
538 cells were incubated for 2 h with control or pre-treated *P. aeruginosa* PA14 at a multiplicity  
539 of infection of 10. Bacterial cytotoxicity was determined by measurement of lactate  
540 dehydrogenase (LDH) release. LDH is a stable cytosolic enzyme that diffuses into the culture  
541 medium upon cell lysis. This marker of cytotoxicity was assayed using the Cytotox 96  
542 enzymatic assay (Promega, Charbonnières, France) as previously described (17).

543

#### 544 ***P. aeruginosa* AmiC purification.**

545 To express *P. aeruginosa* AmiC in *E. coli*, a synthetic codon optimized gene was prepared  
546 (MWG Operon) with a leader sequence of MHHHHHHSSGVLDLTENLYFQS to provide a  
547 cleavable 6\*His tag. This was cloned into the *Nco*I and *Hind*III restriction sites of the pET-

548 Duet1 vector (Novagen). This construct was transformed into Rosetta2 (DE3) cells  
549 (Novagen), and grown in 500 mL of ZYM-5052 media (66) at 37°C until mid-log phase, and  
550 then at 20°C overnight. Cells were harvested by centrifugation at 4,750 ×g for 30 min, and  
551 resuspended in 20 mM Tris-HCl, 0.5 M NaCl, 20 mM imidazole pH 8.0 (buffer A). Cells  
552 were lysed using a sonicator, and clarified by centrifugation at 20,000 × g. The protein was  
553 purified using a 1 mL HisTrap crude FF column and a Superdex 200 16/600 hr column (GE  
554 Healthcare), using an ÄKTExpress system (GE Healthcare) at 4°C. Lysate was loaded over  
555 the IMAC column at 0.25 ml.min<sup>-1</sup>, and washed with buffer A. AmiC was eluted in buffer A  
556 supplemented with 250 mM imidazole pH 8.0 and loaded onto the gel filtration column. The  
557 protein was then eluted isocratically in 10 mM Hepes-NaOH, 0.5 M NaCl pH 7.0. DMSO was  
558 added to the purified protein to 1% (v/v), and the protein was concentrated using a Vivaspin  
559 20 centrifugal concentrator (Sartorius; 10 kDa molecular weight cut-off) at 4°C.

560

#### 561 **Microscale thermophoresis.**

562 AmiC was labeled using the RED-NHS Labeling kit (NanoTemper Technologies). The  
563 labeling reaction was performed according to the manufacturer's instructions in the supplied  
564 labeling buffer applying a concentration of 20 μM protein (molar dye:protein ratio ≈ 2:1) at  
565 RT for 30 min. Unreacted dye was removed with the supplied dye removal columns  
566 equilibrated with MST buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM MgCl<sub>2</sub>). The  
567 label:protein ratio was determined using photometry at 650 and 280 nm. Typically, a ratio of  
568 0.8 was achieved.

569 AmiC, either labelled (CNP, isatin, BNP and cANF<sup>4-23</sup>) or unlabeled (sANP, osteocrin), was  
570 adjusted to 100 nM with MST buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM  
571 MgCl<sub>2</sub>) supplemented with 0.05 % Tween-20. CNP, isatin, sANP or osteocrin were dissolved  
572 in MST buffer supplemented with 0.05 % Tween-20. A series of 1:1 dilutions were prepared

573 in the identical buffer, producing ligand concentrations ranging from 13 nM to 434  $\mu$ M  
574 (CNP), 3.12  $\mu$ M to 3 mM (isatin), 892 nM to 1.8 mM (sANP), 28 nM to 450  $\mu$ M (BNP), 68  
575 nM to 1.1 mM (cANF<sup>4-23</sup>) and 120 pM to 4  $\mu$ M (osteocrin). For thermophoresis, each ligand  
576 dilution was mixed with one volume of AmiC, which leads to a final concentration of AmiC  
577 of 50 nM and final ligand concentrations at half of the ranges above. After 5 min incubation,  
578 followed by centrifugation at 10,000  $\times g$  for 10 min, approximately 4  $\mu$ L of each solution was  
579 filled into Monolith NT Standard Treated Capillaries (NanoTemper Technologies GmbH).  
580 Thermophoresis was measured using a Monolith NT.115 or Monolith NT.LabelFree  
581 instrument (NanoTemper Technologies GmbH) at an ambient temperature of 25°C with 5  
582 s/30 s/5 s laser off/on/off times, respectively. Instrument parameters were adjusted with 50 %  
583 LED power and 20-40% MST power. Data of three independently pipetted measurements  
584 were analyzed (NT.Analysis software version 1.5.41, NanoTemper Technologies) using the  
585 signal from Thermophoresis + T-Jump. Following data analysis, the thermophoresis signals  
586 were fitted to formula for  $K_D$  from the law of mass action:

$$587 \quad f(c) = \text{unbound} + \frac{(\text{bound} - \text{unbound}) \times ([\text{AmiC}] + c + K_D - \sqrt{([\text{AmiC}] + c + K_D)^2 - (4 \times [\text{AmiC}] \times c)})}{2 \times [\text{AmiC}]}$$

588 Where  $f(c)$  is the observed thermophoresis signal, *unbound* is the signal in the absence of  
589 ligand (normalized to 0), *bound* is the signal at infinite ligand concentration,  $c$  is the  
590 concentration of ligand in the same units as AmiC concentration, and  $K_D$  is the dissociation  
591 constant. The data were fitted using GraphPad Prism version 5, and figures for  
592 thermophoresis were generated using this software. For cANF<sup>4-23</sup>, incubation with AmiC  
593 resulted in a dose-dependent increase in AmiC fluorescence, which confounds the MST  
594 signal. This increase was therefore measured using the MST device, and the data fitted to the  
595 above equation. To confirm that the fluorescence increase was related to binding, samples  
596 were heated to 95°C for 10 min with 2% (w/v) SDS and 20 mM DTT, and retested: addition  
597 of cANF<sup>4-23</sup> no longer increased fluorescence.

