Antibiotic capture by bacterial lipocalins uncovers an extracellular mechanism of intrinsic antibiotic resistance

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8   Omar M. El-Halfawy[a,b], Javier Klett[c], Rebecca J. Ingram[d], Slade A. Loutet[e], Michael E. P.

9   Murphy[e], Sonsoles Martín-Santamaría[c], and Miguel A. Valvano[a,d]

10

11   [a] Department of Microbiology and Immunology, University of Western Ontario, London,

12   Ontario, N6A 5C1, Canada; [b] Microbiology and Immunology Department, Faculty of Pharmacy,

13   Alexandria University, Egypt; [c] Department of Chemical and Physical Biology, Centre for

14   Biological Research, CIB-CSIC, Madrid 28040, Spain; [d] Centre for Experimental Medicine,

15   Queen's University Belfast, BT9 7BL, United Kingdom; [e] Department of Microbiology and

16   Immunology, University of British Columbia, Vancouver, BC, V6T 1Z3, Canada

17

18   Address correspondence to Miguel A. Valvano, m.valvano@qub.ac.uk.

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20   Running title: Extracellular antibiotic capture by bacteria

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25 **ABSTRACT** The potential for microbes to overcome antibiotics from different classes before

26 they reach the bacterial cells is largely unexplored. Here we show that a soluble bacterial

27 lipocalin produced by *Burkholderia cenocepacia* upon exposure to sublethal antibiotic

28 concentrations increases resistance against diverse antibiotics *in vitro* and *in vivo*. These

29 phenotypes were recapitulated by heterologous expression in *B. cenocepacia* of lipocalin genes

30 from *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis* and methicillin-resistant

31 *Staphylococcus aureus*. Purified lipocalin bound different classes of bactericidal antibiotics and

32 contributed to bacterial survival *in vivo*. Experimental and X-ray crystal structure-guided

33 computational studies revealed that lipocalins counteract antibiotic action by capturing

34 antibiotics in the extracellular space. We also demonstrated that fat-soluble vitamins prevent

35 antibiotic capture by binding bacterial lipocalin with higher affinity than antibiotics. Therefore,

36 bacterial lipocalins contribute to antimicrobial resistance by capturing diverse antibiotics in the

37 extracellular space at the site of infection, which can be counteracted by known vitamins.

38

39 **IMPORTANCE** Current research on antibiotic action and resistance focuses on targeting

40 essential functions within bacterial cells. We discovered a previously unrecognized mode of

41 general bacterial antibiotic resistance operating in the extracellular space, which depends on

42 bacterial protein molecules called lipocalins. These molecules are highly conserved in most

43 bacteria and have the ability to capture different classes of antibiotics outside bacterial cells. We

44 also discovered that liposoluble vitamins, such as vitamin E, overcome *in vitro* and *in vivo*

45 antibiotic resistance mediated by bacterial lipocalins, providing an unexpected new alternative to

46 combat resistance by using this vitamin or its derivatives as antibiotic adjuvants.

47

48   Treating infections is becoming increasingly difficult since microbes often show intrinsic, high-

49   level resistance to virtually all clinically approved antibiotics (1). Ineffective microbial killing

50   and exposure to sublethal antibiotic concentrations elicit adaptive bacterial stress responses

51   enhancing antibiotic resistance and tolerance (2-7). Much has been learned about antibiotic

52   resistance mechanisms at the cellular level (8, 9), but whether microbes subvert the action of

53   antibiotics before they come in contact with the bacterial cells has remained largely unexplored

54   with the exception of β-lactamases, which are often trapped into released membrane vesicles (10-

55   13).

56   *Burkholderia cenocepacia* is a highly multidrug resistant, opportunistic Gram-negative

57   bacterium that causes serious respiratory infections in patients with cystic fibrosis (14). Bacteria

58   of the genus *Burkholderia* are notorious for their ability to resist the action of multiple classes of

59   antimicrobials (15), representing an attractive model to understand intrinsic mechanisms of

60   resistance in opportunistic bacteria. Recently, we showed that in response to sublethal antibiotic

61   concentrations, *B. cenocepacia* produce and release molecules such as the polyamine putrescine

62   and YceI, a conserved hypothetical protein of unknown function (16). YceI proteins comprise a

63   family of bacterial lipocalins (herein abbreviated as BCNs), which are small proteins widely

64   conserved in Gram-negative and Gram-positive bacteria, but whose physiological role is unclear

65   (17, 18). In co-culture experiments, *B. cenocepacia* protects *Pseudomonas aeruginosa* from

66   killing by different bactericidal antibiotics (16). This effect was abrogated in the *B. cenocepacia*

67   double deletion mutant Δ*bcnA-bcnB* (16), but the individual contribution of each BCN paralog

68   and their mechanisms remained unknown.

69   Here, we show that secreted BcnA contributes to increased resistance of *B. cenocepacia* to

70   various classes of antibiotics *in vitro* and *in vivo*. The expression of BCN orthologs from *P.*

71   *aeruginosa, Mycobacterium tuberculosis* and methicillin-resistant *Staphylococcus aureus* in *B.*

72   *cenocepacia* Δ*bcnA* recapitulated this function. Experimental and computational studies revealed

73   that BCNs bind to a range of antibiotics, thus preventing their antibacterial activity and

74   contributing to resistance. X-ray crystallography studies of BCN structures, in combination with

75   docking and MD simulations, have helped us to rationalize plausible binding modes. We also

76   discovered that fat-soluble vitamins bound BcnA with a higher affinity than antibiotics, enabling

77   them to outcompete antibiotics. This finding provides a clinically applicable strategy whereby

78   known vitamins could become antibiotic adjuvants by increasing the concentration of free

79   antibiotics in the proximity of bacterial cells, thereby boosting their microbicidal activity.

80

81   **RESULTS**

82

83   **BcnA is a secreted bacterial lipocalin required for full resistance of *B. cenocepacia* to**

84   **different classes of antibiotics.** We investigated the role of *B. cenocepacia* BcnA (BCAL3311)

85   and BcnB (BCAL3310) by constructing individual deletion mutants in their corresponding genes

86   and assessing bacterial susceptibility to model bactericidal antibiotics representing different

87   classes including rifamycins (mRNA transcription inhibitors), fluoroquinolones (DNA

88   replication inhibitors), several β-lactams (cell wall peptidoglycan synthesis inhibitors), and

89   cationic antimicrobial peptides (cell membrane active agents). The Δ*bcnA* mutant, but not

90   Δ*bcnB*, had increased susceptibility (4-fold MIC reduction) to rifampicin, norfloxacin,

91   ceftazidime, and the cationic antimicrobial peptide polymyxin B (PmB), and 2-fold MIC

92   reduction to meropenem. No effect was observed with the aminoglycoside gentamicin (protein

93   synthesis inhibitor) (Fig. 1A; see antibiotic chemical structures in Fig. S1). Similarly, we also

94   tested model bacteriostatic antibiotics representing different classes. The Δ*bcnA* mutant, but not

95   Δ*bcnB*, had increased susceptibility to minocycline (tetracycline family protein synthesis

96   inhibitor; 4-fold MIC reduction), and trimethoprim (pyrimidine inhibitor of bacterial

97   dihydrofolate reductase; 2-fold MIC reduction), while no effect was observed with the macrolide

98   (protein synthesis inhibitor) azithromycin (Fig. 1A and Fig. S1).

99   The expression of *bcnA* and *bcnB* genes in response to antibiotics at near-MIC (sublethal)

100   concentrations was characterized by constructing chromosomal *lux* fusions. Expression of

101   *bcnA*::*luxCDABE* was upregulated upon exposure to PmB, rifampicin, and norfloxacin, but not

102   ceftazidime (Fig. 1B). In contrast, *bcnA*::*luxCDABE* expression was slightly reduced in response

103   to gentamicin, likely due to protein synthesis inhibition at near-MIC concentrations (Fig. 1B).

104   Expression of *bcnB*::*luxCDABE* only increased by exposure to norfloxacin and ceftazidime, and

105   was slightly reduced by rifampicin and gentamicin (Fig. S2A). Thus, *bcnA* and *bcnB* genes

106   respond to antibiotic stress, but they appeared to be differentially regulated. *bcnA* and *bcnB* are

107   located on the same strand and apart by 63 base pairs (Fig. 1C). Immediately upstream of *bcnA*

108   there is an open reading frame (BCAL3312), also transcribed in the same strand, which encodes

109   a predicted cytochrome b651 protein. Putative promoter regions are found upstream from

110   BCA3312 and downstream from *bcnB* next to a predicted Rho-independent transcription

111   termination sequence. The genomic organization of the *bcn* region suggest BCAL3312-*bcnA*-

112   *bcnB* are cotranscribed and may form an operon. However, the transcribed 63-bp intergenetic

113   region between *bcnA* and *bcnB* has the potential to form strong RNA secondary structures, which

114   might explain the differential regulation of both genes by antibiotics. The secretion of BcnA and

115   BcnB proteins was also investigated using FLAG-tagged derivatives; only BcnA was secreted

116   extracellularly into the growth medium (Fig. S2B). From these experiments, we concluded that

117   BcnA is the major contributor to intrinsic antibiotic resistance upon antibiotic stress and is

118   secreted to the extracellular bacterial milieu.

