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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 cANF⁴⁻²³ 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 AI₃, 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- γ (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 cANF⁴⁻²³, a specific NPR-C agonist, on bacterial cytotoxicity
156 towards lung cells. The peptide cANF⁴⁻²³ (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 cANF⁴⁻²³, 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 ± 0.2 , 3.66 ± 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 ± 30
238 kDa and 136 ± 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 ± 0.3 μ 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 ± 200 μ 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 μ 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 μ 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, cANF⁴⁻²³, 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*⁻ mutant strain (Figure S4D). It is interesting to note that the strain over-expressing
279 AmiC (AmiC⁺) 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 μm). 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]. 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]. 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;
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- γ , 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 cANF⁴⁻²³, 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 cANF⁴⁻²³, 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 cANF⁴⁻²³ (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 μM (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 μM , 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 cANF⁴⁻²³
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 cANF⁴⁻²³
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 OD₆₀₀ = 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 OD₆₀₀ = 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 OD₆₀₀ = 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 OD₆₀₀ = 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% CO₂ 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 3×10^5 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 MHHHHHHSSGVLDGTENLYFQS 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⁻¹, 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 MgCl₂). 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 cANF⁴⁻²³) 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 MgCl₂) 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 μ M
574 (CNP), 3.12 μ M to 3 mM (isatin), 892 nM to 1.8 mM (sANP), 28 nM to 450 μ M (BNP), 68
575 nM to 1.1 mM (cANF⁴⁻²³) and 120 pM to 4 μ 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 μ 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 cANF⁴⁻²³, 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 cANF⁴⁻²³ 