598

599 **Statistical analysis.**

600 The non parametric Mann-Whitney test was used to compare the means within the same set of  
601 experiments.

602

603 **Supplemental material figures and tables**

604 **Supplemental Table S1.** List of amino acids potentially involved in the binding of CNP to  
605 NPR-C and AmiC.

606

607 **Supplemental Table S2.** List of Strains and plasmids used in this study.

608

609 **Supplemental Table S3.** Primers used in this study.

610

611 **Supplemental Figure S1.** 2D ligand interaction diagrams. 2D representation of the CNP  
612 surrounded by amino acids of hNPR-C (a) and AmiC (b) located within 4 Å of the peptide.

613

614 **Supplemental Figure S2.** Modelisation of AmiC sensor and CNP or isatin interactions by  
615 molecular docking approach.

616

617 **Supplemental Figure S3.** Analytical native gel electrophoresis and size exclusion  
618 chromatography.

619

620 **Supplemental Figure S4.** Involvement of AmiC protein on PA14 cytotoxicity.

621

622 **Supplemental Figure S5.** Involvement of the transporter PA14\_64270 (gene PA14\_64270)  
623 orthologs of the protein PA4858 in PAO1, in the effect of CNP on both biofilm and virulence  
624 activity.

625

626 **Supplemental Figure S6.** AmiC location using Dot-Blot analysis.

627

628

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630

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637

639

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855  
856

857 **Figure legends**

858

859 **Figure 1. Biofilm formation by *Pseudomonas aeruginosa* PA14 exposed to C-type**

860 **Natriuretic Peptide (CNP) or Brain Natriuretic Peptide (BNP) in dynamic condition. (A)**

861 COMSTAT analyses of biofilms of *P. aeruginosa* PA14 control (PA14) or exposed to CNP

862 ( $10^{-6}$  M) or BNP ( $10^{-6}$  M). (\*\*\* =  $p < 0.001$ ). Data are the means of twelve samples from

863 six independent experiments for control bacteria, ten samples from six independent

864 experiments for CNP-exposed bacteria and six samples from two independent experiments for

865 BNP-exposed bacteria. (B) 3D-shadow representations of the biofilm structures developed

866 under dynamic condition by *P. aeruginosa* PA14 control, exposed to CNP or exposed to BNP

867 at 37 °C for 24 h in LB broth. Biofilms were stained with the Syto 61 Red dye or SytoX dye

868 and observed by confocal laser scanning microscopy.

869

870 **Figure 2. Effect of antagonists and agonists of natriuretic peptide receptors. (A) 2D black**

871 **and white images resulting from CLSM observations of MPAO1 24-h biofilms obtained in**

872 **static condition after growth in control condition, exposed to CNP ( $10^{-6}$  M), exposed to isatin**

873 **( $10^{-5}$  M) or exposed to both isatin and CNP. (B) COMSTAT analyses of biofilms of *P.***

874 ***aeruginosa* MPAO1 exposed to CNP (+ CNP), exposed to isatin (+ Isa) or exposed both to**

875 **isatin and CNP (+Isa + CNP). Data are the mean of three independent experiments for CNP**

876 **exposition, and two independent experiments for isatin and isatin + CNP exposition. (\*\*\*)=**

877  **$p < 0.001$ ; NS: Not significantly different). (C) Cytotoxic activity of PA14 alone (control),**

878 **PA14 exposed to NPR-C agonist cANF<sup>4-23</sup> ( $10^{-6}$  M) or PA14 exposed to NPR-A agonist sANP**

879 **( $10^{-6}$  M). The cytotoxic effect of the bacterium was determined by measuring lactate**

880 **dehydrogenase (LDH) accumulation in the medium. Data are the means of nine samples from**

881 **three independent experiments for cANF<sup>4-23</sup> and six samples from two independent**

882 **experiments for sANP. \*\*\*: Significantly different ( $p < 0.001$ ). \*\*: Significantly different**

883 **( $p < 0.01$ ). \*: Significantly different ( $p < 0.05$ ).**

884

885 **Figure 3. Cartoon representation of AmiC (pdb:1PEA), in red, and hNPR-C**

886 **(pdb:1JDP), in blue, superimposed using STAMP algorithm. (A) Overall superimposition**

887 **of AmiC and NPR-C, shown in cartoon form. CNP peptide is colored green. (B)**

888 **Superimposition by lobes of AmiC and hNPR-C. The lobe on the left corresponds to amino**

889 **acids 8 to 123 and 261 to 338 of AmiC. The lobe on the right corresponds to amino acids 124**

890 to 260 and 339 to 375 of AmiC. These images were made using VMD (Humphrey et al.,  
891 1996).