119   **BCN orthologs from different species restore BcnA function in *B. cenocepacia*.** To

120   demonstrate whether BCN orthologs from other bacteria could restore BcnA function in the

121   Δ*bcnA* strain, we tested BCNs of *P. aeruginosa* PAO1 [PA0423, PA4340 and PA4345, herein

122   BcnA1(Pa), 2 (Pa), and 3(Pa), respectively], *Mycobacterium tuberculosis* H37Rv [Rv1890c,

123   BcnA(MTb)] and the community-acquired methicillin-resistant *Staphylococcus aureus* USA300

124   [SAUSA300_2620, BcnA(Sa)]. CFU counts on PmB-containing plates (Fig. 2A), and Etest

125   MICs against rifampicin, ceftazidime, and ciprofloxacin (a fluoroquinolone closely related to

126   norfloxacin) (Fig. 2B) showed that heterologous expression of BCNs from these different

127   bacteria restores antibiotic resistance to parental levels, indicating these proteins have a

128   conserved function.

129   **BcnA sequesters antibiotics.** BCNs bind to diverse hydrophobic molecules (19-21); hence

130   we hypothesized that BCNs could capture antibiotics and reduce their effective concentration in

131   the bacterial milieu. An antibiotic bioassay demonstrated that BcnA sequestered rifampicin,

132   PmB, norfloxacin and ceftazidime, in descending order of magnitude, but not gentamicin (Fig.

133   3A and Fig. S3, A-E). Further, the relative affinity of BcnA for antibiotics was determined *in*

134   *vitro* by binding competition of antibiotics with Nile Red in complex with BcnA. Nile Red is a

135   fluorophore used to test hydrophobic binding sites in proteins (22). The calculated binding

136   inhibitory constants (K_i values) for each antibiotic (Fig. 3B and Fig. S3, F-M) mirrored their

137   relative ability to be sequestered by BcnA (Fig. 3A) and the antibiotic susceptibility phenotypes

138   of Δ*bcnA* (Fig. 1A). Notably, the binding of BcnB to Nile Red occurred at ~20-fold lower

139   affinity than that of BcnA (Fig. S3, N-Q) agreeing with the lesser role of BcnB in antibiotic

140   resistance. The involvement of hydrophobic moieties in the interaction of ligands with BcnA was

141   suggested by the significantly higher K_i value of PmB nonapeptide, which lacks the hydrophobic

142   N-terminal tail of PmB (23), relative to PmB (Fig. 3B, Fig. S3, F-I). Notably, azithromycin and

143   gentamicin, the only antibiotics not showing antibiotic sensitivity reduction phenotype with

144   Δ*bcnA* (Fig. 1A), are the only tested antibiotics lacking aromatic or hydrophobic moieties (Fig.

145   S1). To test polar hydrophilic binding sites in BcnA compared to hydrophobic sites, we used two

146   related BODIPY dye-labelled phospholipids. BcnA increased the intensity of fluorescence of

147   BODIPY fluorophore when attached to fatty acyl chain of the phospholipid in BODIPY-

148   phosphocholine, but not when attached to the hydrophilic polar head group (BODIPY-

149   phosphoethanolamine) (Fig. S3, R-T). This further supported a role for fatty acyl chains in the

150   interaction with hydrophobic sites in BcnA.

151   **Structure and molecular modeling reveal distinct docked binding modes for BcnA.** To

152   elucidate the mode of binding of antibiotics within BCNs, we first solved the X-ray crystal

153   structures of BcnA and BcnB to 1.4- and 1.6-Å resolution, respectively (*Supplemental Results*,

154   Table S1 and Fig S4A-E). Visual inspection and structural alignments by the DALI server (24)

155   confirmed a barrel-shaped lipocalin fold for both proteins (Fig. 4A and B). An octaprenyl

156   pyrophosphate was bound within a long, hydrophobic tunnel extending from one end of the

157   barrel in each structure (Fig. S4A and B). The binding of octaprenyl pyrophosphate potentially

158   occurred during recombinant expression in *Escherichia coli*. Analysis with the PDBePISA server

159   (25) predicted that BcnA is a monomer whilst BcnB is a dimer by crystallographic symmetry

160   (~2840 Å^2 of buried surface area including a portion of the tunnel opening). These oligomeric

161   states were confirmed in solution using size exclusion chromatography-multiangle light

162   scattering (SEC-MALS, Fig. S4C and D). Superposition of BcnA chain A and BcnB chain C

163 (148 Cα atoms, 22% sequence identity) resulted in a root mean squared deviation of 1.66 Å (Fig.

164 4C). The largest structural differences observed were located in two of the loops that make up

165 the tunnel opening (Fig. 4C). These differences may play a role in the antibiotic binding potential

166 of BcnA and BcnB.

167 Using the established crystallographic structures, we applied molecular modeling and

168 docking calculations to predict BCN binding modes for antibiotics (*Supplemental Results*). These

169 studies suggested two distinct docked binding modes for BcnA. One binding mode involved

170 residues in the rim of the lipocalin pocket (Fig. 4D and Fig. S4F-I). Polar interactions, mainly

171 with polar residues, were observed with all tested antibiotics. There were also interactions

172 between the aromatic moieties in PmB, rifampicin, norfloxacin, and ceftazidime, and lipophilic

173 residues. These interactions were not observed with gentamicin, as it lacks aromatic moieties;

174 that may contribute to weak binding to BcnA. The second binding mode was predicted for more

175 lipophilic molecules (e.g. Nile Red), occurring deeper inside the lipophilic tunnel (Fig. 4E).

176 Further, analyses from molecular dynamics (MD) simulations suggested both structural and

177 ligand recognition roles for residues D82 and D93 (*Supplemental Results*). These residues are

178 located in the loops at the top of the tunnel opening of BcnA. Site-directed mutagenesis was

179 performed and the BcnA$_{D82A-D93A}$ mutant showed decreased binding affinity for Nile Red (Fig.

180 S5A-B), which we propose is due to structural changes (*Supplemental Results*, Fig. S6A-E, and

181 Fig. S7). Docking Nile Red into the BcnA$_{D82A-D93A}$ mutant (following the general docking

182 protocol with the minimized average structure from the ns 2.5 to the ns 5 of the MD simulation)

183 did not lead to any binding position inside the lipophilic tunnel (Fig. S7). Interestingly, D93 is a

184 highly conserved residue in the consensus motif of the BCN protein family (Fig. S5C),

185 exemplified in the alignment of BCN homologs (Fig. S5D). Further, Asp is found in equivalent

186 positions to D82 in homologs of *B. cenocepacia* BcnA. Thus, it is credible that the mode of

187 interaction between BcnA and antibiotics is common among this large family of conserved

188 bacterial proteins.

189 **Exogenous BcnA protects different bacterial species from antibiotic killing *in vitro* and**

190 ***in vivo*.** Since *B. cenocepacia* BcnA is secreted (Fig. S2D), as predicted for most other BCNs, we

191 therefore hypothesized that exogenous BCNs produced by one bacterial species have the

192 potential to protect other bacteria from the action of antibiotics including antimicrobial peptides.

193 This was investigated using purified recombinant BCNs from *B. cenocepacia*. *In vitro* antibiotic

194 protection assays showed that *P. aeruginosa* PAO1 treated with 1.5 µM purified BcnA had

195 reduced sensitivity to PmB, but not with BcnB (Fig. 5A). In contrast, at 8- to 16-fold lower PmB

196 concentrations, both proteins protected *Salmonella enterica* Typhi, *Shigella flexneri*,

197 *Acinetobacter baumannii*, *Acinetobacter lwoffii*, and *Acinetobacter junii* strains (Fig. S7A). This

198 disparity in the PmB concentrations at which protection by BcnA and BcnB occurs correlates

199 with their relative affinities to Nile red (Fig. S3N-Q, see above). *In vivo* infections in C57BL/6

200 mice demonstrated that BcnA protected *P. aeruginosa* Q502, a virulent cystic fibrosis clinical

201 isolate (26), from PmB killing in an intraperitoneal sepsis model (Fig. 5B). We also employed

202 the *Galleria mellonella* larvae infection model. The Δ*bcnA* and Δ*bcnB* mutants had reduced

203 virulence in *G. mellonella* relative to the parent strain (Fig. S7B). However, significantly lower

204 numbers of Δ*bcnA* bacteria than parental and Δ*bcnB* strains were recovered from the hemolymph

205 of infected larvae at 200 min post-infection (Fig. S6B), suggesting Δ*bcnA* bacteria were more

206 susceptible than Δ*bcnB* to larvae's humoral immune response, which is mainly driven by host

207 antimicrobial peptides (27, 28), mirroring the different *in vitro* susceptibility of Δ*bcnA* and

208 Δ*bcnB* to PmB. Similarly, infection of BcnA-treated *Galleria mellonella* larvae with *P.*

209   *aeruginosa* PAO1, resulted in more rapid killing of the larvae than control or BcnB-treated

210   larvae (Fig. 5C). We recovered significantly higher bacterial CFUs from the hemolymph of

211   BcnA-treated infected larvae (Fig. 5D), suggesting that exogenous BcnA provides a survival

212   advantage to infecting bacteria. Enhanced bacterial killing of BcnA-treated larvae was also

213   observed for *Klebsiella pneumonia*, *A. baumannii*, and *S. aureus* USA300 (Fig. S7C). Together,

214   the *in vivo* infections results underpin a biological role for BCNs in providing a survival

215   advantage to infecting bacteria from different species.