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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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 cANF⁴⁻²³ (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 cANF⁴⁻²³ 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.

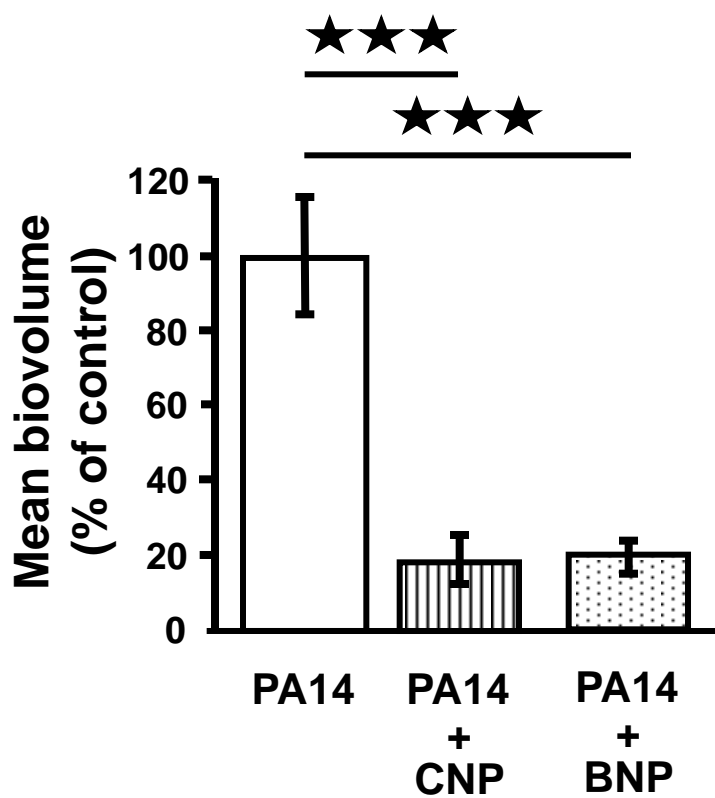
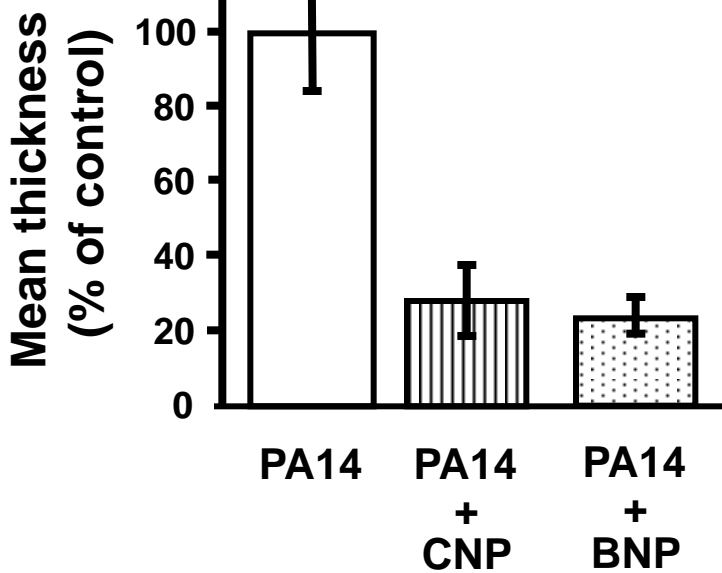
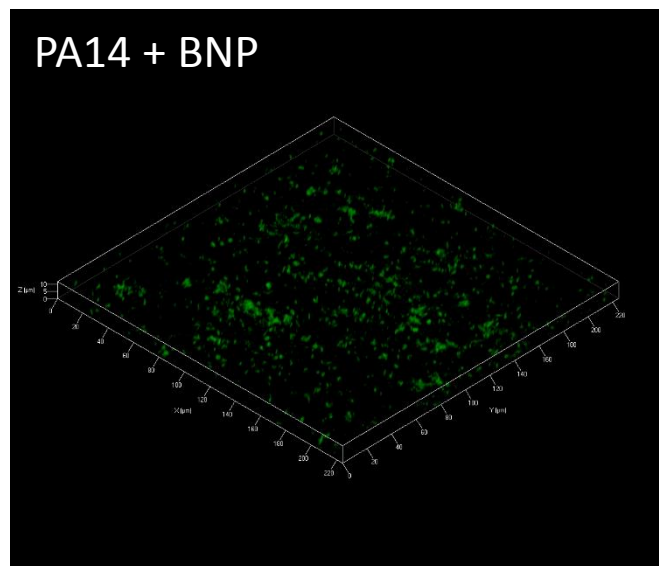
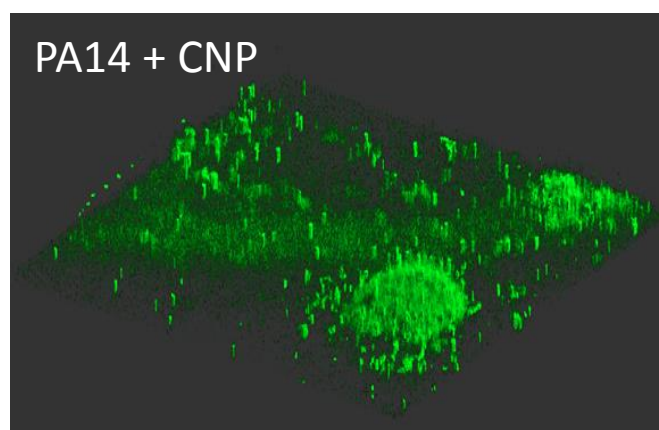
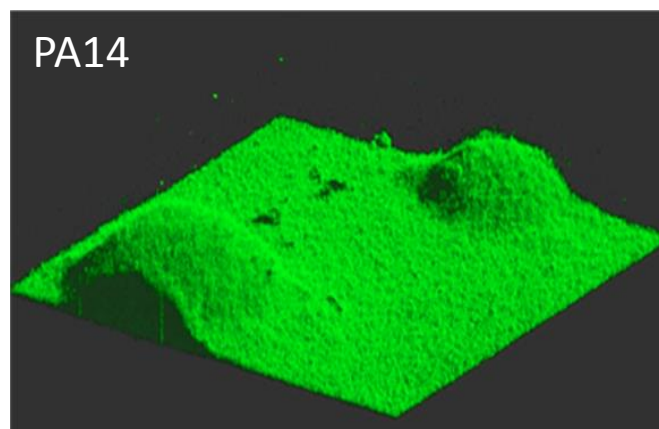
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900 **Figure 5. CNP, BNP , NPR agonists and isatin affinities for AmiC *in vitro*.** Recombinant