892

893 **Figure 4. Effect of C-type Natriuretic Peptide (CNP) on expression level of genes from**  
894 **the *ami* operon.** Expression levels of *ami* genes in PAO1-treated bacteria relative to those in  
895 PAO1 non-treated (control). RNAs were extracted 1 h after PAO1 exposure to physiologic  
896 solution (control) or CNP ( $10^{-6}$  M) and assayed by quantitative real-time reverse transcription-  
897 PCR (qRT-PCR). These data are the mean of two independent experiments. \*\*\* =  $p <$   
898 0.001; NS: Not significantly different.

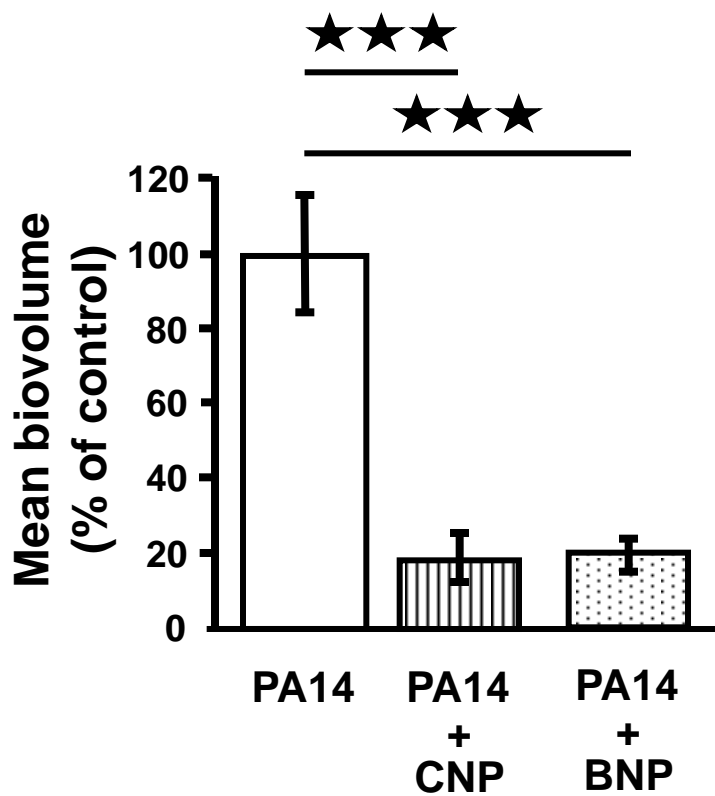
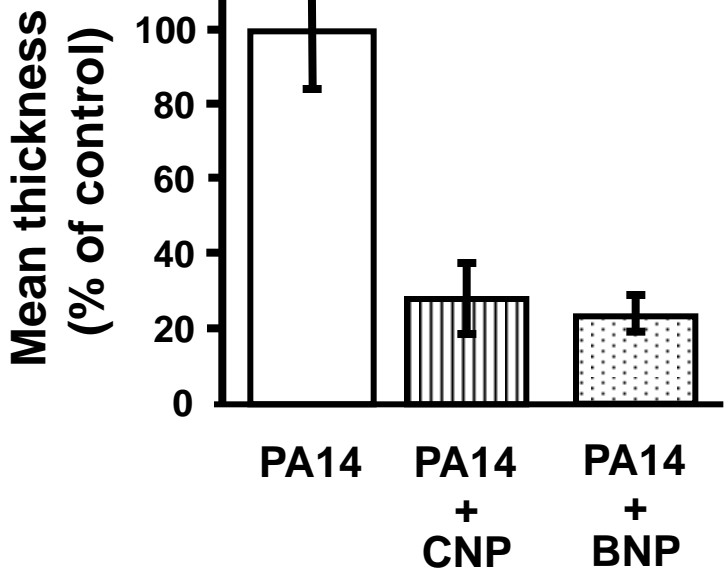
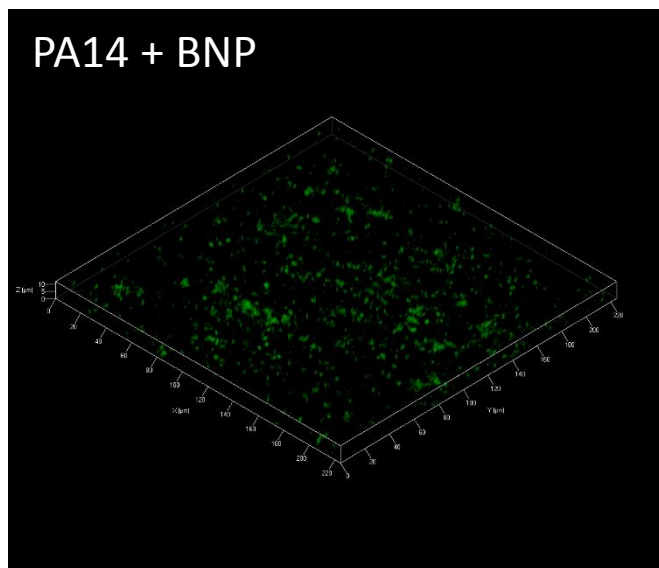
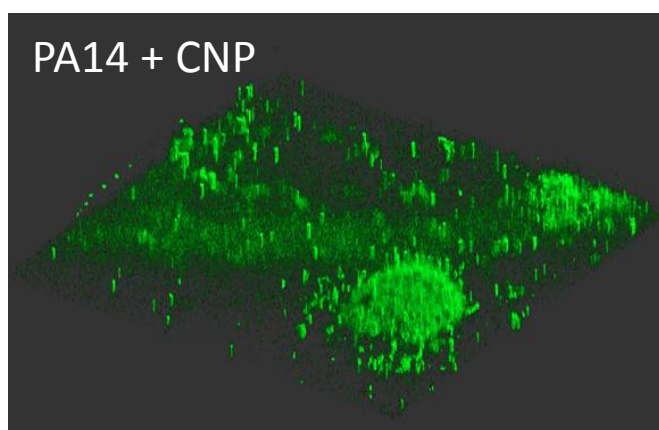
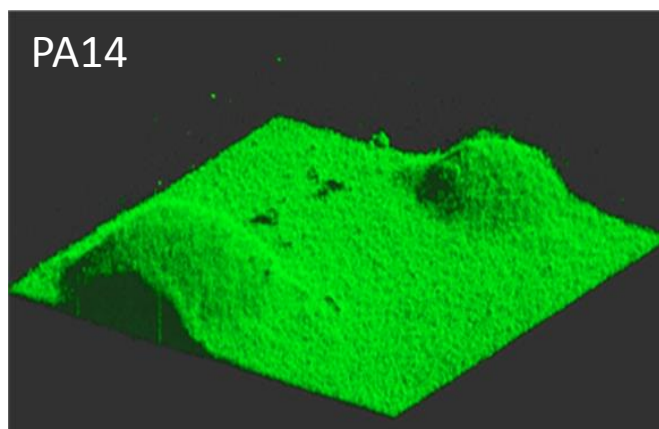
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900 **Figure 5. CNP, BNP , NPR agonists and isatin affinities for AmiC *in vitro*.** Recombinant  
901 AmiC was fluorescently labeled and incubated with varying concentrations of natriuretic  
902 peptides receptor agonists and isatin(A). These mixtures were then analysed using by  
903 microscale thermophoresis. The results were normalized to a fitted zero for unbound AmiC,  
904 and the thermophoresis signal shown as parts per thousand. Addition of cANF<sup>4-23</sup> caused  
905 AmiC fluorescence to increase in a dose dependent fashion: this increase was used as the  
906 signal rather than thermophoresis, as the effect confounds the thermophoresis signal. The  
907 results were fit to the dissociation constant formula from the law of mass action. The fitted  
908 values for  $K_D$  are shown for isatin, BNP, sANP, cANF<sup>4-23</sup> and osteocrin (B). We showed that  
909 these compounds bound either too weakly (sANP), more tightly (osteocrin) or presented no  
910 interaction (BNP). For each compound tested, data are the mean of three independent  
911 experiments.