216   **Liposoluble vitamins inhibit BcnA-mediated antibiotic capture.** Conceivably, molecules

217   with superior binding affinity to BCNs than that of antibiotics should prevent BCN mediated

218   resistance. Since lipophilic moieties are predicted to bind deep within the BCN pocket, we tested

219   normal dietary hydrophobic supplements, recommended for several patient groups including

220   cystic fibrosis patients, such as the fat-soluble vitamins α-tocopherol (vitamin E) and

221   menaquinone (vitamin K2). Docking of α-tocopherol showed its alkyl chain buried into the

222   BcnA tunnel and its cyclic head placed towards the entrance, similar to the Nile Red binding

223   pose (Fig. 4E). Of note, α-tocopherol followed by menaquinone exhibited very low K_i values in

224   Nile Red displacement assays (~2-4 orders of magnitude lower than the K_i values of antibiotics),

225   indicative of their high affinity to BcnA (Fig. 3B). This prompted us to test the BCN-inhibitory

226   activity of fat-soluble vitamins *in vivo*. Using *G. mellonella* larvae infected with *P. aeruginosa*

227   PAO1, 10 μM of either α-tocopherol or menaquinone significantly reduced the survival

228   advantage of *P. aeruginosa* in BcnA-treated larvae (Fig. 6A). This supports the notion that the

229   protective function of BCN on infecting bacteria can be inhibited *in vivo* (Fig. 6B).

230

231   **DISCUSSION**

232   Lipocalins are an ancient family of small proteins found in all kingdoms of life with the ability to

233   bind hydrophobic ligands, but with different functions depending on the cell types and organisms

234   (29, 30). In many cases and particularly in bacteria (17), their function is unclear although BCN

235   orthologs can be found by data mining in the majority of completed genomes. In this study, we

236   have demonstrated for the first time that secreted BCNs contribute to antibiotic resistance by

237   capturing and neutralizing antibiotics in the bacterial milieu. BCNs bind a range of antibiotics

238   with diverse chemical structures, increasing antibiotic resistance *in vitro* and enhancing bacterial

239   survival *in vivo*. Our structural work suggests that BCNs have two binding modes. Hydrophobic

240   molecules like Nile Red and Vitamin E and K2 can bind in the interior of the lipocalin tunnel,

241   while antibiotic molecules interact with the rim and their binding properties are much weaker.

242   This suggests that antibiotic binding and scavenging is not a primary function of secreted BCNs

243   but these proteins may also have other yet undiscovered roles.

244   We propose that the antibiotic binding ability of BCNs becomes particularly relevant under

245   conditions whereby antibiotics cannot effectively kill bacteria and their presence in sublethal

246   concentrations elicits protective bacterial stress responses. Exposure to antibiotics triggers

247   complex and multi-factorial bacterial processes involving changes in regulation, metabolism and

248   energy generation (31-35). There is a body of evidence indicating that antibiotics at sublethal

249   concentrations can stimulate the production of reactive oxygen intermediates (36-40), and also

250   that oxidative stress associated with pathological inflammation reduces the efficacy of antibiotics

251   (33). The increased *bcnA* transcription upon antibiotic treatment indicates this gene responds to

252   antibiotic-induced stress. Recent work in the model plant *Arabidopsis thaliana* shows that two

253   lipocalins that are related to bacterial orthologs have distinct but overlapping functions essential

254   for protection from lipid peroxidation (41). Further, the mammalian odorant-binding protein, a

255   soluble lipocalin, protected the bacterial cells from hydrogen peroxide-induced stress when

256   overexpressed in *E. coli* (42), whereas *P. aeruginosa* PAO1 BcnA1(Pa) was overexpressed in

257   response to hydrogen peroxide and paraquat (43). Donnarumma *et al*. (18) have recently

258   suggested that the highly conserved *Neisseria* BCN (GNA1030) is a ubiquinone-8 binding

259   protein. Since ubiquinone-8 is a cofactor mainly involved in the electron transport chain (44) and

260   with antioxidant properties(45), these authors propose a role for this protein in antioxidant

261   defense, perhaps by delivering ubiquinone-8 to the bacterial membrane or the periplasmic space.

262   Ubiquinones are prenylated benzoquinones. Interestingly, the majority of known crystal

263   structures of bacterial lipocalins including BcnA and BcnB have been solved with octaprenyl-

264   like molecules bound to the lipocalin tunnel, although it is not certain if the presence of this

265   molecule corresponds to a physiological substrate or is an artefact associated with the protein

266   purification prior to crystallization. Therefore, while it may be reasonable to propose that BCNs

267   could play a role in oxidative stress responses the mechanisms involved remain to be elucidated.

268   In summary, we have uncovered a new bacterial strategy for general antibiotic resistance

269   operating extracellularly based on BCN-mediated antibiotic capture, which provide bacteria with

270   a selective advantage to overcome antibiotic toxicity particularly in chronic infections where

271   antibiotic treatment often fails. Further, we reveal a strategy to disrupt antibiotic capture and

272   propose liposoluble vitamins as clinically usable BCN inhibitors.

273

274   **MATERIAL AND METHODS**

275   **Strains and reagents.** Table S2 lists bacteria and plasmids used in this study. Bacteria were

276   grown in LB (supplemented with 0.4% rhamnose when required) at 37°C. *Escherichia coli*

277   cultures were supplemented as required with the following antibiotics (final concentrations):

278 tetracycline (30 µg/ml), kanamycin (40 µg/ml), and trimethoprim (50 µg/ml). *B. cenocepacia*

279 cultures were supplemented as required with trimethoprim (100 µg/ml), and tetracycline (100

280 µg/ml). Antibiotics (Sigma) were diluted in water except for PmB, which was diluted in 0.2%

281 bovine serum albumin/0.01% glacial acetic acid buffer. Rifampicin was dissolved in dimethyl

282 sulfoxide (DMSO).

283 **General molecular techniques.** DNA manipulations were performed as previously

284 described (46). T4 DNA ligase (Roche Diagnostics), Antarctic phosphatase (New England

285 Biolabs) and restriction endonucleases were used as recommended by the manufacturers.

286 Transformation of *E. coli* GT115 and DH5α was performed using the calcium chloride method

287 (47). Mobilization of plasmids into *B. cenocepacia* was conducted by triparental mating (48)

288 using *E. coli* DH5α carrying the helper plasmid pRK2013 (49). DNA amplification by

289 polymerase chain reaction (PCR) was performed using a C1000 Thermal cycler (Bio-Rad

290 Laboratories Ltd., Mississauga, Ontario, Canada) with Taq or HotStar HiFidelity DNA

291 polymerases (Qiagen) and optimized for each primer pair. DNA sequencing was carried out at

292 Eurofins, Huntsville, Alabama, USA. The DNA sequences were analyzed with the BLAST

293 computer program and compared to the sequenced genome of *B. cenocepacia* strain J2315. The

294 sequence of *S. aureus* gene SAUSA300_2620 was optimized for *B. cenocepacia* codon usage

295 and custom synthesized at Eurofins. Cloning, expression, and purification of bacteriocalins was

296 performed as previously described (16). Transcriptional fusions to *luxCDABE* and the

297 subsequent luminescence expression assays were performed as previously described (36)*.* For

298 site-directed mutagenesis, pOE16 was amplified with Pfu polymerase using the appropriate

299 primer pairs; the PCR products were digested overnight with 1 U *Dpn*I at 37°C, and then

300 introduced into *E. coli* DH5α competent cells by transformation. Transformants were selected on

301 LB agar plates supplemented with kanamycin; amino-acid replacements were confirmed by

302 DNA sequencing.

303 **Protein analysis and Western Blotting.** Overnight cultures were diluted to OD_{600} 0.03 in 30

304 ml fresh LB medium with or without PmB and incubated for 3.5 h at 37^oC, 200 rpm. Following

305 incubation, cells equivalent to OD_{600} ~0.2 were pelleted, resuspended in 30 µl SDS-PAGE

306 protein loading dye, and boiled to obtain whole cell lysates. Secreted proteins were precipitated

307 from the supernatant of the rest of the cultures using 10% trichloroacetic acid as previously

308 described (50). Precipitated proteins were resuspended in Tris buffer, 1 M, pH 7.5. The volume

309 of protein samples loaded to the 16% SDS-polyacrylamide gel was normalized to the OD_{600}

310 value. After SDS–PAGE, proteins were transferred onto nitrocellulose membranes and the

311 membranes were blocked overnight at 4°C with Western blocking reagent (Roche Diagnostics,

312 Laval, QC, Canada) in TBST (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1 % Tween-20). The

313 primary antibodies, anti-FLAG M2 monoclonal antibody (Sigma) or anti-α-subunit RNA

314 Polymerase (*E. coli*) (Neoclone, Madison, WI, USA), were diluted to 1:15,000 in TBST and

315 applied for 1.5 h. Secondary antibody, goat anti-mouse Alexa Fluor 680 IgG antibodies

316 (Invitrogen), was diluted to 1:15,000 and applied for 1 h. Western blots were developed using

317 LI-COR Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA).

318 **Antibiotic susceptibility testing.** The inoculum of *B. cenocepacia* K56-2, the appropriate

319 mutants and other bacterial species was prepared by the direct colony suspension method

320 according to CLSI (51). Cultures of OD_{600} of 0.0008 in fresh cation-adjusted MHB with or

321 without the antibiotic were incubated at 37°C with medium continuous shaking in a Bioscreen C

322 automated growth curve analyzer (MTX Lab Systems, Vienna, VA, USA). Bacterial growth was

323 assessed turbidimetrically at 600 nm. Etest strips (AB bioMérieux, Solna, Sweden) were applied

324   to agar plates (17 ml agar in 85 mm Petri dish) inoculated with test bacteria by swabbing

325   overnight cultures diluted to OD_{600} of 0.04; plates were then incubated at $37^{\circ}C$ for 24 h.