901 AmiC was fluorescently labeled and incubated with varying concentrations of natriuretic
902 peptides receptor agonists and isatin(A). These mixtures 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 cANF⁴⁻²³ 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, cANF⁴⁻²³ 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**

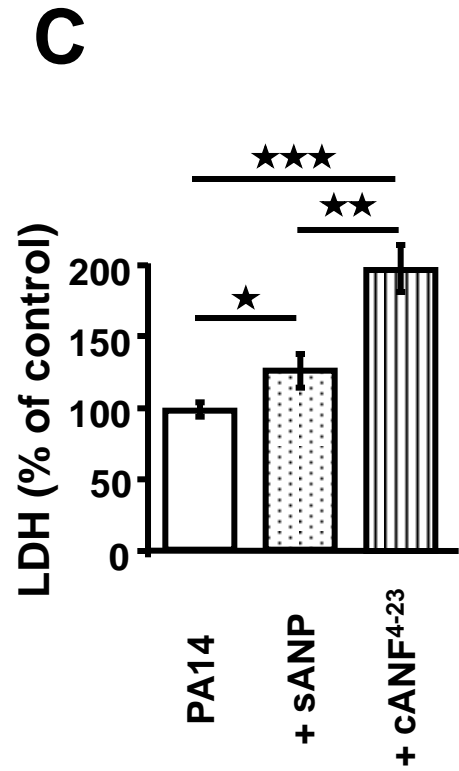
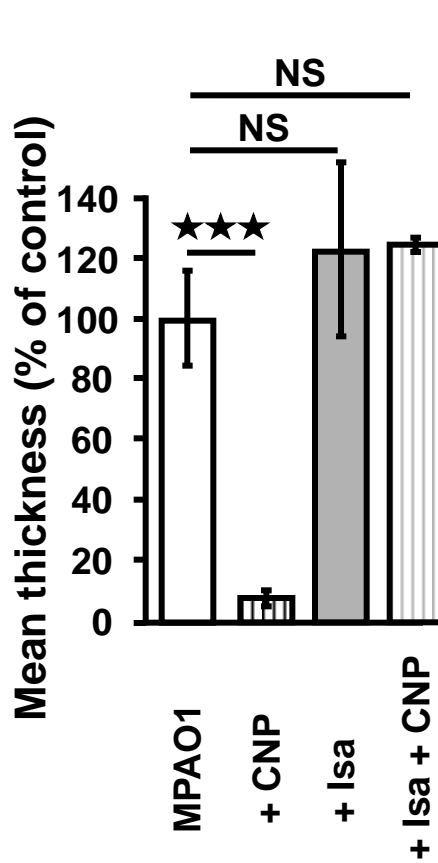
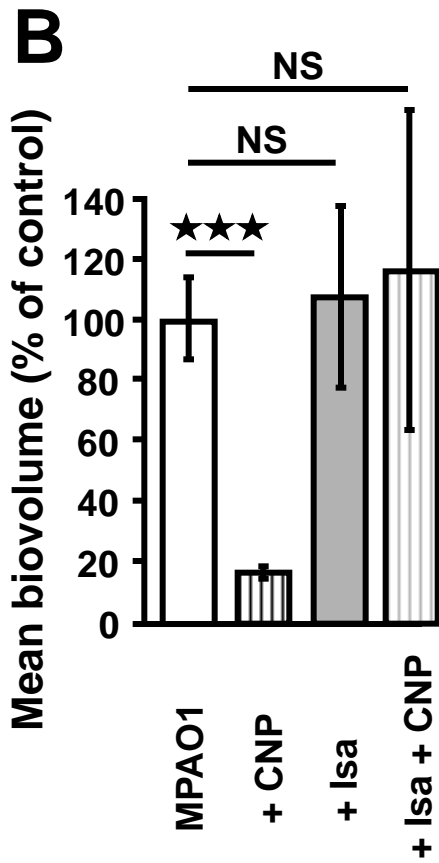
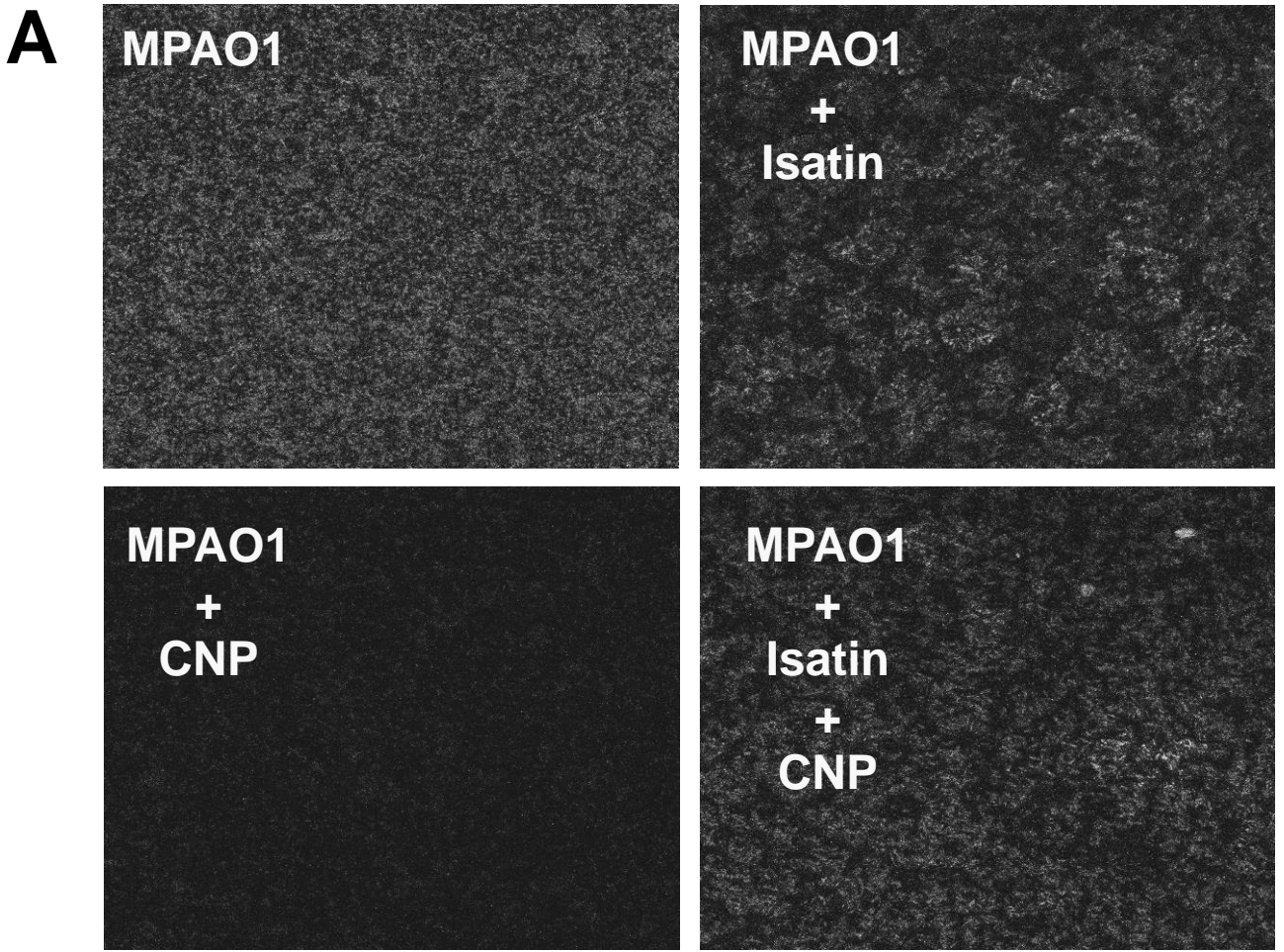
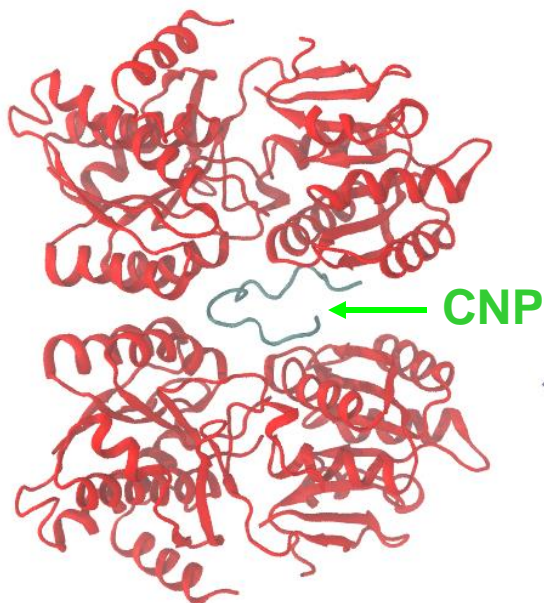