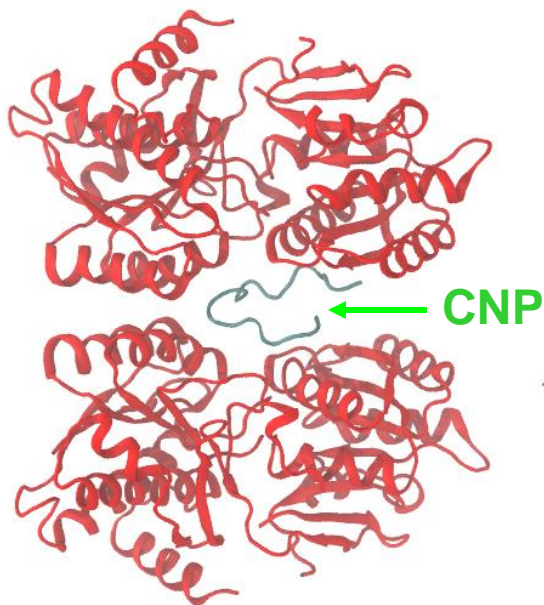
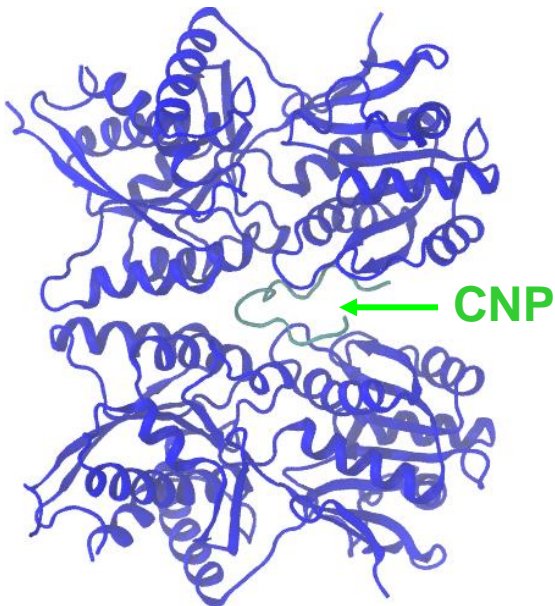
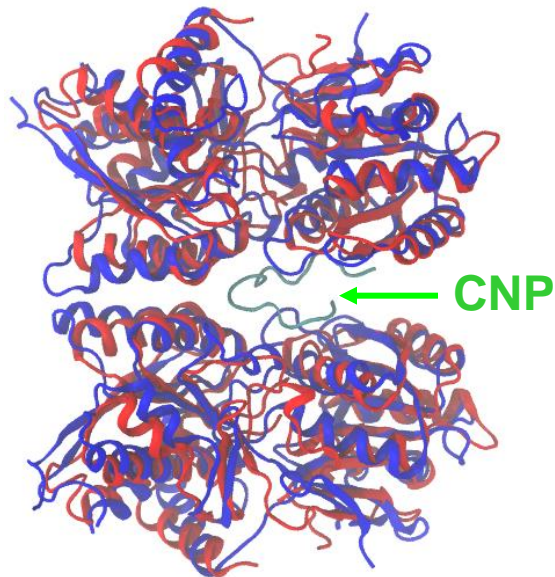
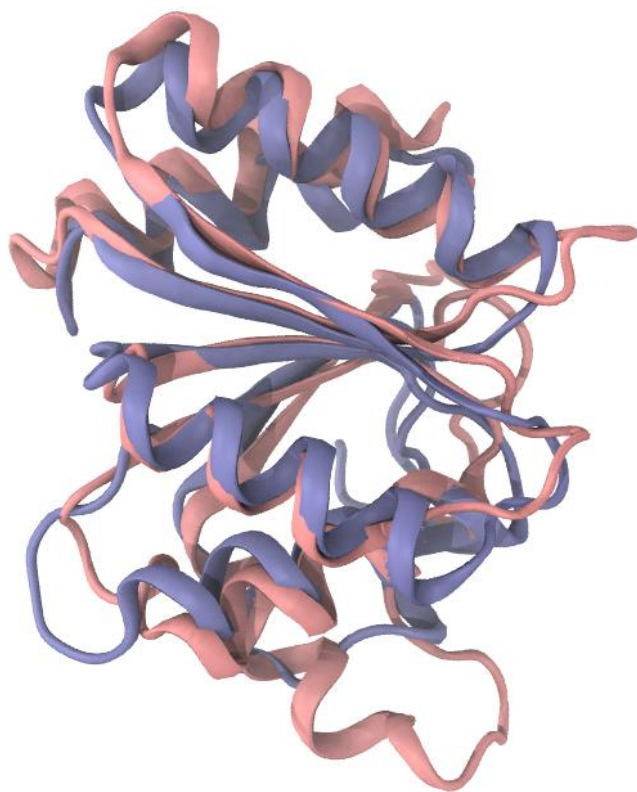
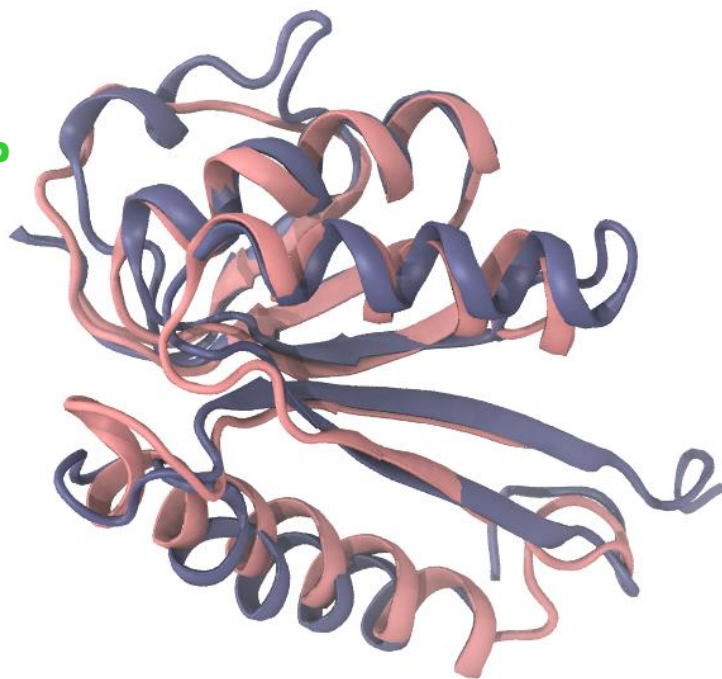
912