326   Alternatively, population analysis profiling (PAP) was performed turbidimetrically or by cfu

327   counting as previously described (16). For *in vitro* protection assays, *B. cenocepacia*

328   bacteriocalins were added to LB broth at a final concentration of 1.5 µM.

329   ***In vitro* binding assays.** These assays were performed as previously described (22) with few

330   modifications. Purified BCNs were prepared in phosphate buffered saline (PBS, pH 7.4).

331   Phospholipids and Nile Red were prepared in DMSO. The binding of each fluorescent probe to

332   BCNs was measured by titrating 100 µl of BCNs (1.5 µM) in a flat bottom 96-well microtiter

333   plate (LUMITRAC 200 White, Greiner bio-one, Monroe, NC, USA) with aliquots of increasing

334   concentrations of probe until fluorescence intensity reached plateau indicating all binding sites

335   were occupied. All spectra were corrected for background fluorescence determined from probe

336   into buffer titrations. Fluorescence was measured using a Cary Eclipse Fluorescence

337   spectrophotometer (Varian) set at an excitation wavelength (λ_{ex}) specific for each probe, as

338   follows: Nile Red (550 nm), and BODIPY phospholipids (500 nm for fatty acyl BODIPY labeled

339   phosphocholine and 505 nm for head group BODIPY labeled phosphoethanolamine). The

340   emission spectrum for each probe was collected across the following wavelengths (λ_{em}): Nile

341   Red (590–750 nm), and BODIPY phospholipids (510–665 nm). The background-corrected

342   binding fluorescence with each probe was fitted to a one-site binding model as previously

343   described for human AGP (22). The equilibrium binding affinity constant for the probe–BCN

344   complex (K_D), the probe concentration needed to achieve a half-maximum binding at

345   equilibrium, was determined by non-linear least square regression analysis of the binding

346   isotherms using GraphPad Prism V5.0 software (GraphPad software).

347   For probe displacement experiments, antibiotic solutions and fat-soluble vitamins (prepared

348   in DMSO) diluted in PBS, pH 7.4 were titrated against BCN–probe complex at a saturating

349   concentration necessary to obtain the maximum fluorescence when bound. Displacement of

350   probe was measured as the corresponding decrease in fluorescence upon the progressive increase

351   of antibiotic concentration. The binding inhibitory constants (K_i) for the test compounds were

352   determined by nonlinear regression analysis using competition-binding equations for one site

353   binding calculated by GraphPad Prism V5.0 software. The lower the K_i values, the higher the

354   affinity of the molecule to BcnA. All fluorometric assays were conducted in duplicate 3

355   independent times.

356   *Galleria mellonella* **larvae** *in vivo* **infection.** These assays were performed as described (52)

357   with modifications. Overnight cultures were diluted in PBS, pH 7.4 with or without *B.*

358   *cenocepacia* BCNs at 1.5 µM final concentration to OD_{600} as follows: *B. cenocepacia* and *P.*

359   *aeruginosa* PAO1 to 0.00004, *K. pneumoniae* Kpn18 to 0.04, *A. baumannii* AB1 to 0.4 and *S.*

360   *aureus* USA300 to 0.004. The larvae were injected with 10 µl of the bacterial suspensions or

361   sterile PBS (10 larvae/group in each experiment) using 10 µl Microliter syringes (Hamilton). The

362   larvae were incubated at $30^{o}C$ and their viability was checked at regular time intervals. In similar

363   assays, 5 larvae/group were sacrificed at 200 min post-infection and the hemolymph was

364   extracted as previously described (52). The hemolymph was immediately serially diluted in PBS,

365   plated on LB agar supplemented with 0.3% cetrimide or 200 µg/ml Ampicillin- 25 µg/ml PmB to

366   quantify the cfu of *P. aeruginosa* PAO1 or *B. cenocepacia* respectively recovered from the

367   infected larvae.

368

369   **Intraperitoneal infection in mice.** A clinical isolate of *Pseudomonas aeruginosa* (strain

370   Q502) was grown overnight in nutrient broth at 37°C with constant agitation. The bacteria were

371   centrifuged at 2000 ×*g* and washed 3 times in sterile endotoxin-free PBS. The bacteria were

372   resuspended in sterile injection-grade saline and the inoculum adjusted to an optical density of

373   0.5 (A_{550}). Female, adult (8-12-week old) C57BL6 mice were infected intraperitoneally with 100

374   µl of the bacterial suspension, subsequent growth of the inoculum on nutrient agar demonstrated

375   that each animal received 10^6 CFU. A sample size of n=6 mice per treatment was used. This was

376   determined by GraphPad StatMate 2.0 to ensure 80% power to detect statistically significant

377   effects between antibiotic treated and untreated animals at significance level (alpha) of 0.05,

378   two-tailed. The actual power was >99%. Mice were selected at random from open stock cages (n

379   = 10 per cage), ear marked to allow individual identification and then sequentially placed into

380   treatment groups. During the course of the experiment mice were housed in individually

381   ventilated cages "IVCs". This method of assigning animals to groups ensures that there is

382   approximately equal distribution of mice from different stock cages in each group to minimize

383   the influence of cage-to-cage variability. At the time of inoculation, the mice were treated with

384   PmB at the standard pediatric dose of 20,000U/kg (n = 6), PmB and 100 µl of 25 µM BCN (n =

385   6), BCN only (n = 6), or a saline control (n = 6) by intraperitoneal injection. The individual

386   components injected into mice were added to the same syringe immediately before the IP

387   injection. Animals were culled by cervical dislocation 4 h post inoculation. The time point was

388   selected since by this time point and under the infection conditions the untreated mice reach the

389   humane end point and need to be culled, as defined within the UK Home Office license under

390   which the experiments were carried out (PLL 2700). Due to the virulence of the clinical isolate,

391   the dose of the bacterial inoculum, and because the bacteria are delivered IP, the mice rapidly

392 succumb to the infection. In contrast, those given effective antibiotic treatment rapidly clear the

393 infection and remain perfectly healthy. The vast divergence in response seen in this model

394 provides us with the statistical power to robustly assess the microbial response to antibiotic

395 therapies without requiring the use of a very large numbers of mice per group. We are therefore,

396 adhering to the reduction principle of the 3Rs. The peritoneal cavity was lavaged with 3.5 mL of

397 ice-cold sterile endotoxin free PBS and the volume recovered recorded. Serial dilutions of the

398 lavage fluid were plated onto cetrimide agar; bacterial colonies were counted after 24 hours of

399 growth at 37°C. Harvesting the samples and quantifying the bacterial burden in the mice was

400 blinded to the treatment groups. Data were not normally distributed and there was not equal

401 variance between groups; therefore, a non-parametric Kruskal-Wallis test was used. Mouse

402 infection experiments carried out were assessed by the Queen's University Belfast animal

403 welfare and ethical review body (AWERB) committee and conducted under a license issued by

404 the UK home office under the Animals (scientific procedures) Act 1986, amended 2012.

405 **Antibiotic bioassay.** Antibiotic test solutions with or without 1.5 μM BcnA were incubated

406 for 30 min at 37°C with rotation. The solutions were filtered through filter units with MWCO 10

407 KDa by centrifugation at 7500g, at 4°C for 10 min. The concentration of antibiotics in the

408 filtrates was determined by spotting 5 μl on sterile filter discs placed on agar plates swabbed

409 with the test bacteria. Petri dishes (15 cm diameter) containing 40 ml LB agar were swabbed

410 with bacterial suspensions of OD_{600} 0.04. The plates were incubated at 37°C for 24 hr. Each

411 plate included 4 discs containing standard concentrations of the antibiotic alongside the discs

412 impregnated with test and control antibiotic solutions. *E. coli* DH5α was used for bioassays of

413 PmB, norfloxacin, ceftazidime and gentamicin and *S. aureus* USA300 for bioassays of

414 rifampicin. The theoretical disc content of the test antibiotic solutions was 10, 5, 2, 30 and 10 μg

415   for PmB, rifampicin, norfloxacin, ceftazidime and gentamicin respectively. Standard antibiotic

416   discs contained 2-fold higher, the same amount, 2 and 4-fold lower than the test antibiotic discs.