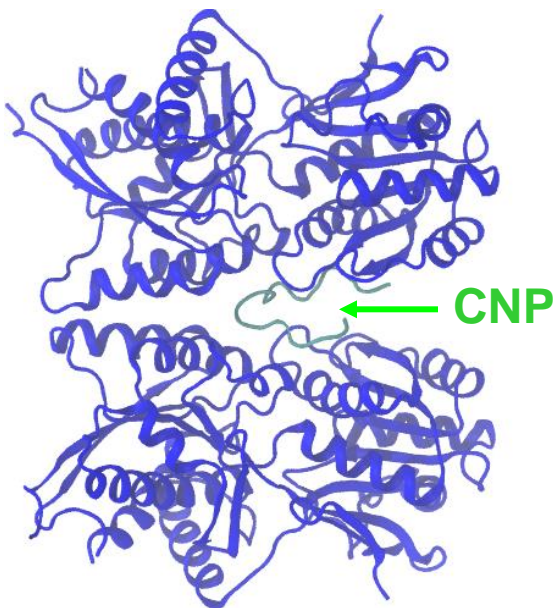
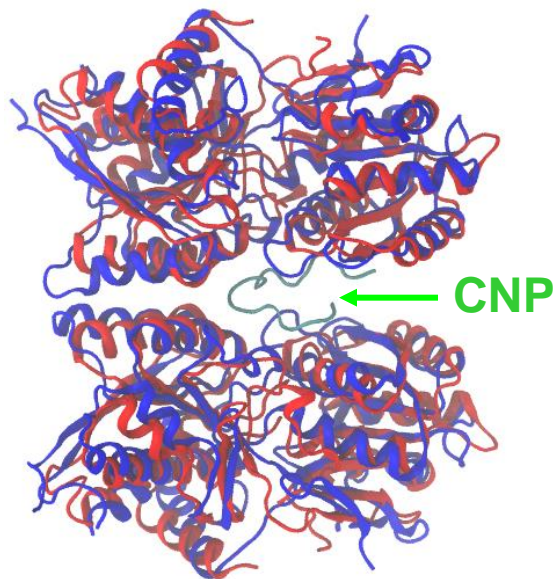
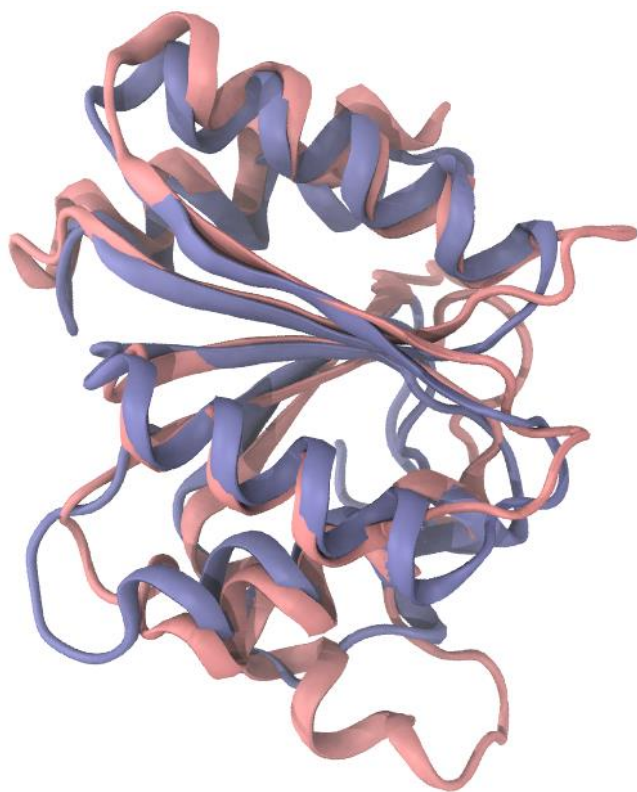
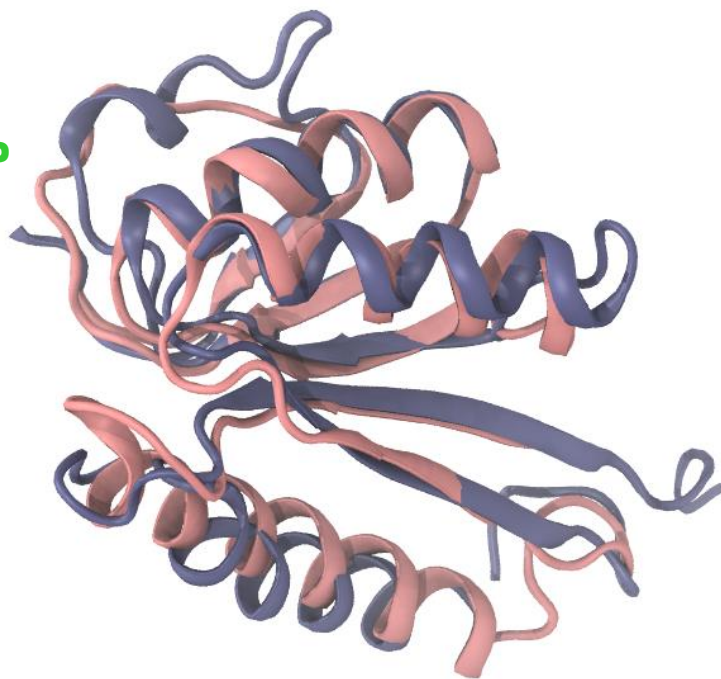


Fig. 2

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

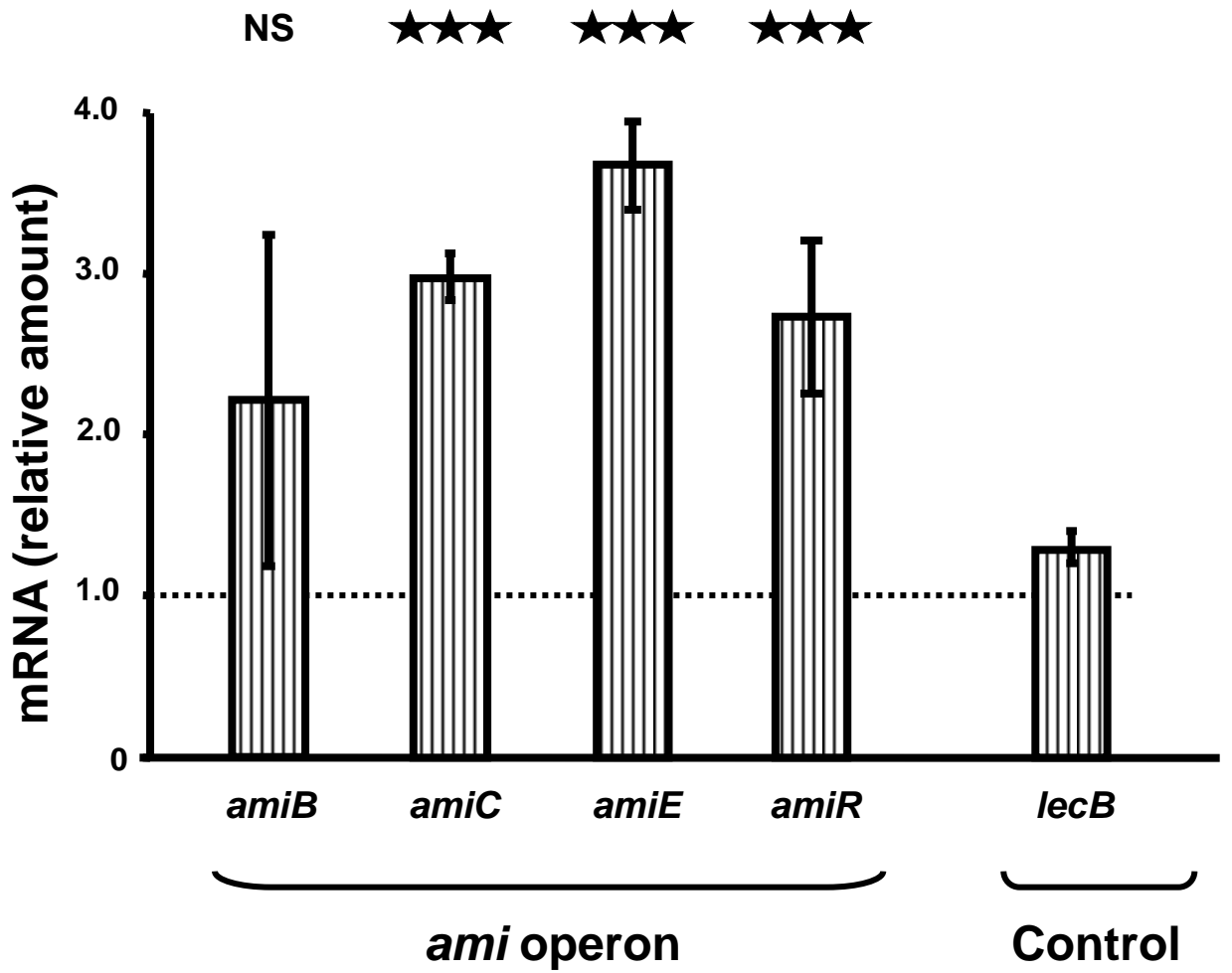
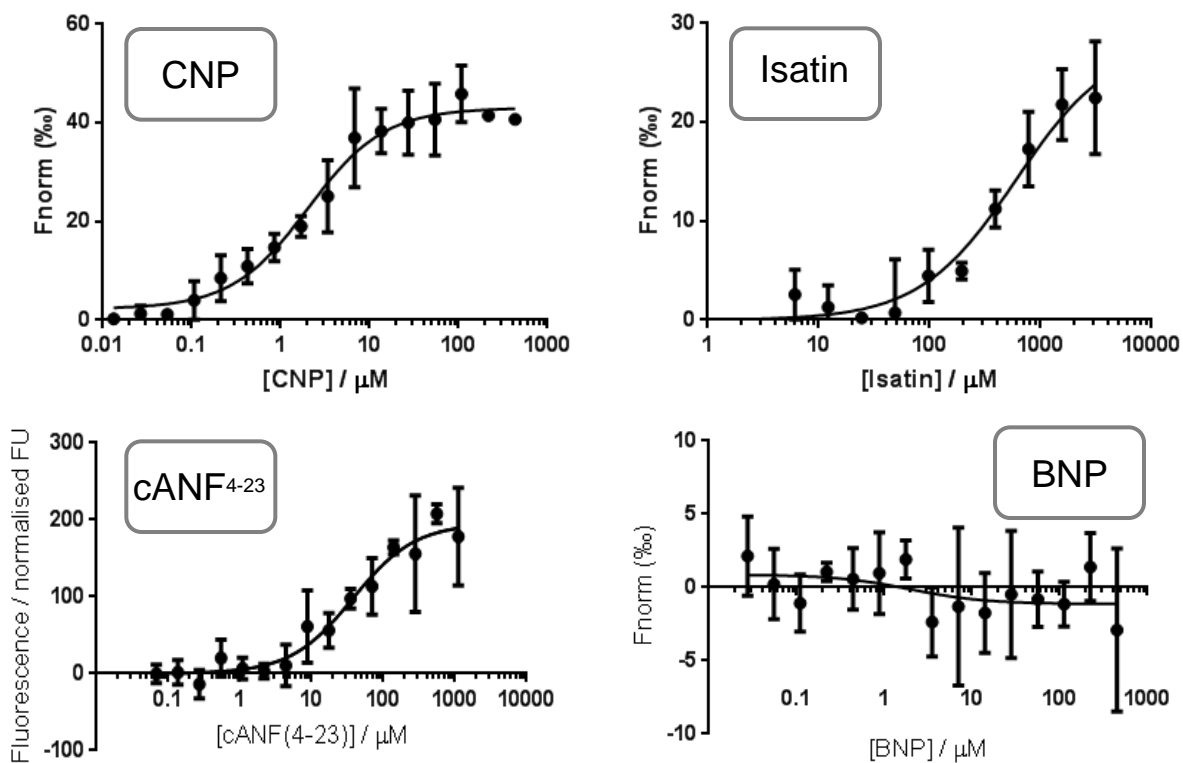
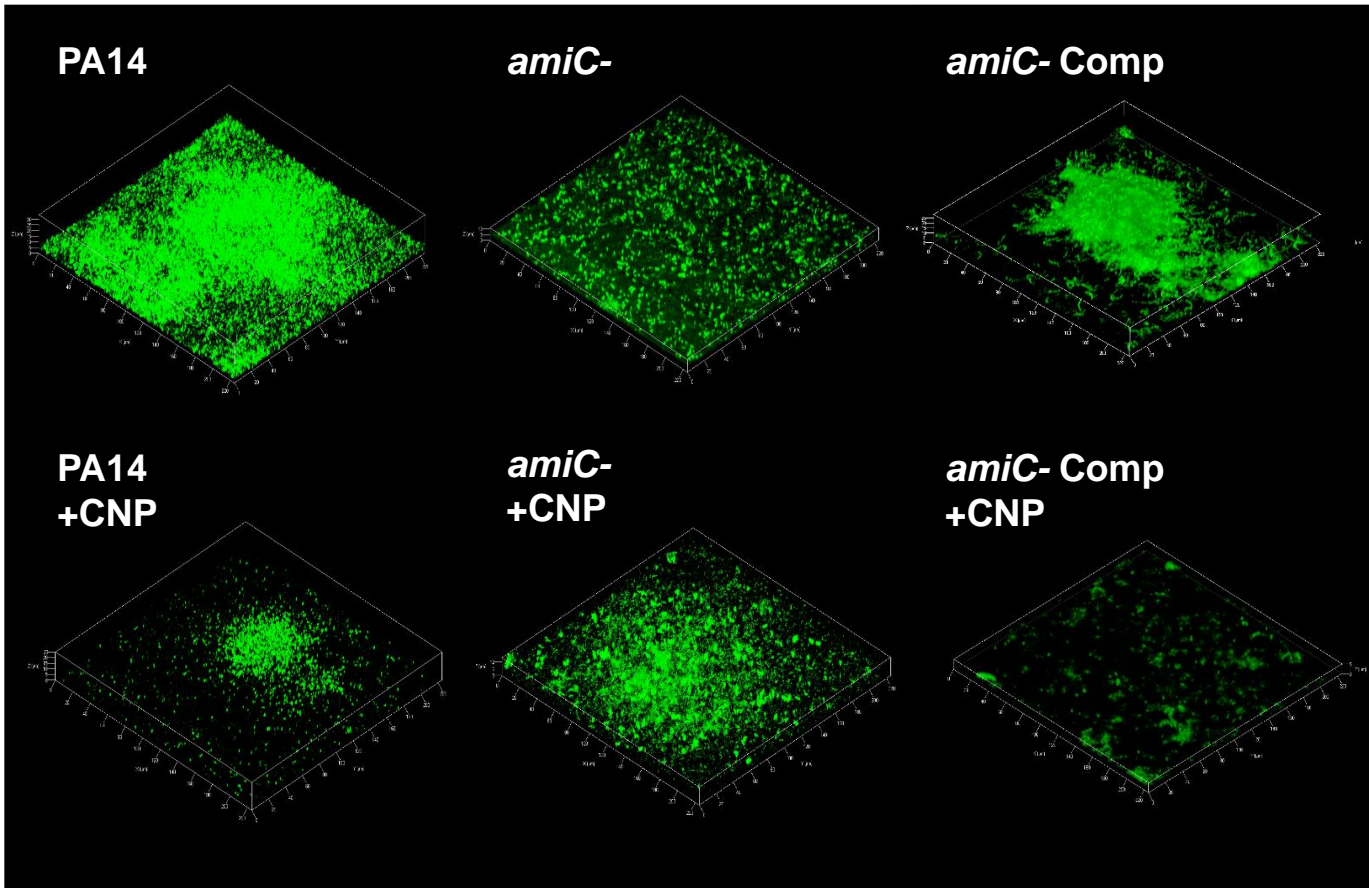
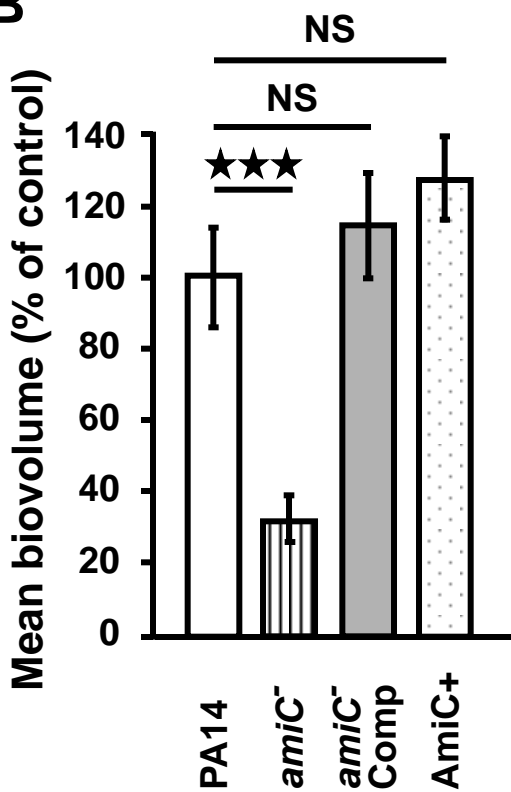
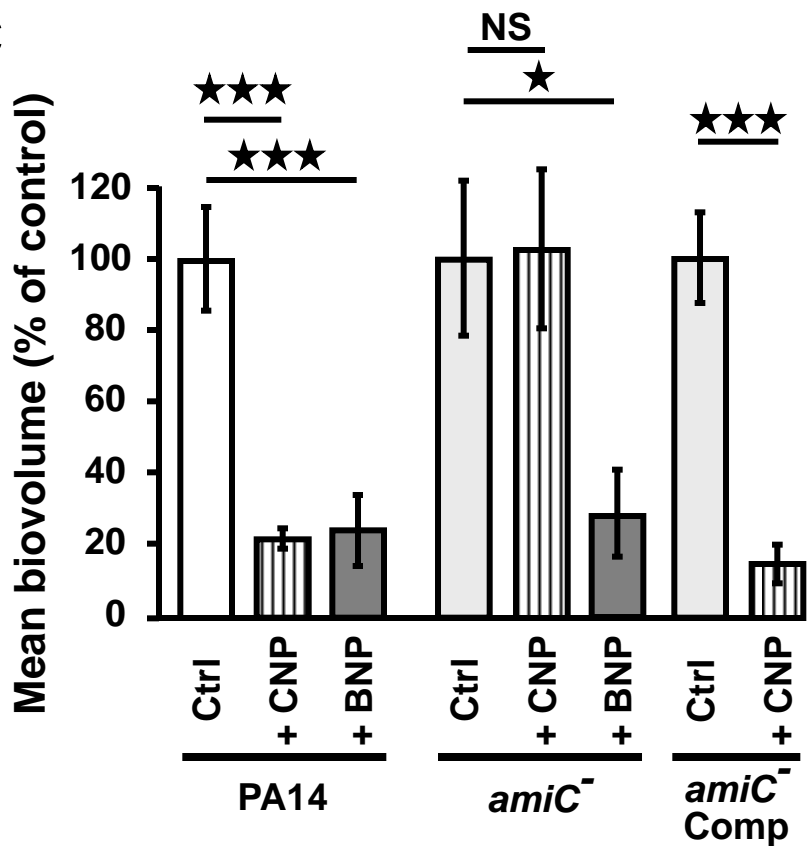


Fig. 4

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 ⁴⁻²³ (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**