913 **Figure 6. Involvement of AmiC protein on *P. aeruginosa* biofilm formation.** (A) 3D-  
914 shadow representations of the biofilm structures developed by *P. aeruginosa* PA14 control  
915 (PA14), *amiC*- strain and *amiC*- complemented strain (*amiC*- Comp), not exposed to peptide  
916 or exposed to CNP ( $10^{-7}$  M) in dynamic condition, at 37 °C for 24 h in LB broth. Biofilms  
917 were stained with the SytoX and observed by confocal laser scanning microscopy. (B)  
918 COMSTAT analyses of biofilms of *P. aeruginosa* PA14 control, *amiC*-, *amiC*- complemented  
919 and AmiC+ strain. (C) COMSTAT analyses of biofilms of *P. aeruginosa* PA14 control,  
920 *amiC*- and *amiC*- complemented exposed to CNP ( $10^{-7}$  M) or to BNP ( $10^{-7}$  M) or neither of  
921 them. Data are the means of twenty-seven samples from eleven independent experiments for  
922 PA14 control, eleven samples from four independent experiments for PA14 exposed to CNP,  
923 nine samples from three independent experiments for PA14 exposed to BNP, nineteen

924 samples from seven independent experiments for *amiC*- control, twelve samples from four  
925 independent experiments for *amiC*- exposed to CNP, eight samples from three independent  
926 experiments for *amiC*- exposed to BNP, twenty four samples from six independent  
927 experiments for *amiC*- Comp control and twelve samples from four independent experiments  
928 for *amiC*- Comp exposed to CNP. Note that the wild-type (left), *amiC* (middle) and *amiC*  
929 Comp (right) strain values are set to 100% independently. \*\*\* =  $p < 0.001$ ; \* =  $p < 0.05$ ;  
930 NS: Not significantly different.