417   After incubation, the clear zones of inhibition were measured and the antibiotic concentrations

418   were determined from standard curves constructed from the standard antibiotic discs.

419   **Structure determination.** BcnA and BcnB was purified by FPLC and concentrated to ~ 20

420   mg/ml in 20 mM Tris, pH 7.5, 100 mM NaCl (1% DMSO for BcnB) with > 95% purity. Protein

421   solutions were mixed 1:1 with mother liquor and crystals were grown at room temperature.

422   BcnA crystals grew with 0.1 M Tris, pH 8.5 and 2.4 M ammonium sulphate (final pH 8.0) as the

423   mother liquor. BcnB crystals grew with 0.1 M HEPES pH 6.5, 26% PEG 6000 as the mother

424   liquor. Datasets were collected on beam line 08ID-1 at the Canadian Light Source (53),

425   integrated using iMOSFLM (54), and scaled with AIMLESS (55) from CCP4. Phases for BcnA

426   and BcnB were obtained using Phaser.MRage (56) from PHENIX using PDB ID 2FGS and

427   1WUB, respectively, as the search models. Both structures were initially built with AutoBuild

428   (57) from PHENIX, and then manually built with Coot (58). The BcnA and BcnB structures

429   were refined with phenix.refine (59) from PHENIX and REFMAC5 (60) from CCP4,

430   respectively, with TLS refinement for both. Figures were generated using Pymol, version 1.8

431   (https://www.pymol.org/). Data collection and refinement statistics are provided in Extended

432   Data Table 1. Size exclusion chromatography-multiangle light scattering (SEC-MALS)

433   experiments were conducted to assess the oligomeric solution state of BcnA and BcnB as

434   previously described (61) with proteins diluted to 1 mg/ml in 20 mM Tris, pH 7.5, 100 mM

435   NaCl.

436

437   **Computational Methods.** All codes can be obtained under License Agreement: AMBER 12

438   and Amber Tools (www.ambermd.org), Maestro suite (www.schrodinger.com), AutoDock 4.2.2

439   and AutoDockTools (http://autodock.scripps.edu, free software). Amber and Maestro have

440   Academic fees. We studied the stability of the two X-ray structures for BcnA and BcnB, as well

441   as the D82A-D93A mutant by molecular dynamic (MD) simulations as implemented in AMBER

442   12. The initial model of D82A-D93A was built with Amber Tools. Missing hydrogen atoms were

443   added and protonation state of ionizable groups was computed by using Maestro Protein

444   Preparation Wizard, version 9.3 (https://www.schrodinger.com/maestro). Atom types and

445   charges were assigned according to AMBER ff10 force field (62). The three molecular systems

446   were hydrated by using cubic boxes containing explicit TIP3P water molecules extending 10 Å

447   away from any protein atom for simulating the aqueous environment with the help of Amber

448   Tools with added counter ions to neutralize the system. Before the MD simulations, the two

449   systems were equilibrated under the following protocol: initial 8000 steps of steepest descent

450   minimization, followed by heating of the system with position restrain (force constant of 20 kcal

451   mol^{-1} $Å^{-2}$) for all protein atoms during 10 ps of MD simulation increasing the temperature from

452   100 K to 300 K plus additional 15 ps at constant temperature of 300 K. Position restrain was

453   gradually decreased during 100 ps at constant 300 K, until the full system was under no restrains

454   with constant temperature (300 K) and pressure (1 atm). After equilibration, 20 ns of MD

455   simulation were run at constant temperature (300 K) and pressure (1 atm). Short and long-range

456   forces were calculated every one and two time steps, respectively (each time step = 2.0 fs),

457   constraining the covalent bonds involving hydrogen atoms to their equilibrium values. Long-

458   range electrostatic interactions were accounted for by means of the particle mesh Ewald

459   approach applying periodic boundary conditions. The root mean square deviation (RMSD) as a

460   function of time with respect to the starting structure for the α-C atoms was computed using

461   CPPTRAJ (63). The 3D coordinates of the structures for BcnA and BcnB subjected to the

462   equilibration protocol described above were used for docking purposes. The two models were

463   prepared for docking calculations by adding Kollman charges (64) with the help of

464   AutoDockTools.

465   3D coordinates of norfloxacin, PmB, ceftazidime, gentamicin, Nile Red and α-tocopherol

466   were built in Corina (65) from the SMILES code. The 3D structure of rifampicin was extracted

467   from the crystallographic structure PDB-ID 1LSV. The structures of the seven ligands were

468   protonated at pH 7.0 using Epik (66) and then optimized with MMFFs force field by using

469   MacroModel version 9.9 (https://www.schrodinger.com/macromodel). Additionally, the

470   structure of polymyxin B was subjected to 100 ps of MD simulation, at 300 K. Ligands were

471   prepared for docking calculations using AutoDockTools by adding Gasteiger charges (67) and

472   setting all rotatable bonds free to move during the docking calculation.

473   Docking calculations of all compounds were performed by means of AutoDock 4.2.2 (68).

474   Analysis was performed with the help of AutoDockTools. The grid point spacing was set at

475   0.375Å, and a hexahedral box was built with x, y, z dimensions 21.00 Å, 26.25 Å, 27.75 Å

476   centred in the binding site of the protein. 200 runs using Lamarckian Genetic algorithm were

477   performed, with a population size of 100, and 250000 energy evaluations. Side chains of residues

478   Y85, W94 and Q41 were considered as flexible during the docking protocol.

479   **BCN consensus motif determination.** A subset of 187 curated 187 BcnA homologues from

480   different bacterial species and families were used to obtain a consensus motif generated by the

481   Gapped Local Alignment Motifs "GLAM2" tool (69). This motif was verified by analyzing an

482   alignment of 1995 BCN homologues using CLUSTAL-omega and visualized using JalView. In

483   addition, upon submitting the predicted motif into the "GLAM2Scan" database (69) against *B.*

484   *cenocepacia*, *P. aeruginosa* PAO1, *M. tuberculosis* H37Rv and *S. aureus* USA300, the correct

485   homologues only were detected as BCNs for each of the organisms.

486   **Statistical Analyses.** Statistical analyses were conducted with GraphPad Prism 5.0. All

487   results are shown as mean ±SEM unless otherwise stated. Unless otherwise stated, data were

488   assumed to follow a Gaussian distribution as determined by D'Agostino-Pearson omnibus K2

489   normality test whenever possible and hence t-tests and ANOVA were used. Unpaired t-test was

490   used to compare the means of two unmatched groups. Paired t-test was used to compare the

491   means of two matched groups, assuming that the distribution of the before-after differences

492   follows a Gaussian distribution. One-way ANOVA followed by Dunn's multiple comparison test

493   was used to compare the means of three or more unmatched groups. The variances were not

494   significantly different among the groups being statistically compared as determined by F-test,

495   except in the *in vivo* mice infection assay (See the "Intraperitoneal infection in mice" section).

496   The sample size (n) was chosen using GraphPad StatMate 2.0 to ensure a minimum of 80%

497   power to detect statistically significant effects at significance level (alpha) of 0.05, two-tailed.

498   However, the actual power of most of the assays was ≥90% and in many cases exceeded 99%. In

499   case of MIC assays, the experiments were repeated 3 independent times and the experiment

500   showing the lowest fold change (if applicable) was reported.

501

502   **SUPPLEMENTAL MATERIAL**

503   Supplemental Results

504   Table S1. X-ray crystallography data collection and refinement statistics

505   Table S2. Strains and Plasmids

515

516

528 Western Ontario for providing the *S. aureus* USA300 strain, and Alice Dubois, Queen's

529 University for assistance with the mouse infection experiments.

530

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721

722

723   **Figure Legends**

724

725   FIG. 1. *B. cenocepacia* BcnA confers resistance to hydrophobic but not hydrophilic antibiotics.

726   (A) MIC by broth microdilution of different antibiotics in cation-adjusted MHB at 18-24 h

727   (representative from 3 independent experiments). (B) Luciferase expression assay of the *B.*

728   *cenocepacia* BcnA (OME61) in response to antibiotics at 3 h. n= 6 from 2 different clones.

729   Results are shown as percentage of relative light units RLU/OD_{600} relative to the control

730   (untreated K56-2 background). The $\%OD_{600}$ are shown in Fig. S2*D*. The mean RLU/OD_{600} of the

731   control is 1.3464. * $p<0.05$ and *** $p<0.001$ from unpaired two-sided Student's t-tests compared

732   to the respective control conditions. (C) Genomic organization of the *B. cenocepacia* K56 region.

733   The asterisk denotes that the transcribed intergenic sequence between *bcnA* and *bcnB* has the

734   potential to form strong secondary structures as determined with mFold

735   (http://unafold.rna.albany.edu/?q=mfold/download-mfold) (70).

736

737   FIG. 2. BCNs from different bacteria can restore full antibiotic resistance in *B. cenocepacia*

738   Δ*bcnA*. (A) cfu count on LB agar containing PmB, from 3 independent experiments, n=6,

739   asterisks denote difference from Δ*bcnA* pSCRhaB2 mutant. * $p<0.05$, ** $p<0.01$ and ***

740    $p<0.001$ determined by unpaired two-sided Student's t-tests. (B) MIC by Etest against rifampicin

741   (Rif), ceftazidime (Cef) and ciprofloxacin (Cipro), a representative of 3 independent

742   experiments. The highest rifampicin concentration on the Etest strips is 32 µg/ml; ≥64 indicates

743   that an MIC could not be detected within the Etest concentration range and would be equal to 64

744   µg/ml or higher.

745

746 FIG. 3. BCNs bind antibiotics and other molecules hydrophobic in nature with high affinity. (A)

747 Antibiotic assay showing reduction in concentration of hydrophobic antibiotics due to

748 sequestration by BcnA, n=3 from three independent experiments. (B) *in vitro* binding assay

749 showing binding inhibition constant (K_i) of antibiotics against 1.5 µM Nile Red binding to 1.5

750 µM BcnA in PBS, 3 independent experiments, n=5.

751

752 FIG. 4. Structural analysis of BcnA and BcnB and ligand docking modeling. (A) BcnA (blue) is

753 a monomer. (B) BcnB (protomer 1, chain C, peach; protomer 2, chain D, grey) is a dimer. (C)

754 Superposition of BcnA and BcnB with dissimilar tunnel opening loops boxed. (D) Docked

755 structure of PmB in complex with the BcnA crystallographic structure. (E) Docking model

756 presenting the superimposition of the two best predicted binding modes of Nile Red (magenta

757 and cyan) and α-tocopherol (yellow) when docked into the BcnA crystallographic structure.

758

759 FIG. 5. *B. cenocepacia* BcnA protects *P. aeruginosa in vitro* and *in vivo*. (A) *in vitro* protection

760 of *P. aeruginosa* PAO1 against PmB with 1.5 µM of BcnA or BcnB, n=8 from 4 independent

761 experiments. (B) Protection of *P. aeruginosa* Q502 from PmB killing in an intraperitoneal

762 infection of C57BL/6 mice, significant difference determined by Kruskal-Wallis test. (C) The

763 survival of *G. mellonella* larvae infected with *P. aeruginosa* PAO1 compared to control group

764 injected with sterile PBS; 10 larvae/group; the results are obtained from 3 independent

765 experiments. The survival of both PAO1 and PAO1-BcnB treated larvae is significantly different

766 from that of PAO1-BcnA treated group at p= 0.0165 and 0.0303 respectively. (D) PAO1 cfu/ml

767 recovered from larval hemolymph 200 min post-infection; n=10 from 2 independent

768   experiments. * p<0.05, ** p<0.01 and *** p<0.001 from unpaired two-sided Student's t-tests

769   compared to the respective control conditions.

770

771   FIG. 6. Fat-soluble vitamins inhibit BCN-mediated antibiotic binding. (A) The survival of *G.*

772   *mellonella* larvae at 20 h post-infection in an *in vivo* protection assay of *P. aeruginosa* PAO1 by

773   1.5 µM BcnA in the presence or absence of 10 µM α-tocopherol (vitamin E) or menaquinone

774   (vitamin K2), 10 larvae per group; the results are obtained from 4 independent experiments. *

775   p<0.05, ** p<0.01 and *** p<0.001 from unpaired two-sided Student's t-tests. (B) Model of the

776   mechanism of resistance by BcnA (top) and its inhibition by the fat-soluble vitamins (bottom).

777

778

A

MIC (μg/ml)

|	K56-2	ΔbcnA	ΔbcnB
PmB	≥4096	2048	≥4096
Rifampicin	256	64	256
Norfloxacin	128	32	128
Ceftazidime	32	8	32
Meropenem	1	0.5	1
Gentamicin	2048	2048	2048
Minocycline	2	0.5	2
Trimethoprim	8	4	8
Azithromycin	64	64	64

B

C
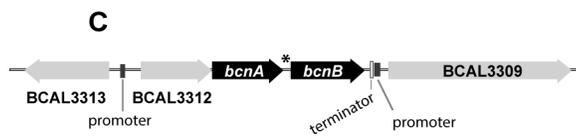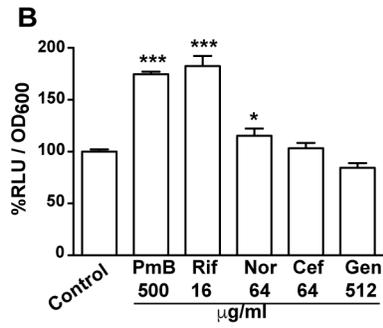
Bcn region in K56-2/J2315

4555 bp

A

B
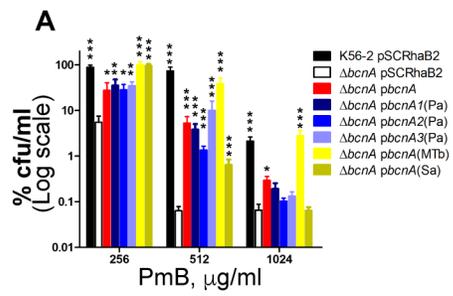
	MIC by Etest, µg/ml		
	Rif	Cef	Cipro
K56-2 pSCRhaB2	≥64	8	4
Δ*bcnA* pSCRhaB2	16	2	2
Δ*bcnA* p*bcnA*	≥64	8	4
Δ*bcnA* p*bcnA1* (Pa)	≥64	8	4
Δ*bcnA* p*bcnA2* (Pa)	32	8	4
Δ*bcnA* p*bcnA3* (Pa)	≥64	8	4
Δ*bcnA* p*bcnA* (MTb)	≥64	8	4
Δ*bcnA* p*bcnA* (Sa)	≥64	8	4

A
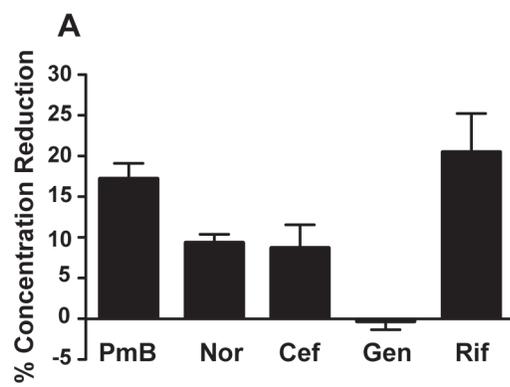
B

	K_i, mM	SE	r2
PmB	0.67	0.15	0.96
PmBN	5.08	2.54	0.35
Rif	$1.65*10^{-2}$	$2.6*10^{-3}$	0.97
Nor	0.40	0.29	0.86
Cef	16.9	8.45	0.33
Gen	115.4	57.7	0.005
Vit E	$4.17*10^{-4}$	$2.04*10^{-4}$	0.98
Vit K2	$5.69*10^{-4}$	$2.8*10^{-4}$	0.94

A

% OD600 vs PmB, mg/ml

- ● control
- □ BcnA
- ▲ BcnB

B

Log cfu/ml

control, BcnA, PmB, PmB + BcnA

ns
p=0.002,**
p=0.046,*

C

% Survival of larvae relative to PBS-treated group vs Time post-infection (h)

- ● PBS
- ■ PAO
- ◆ PAO + BcnA
- ▲ PAO + BcnB

D

cfu/ml of PAO1 recovered from larvae haemolymph

PAO1 control, PAO1 +BcnB, PAO1 +BcnA

p=0.0101,*
p=0.003,**
ns

A

% Survival of larvae relative to PBS-treated group

PAO1	−	+	+	+	+	+	+
BcnA	−	−	+	−	+	−	+
Vit E	−	−	−	+	+	−	−
Vit K2	−	−	−	−	−	+	+

B

Antibiotic   BCN

Protection from lethal
action of antibiotic by BCN

Vitamin K2
Antibiotic   Vitamin E   BCN

Fat-soluble Vitamins abolish BCN Protection
leading to bacterial killing by antibiotic