**A****B****Fig. 1**



**A****AmiC****hNPR-C****AmiC and hNPR-C****B****Fig. 3**



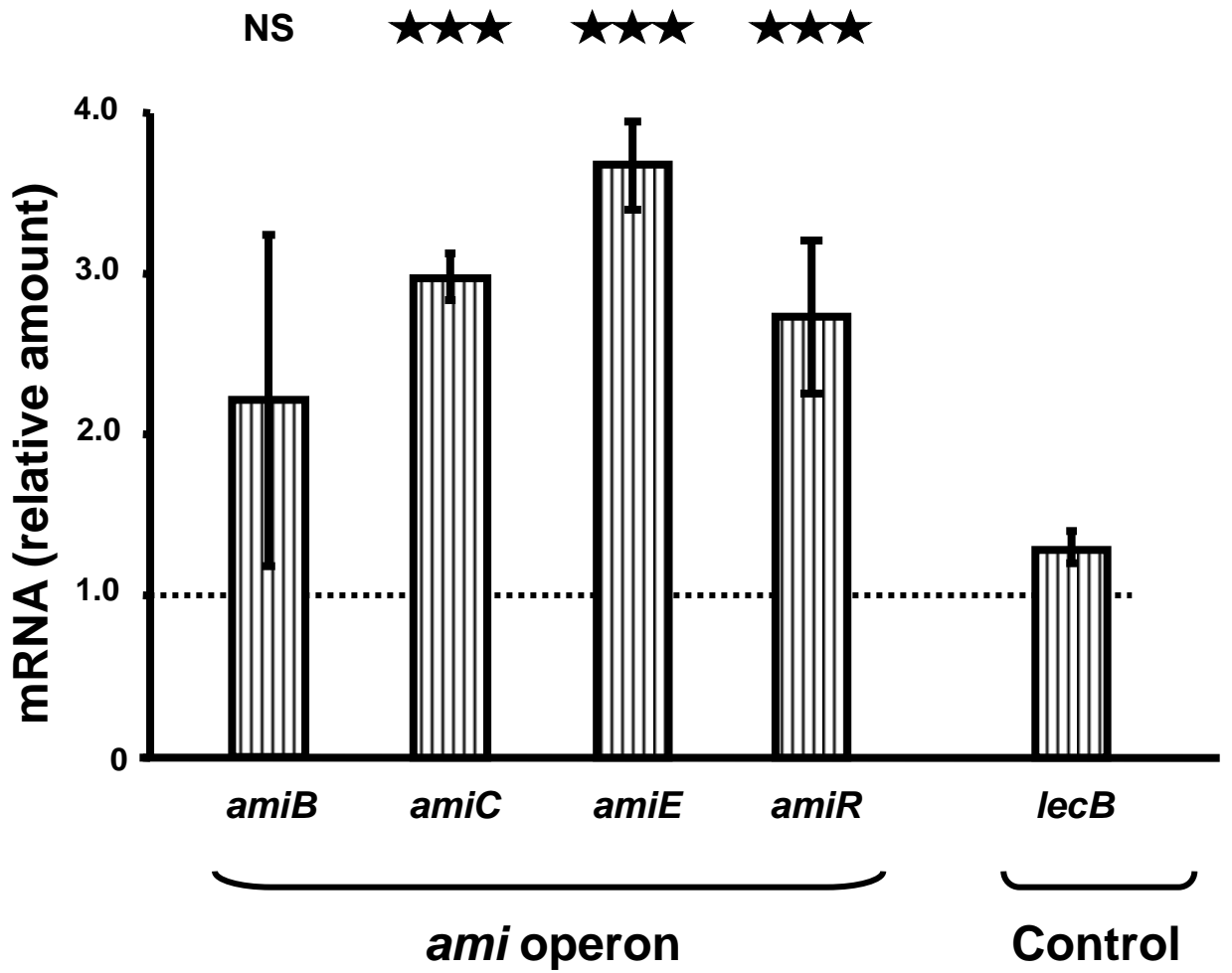
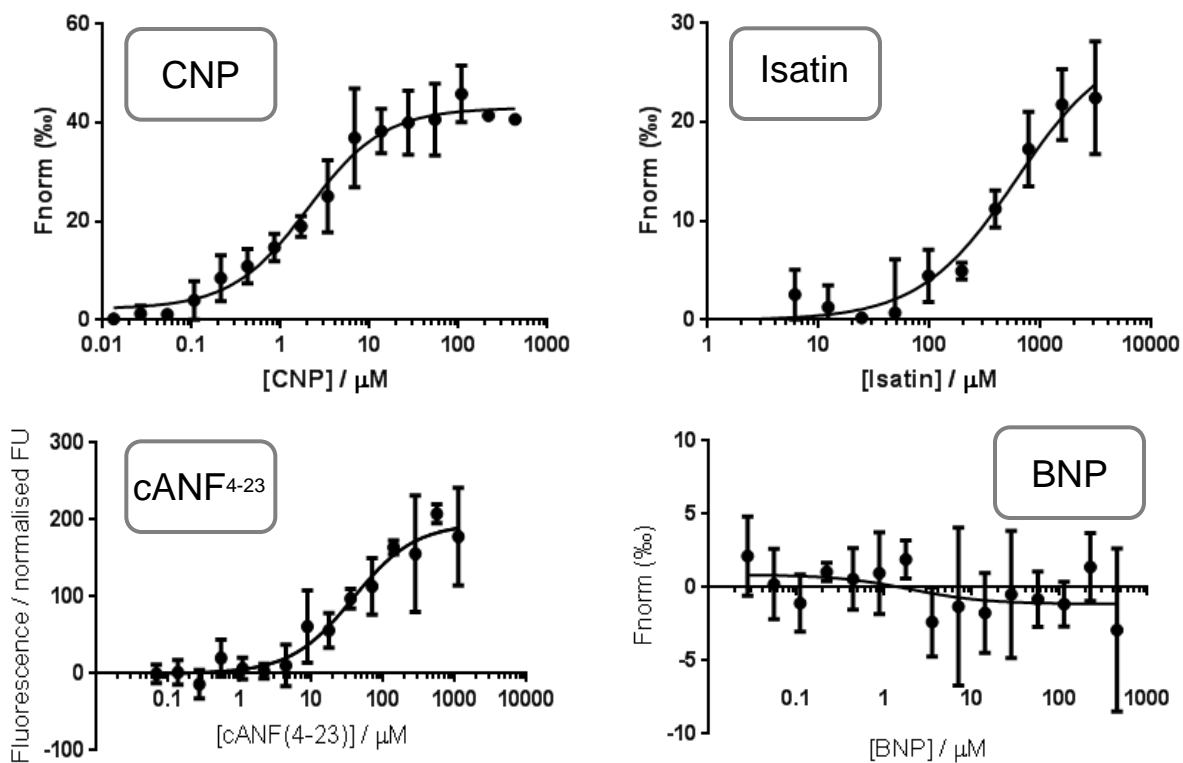
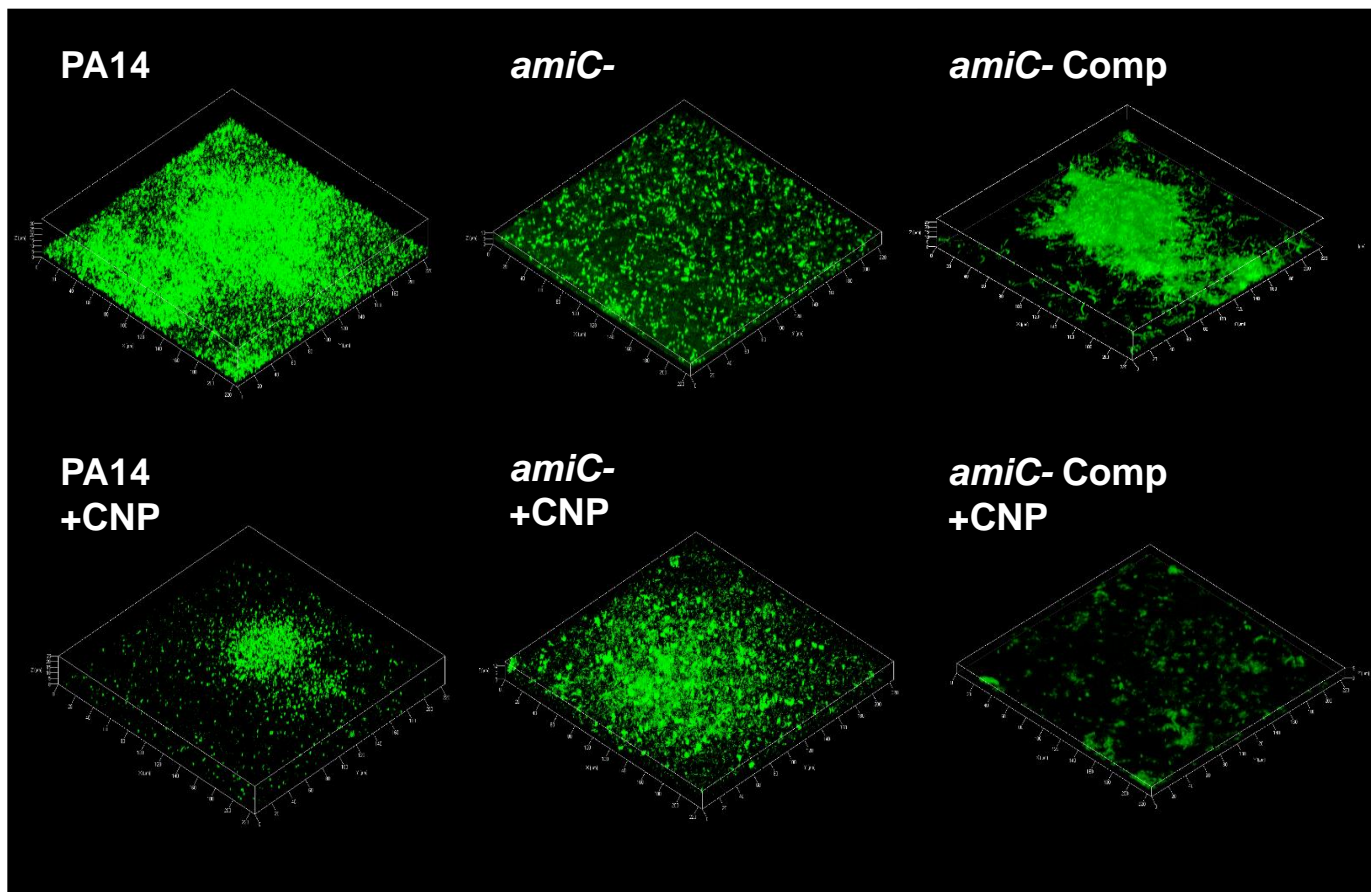