**Fig. S1.** Chemical structures of antibiotics and chemicals used in this study.
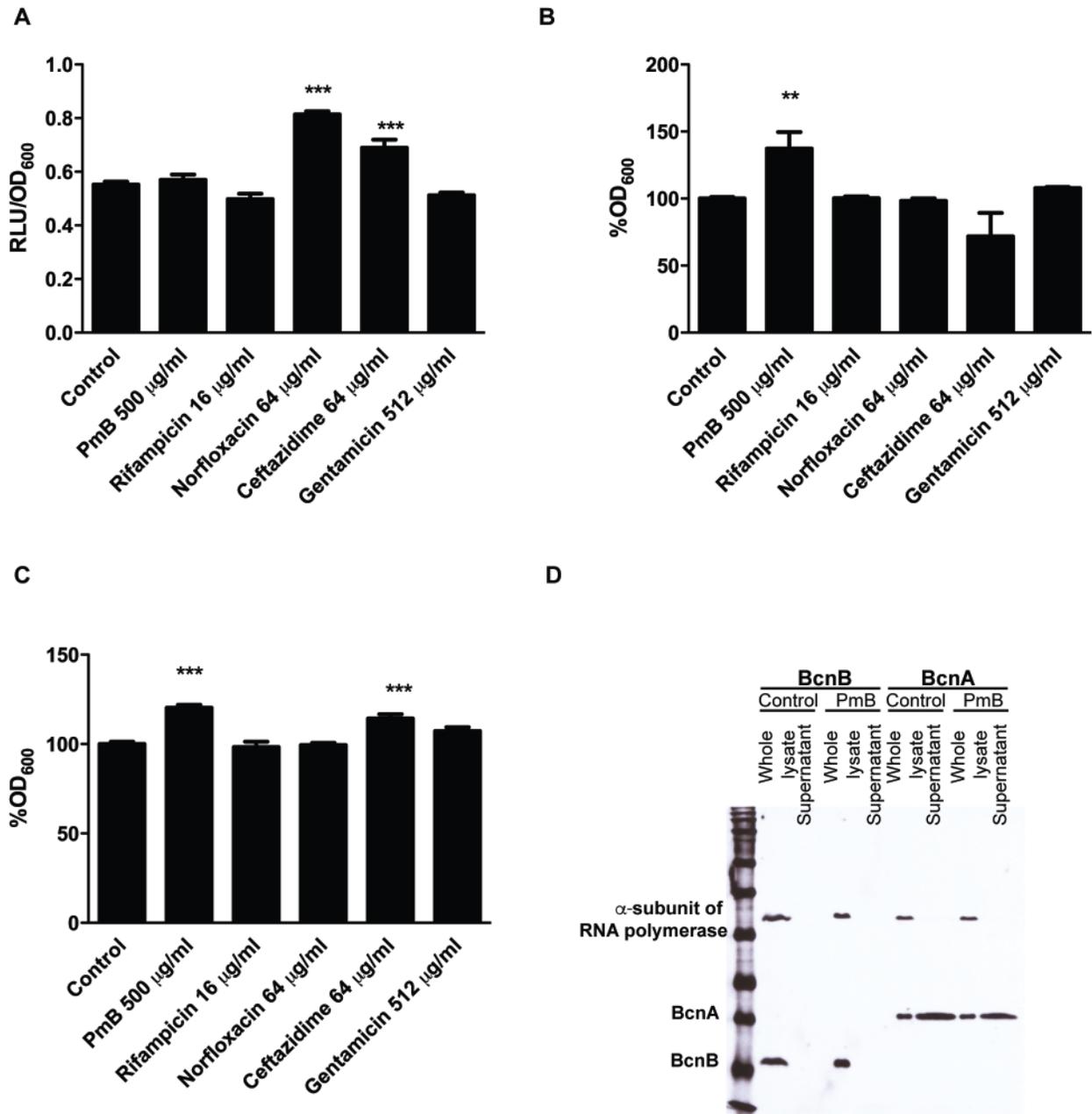
Fig. S2. Expression and secretion profiles of BcnA and BcnB. (A) Luciferase expression assay of BcnB and the relative growth of cells in the luminescence expression assay of the different *B. cenocepacia* BCNs in response to antibiotics at 3 h. Expression of BcnB (OME60). n=6 from 2 different clones. Results are shown as mean of relative light units RLU/OD$_{600}$ ±SEM. (B) Growth in the expression assay of BcnB (OME60). n=6 from 2 different clones. The mean OD$_{600}$ of the control is 0.0963. (C) Growth in the expression assay of BcnA and the associated CybB (OME61) shown in Fig. 1B. n= 6 from 2 different clones. Results are shown as mean of percentage of OD$_{600}$ relative to the control (untreated K56-2 background) ±SEM. The mean OD$_{600}$ of the control is 0.0998. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ from one-way ANOVA tests of overall $p<0.001$ and Bonferroni's post-hoc test compared to the respective untreated control conditions. (D) BcnA is the only secreted *B. cenocepacia* BCN; proteins (carrying a C-terminal FLAG-tag) were detected in whole cell lysates and supernatants of control untreated cultures or cultures treated with 2 µg/ml PmB by Western blot using anti-FLAG antibody. The α-subunit of the RNA polymerase was used as a control for cell lysis. Representative of 3 independent experiments.
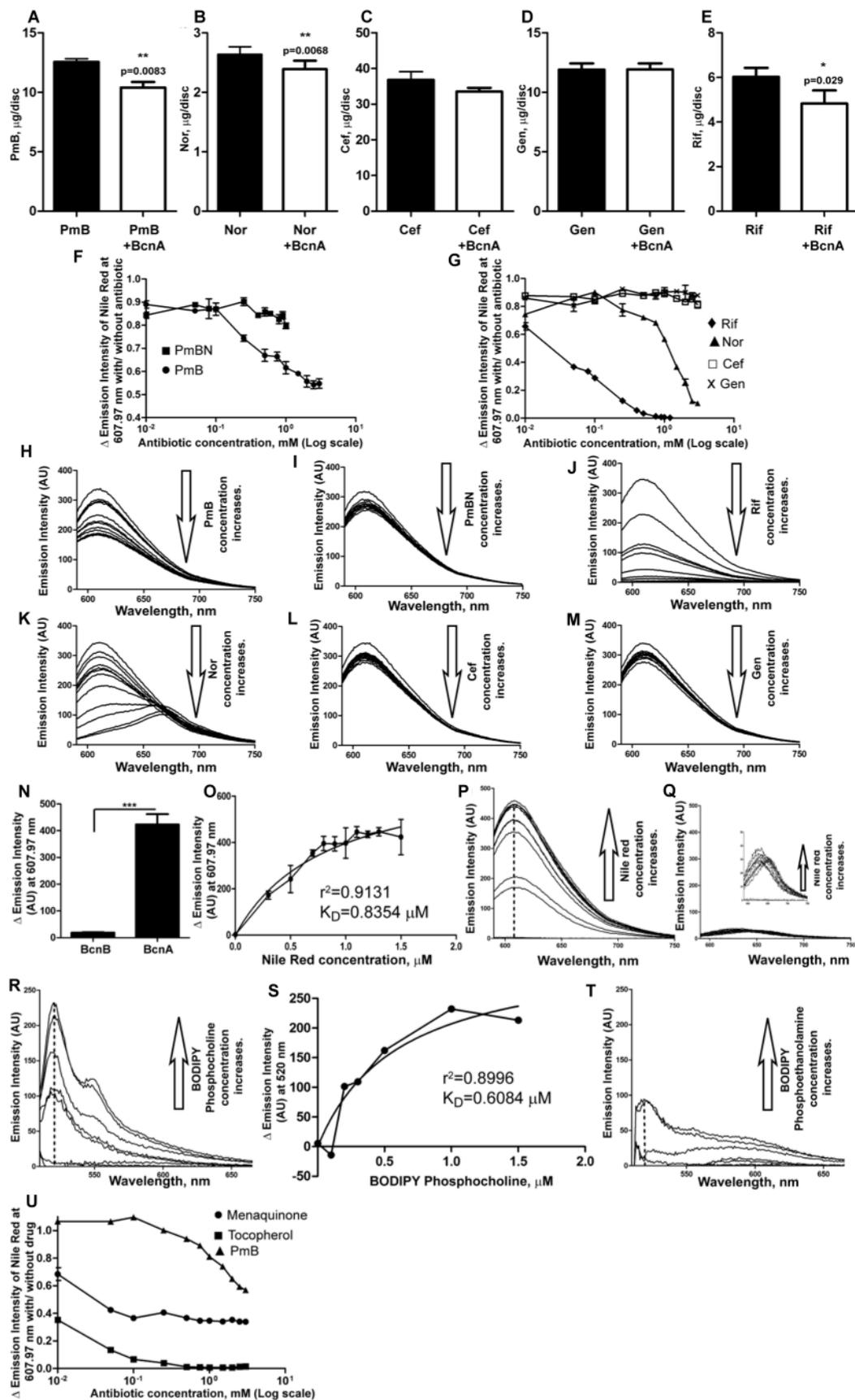
Fig. S3. Binding Assays. (A-E) Antibiotic bioassay results showing mean antibiotic disc contents ±SEM following mixing with BcnA and passing through centrifugal filter units of MWCO 10 KDa compared to control antibiotic solutions. * p<0.05,