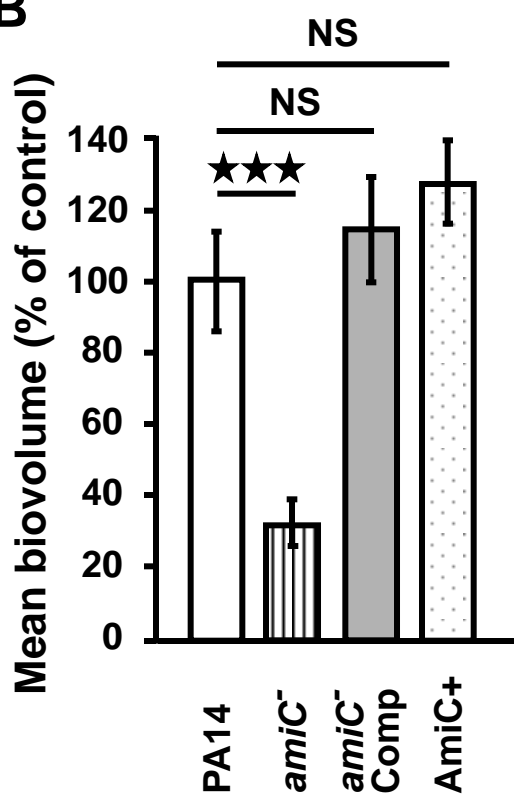
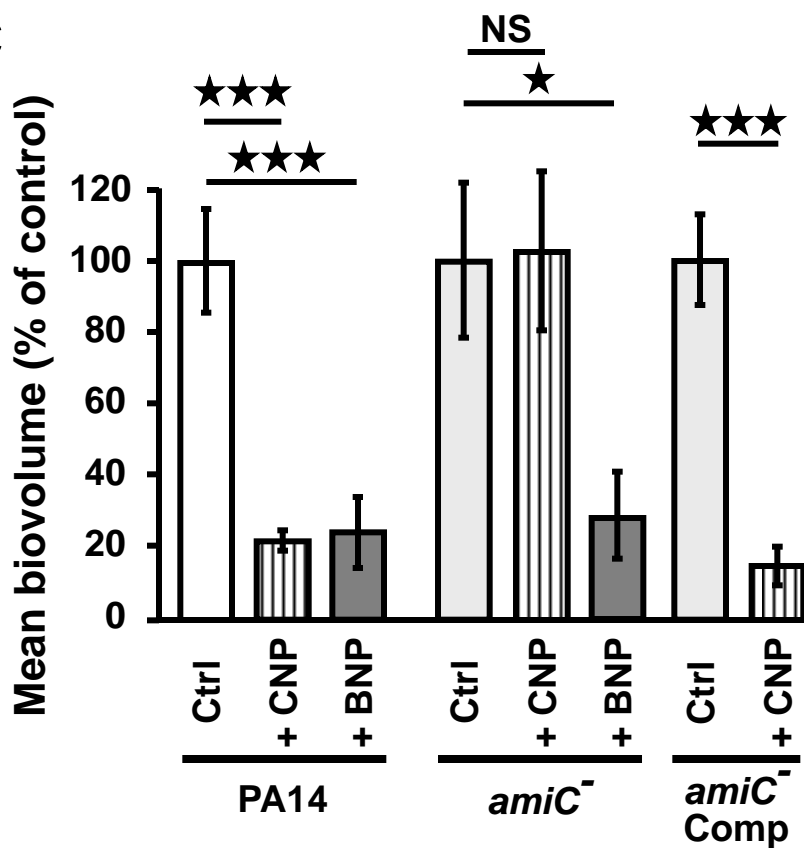


Fig. 4

**A****B**

Ligands	Kd
CNP	$2.0 \pm 0.3 \mu\text{M}$
BNP	No interaction
sANP (NPR-A agonist)	$> 600 \mu\text{M}$
Osteocrin (NPR-C agonist)	$< 100 \text{ nM}$
cANF <sup>4-23</sup> (NPR-C agonist)	$40 \pm 10 \mu\text{M}$
Isatin (NPR-A-NPR-C antagonist)	$600 \pm 200 \mu\text{M}$

**Fig. 5**

**A****B****C****Fig. 6**