and ** $p<0.01$ from paired student's t-tests compared to the respective control conditions. n=3 from 3 independent experiments. At this sample size, the actual power of the assay to detect statistically significant effects at significance level (alpha) of 0.05, two-tailed is >99%. (F-G) Binding displacement assays of Nile Red from 1.5 µM BcnA-1.5 µM Nile Red complex by antibiotics and other chemicals. 3 independent experiments, n=5. (H-M) Binding displacement isotherms showing the displacement of Nile Red by different antibiotics from its complex with BcnA. Mean of n=3 from a representative of 3 independent experiments. (N) The affinity of 1.5 µM BcnA compared to that of 1.5 µM BcnB to bind 1.5 µM Nile Red, n=5 from 3 independent experiments. *** $p<0.001$ from unpaired two-sided Student's t-test. (O) *In vitro* binding assay of 1.5 µM BcnA to Nile Red in PBS, 3 independent experiments, n=5. (P and Q) Binding isotherms from the fluorometric assays showing the interaction between BcnA (P) or BcnB (Q) and Nile Red. Mean of n=3 from a representative of 3 independent experiments. (R-T) Nile red binding affinity of BcnA to BODIPY phosphocholine (R and S) and BODIPY phosphoethanolamine (T), n=4 from 2 independent experiments. (U) Binding displacement assays of Nile Red from 1.5 µM BcnA-1.5 µM Nile Red complex by vitamins. n=5 from 3 independent experiments
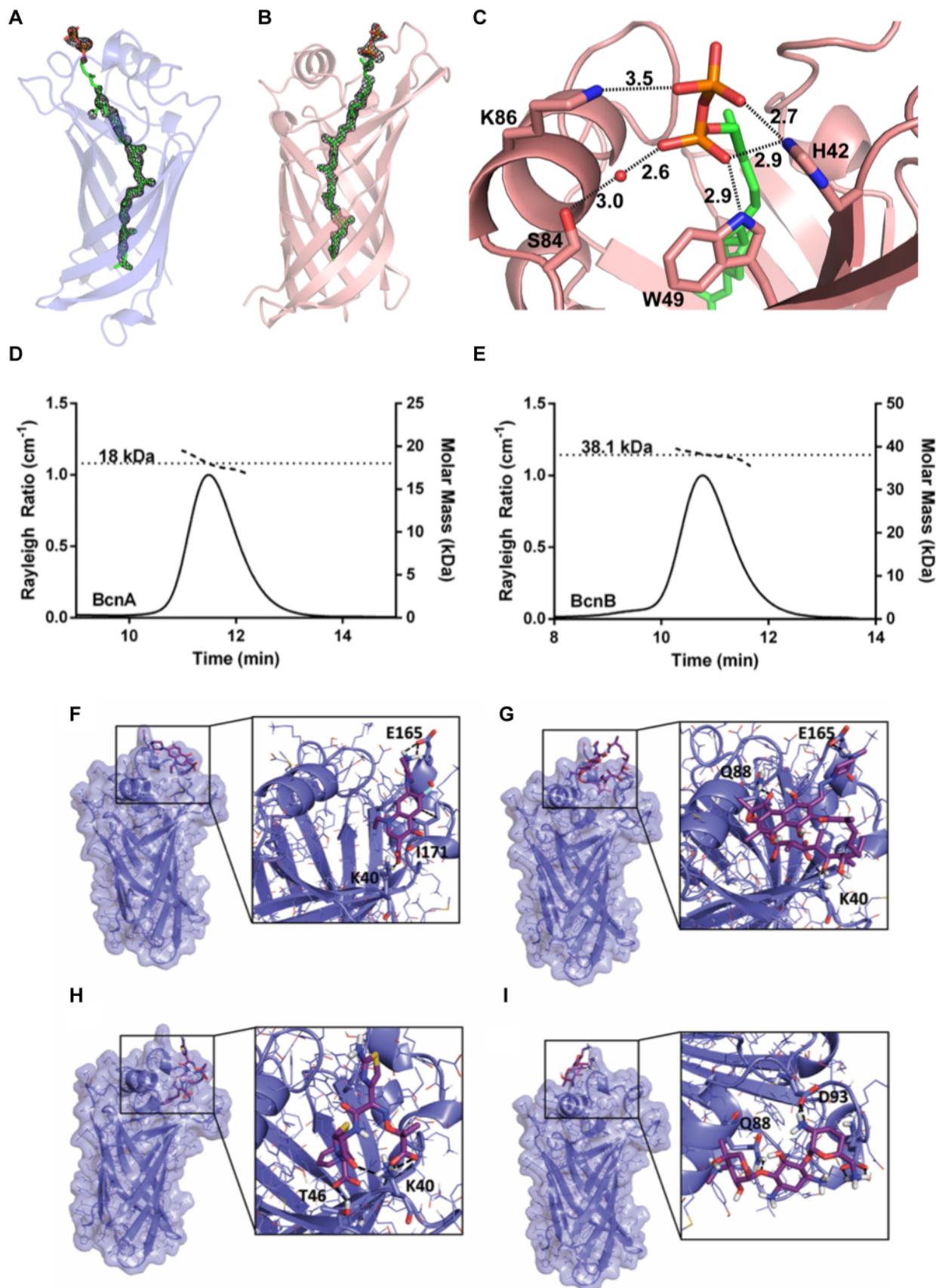
Fig. S4. BcnA and BcnB macromolecular structures and docking binding models of antibiotics into the BcnA structure. BcnA (A) and BcnB (B) possess electron density within the interior of the protein cavities that was modeled as octaprenyl pyrophosphate (OTP; carbon, green; phosphate, brown; oxygen, red). OTP omit maps (Fo-Fc) are contoured at

3.0 σ and shown in black mesh. (C) The cavity opening of BcnB provides a hydrogen-bonding network to the OTP pyrophosphate via amino acids H42, W49, K86, and S84 through a water (red sphere) bridge. These residues are shown as stick figures (carbon, peach; nitrogen, blue; oxygen, red). Hydrogen bonds are represented as dotted lines with distances between the hydrogen bond donor and acceptor atoms shown in Å. The molar mass determination of BcnA (D) and BcnB (E) in solution were determined by SEC-MALS; the elution profiles (solid lines) represent the intensity of scattered light and are expressed as rayleigh ratios. The measured molar masses (dashed line) were constrained to the single elution peak for each protein. The dotted lines represent the final calculated mass for each protein. The BcnA crystallographic structure was used to perform molecular docking experiments. Selected docked binding modes of the antibiotics norfloxacin (F), rifampicin (G), ceftazidime (H), and gentamicin (I) into the BcnA structure (displayed in blue) and main residues interacting with the different ligands (CPK colors) are shown.
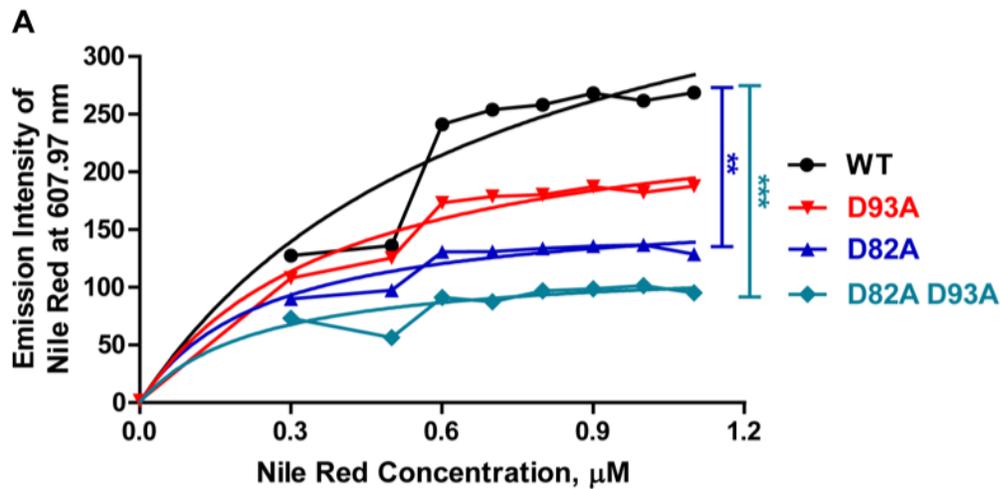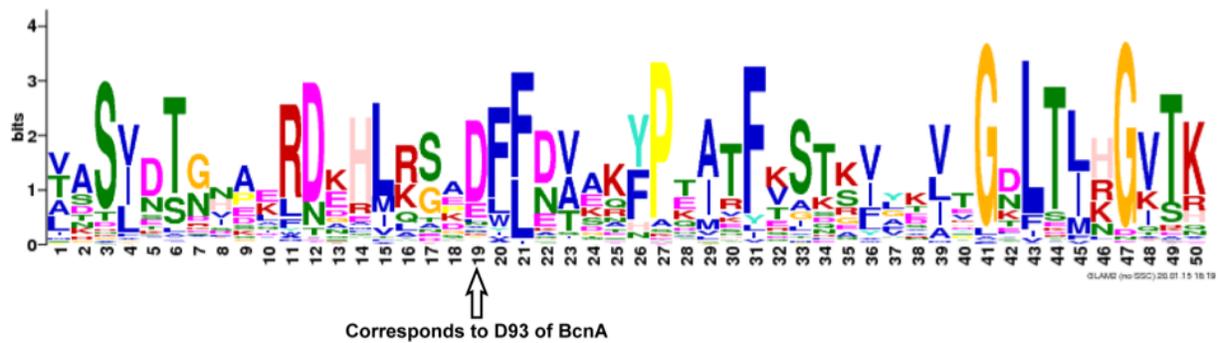
Fig. S5. Nile red binding affinity of *B. cenocepacia* BcnA site-directed mutants compared to the wild type, n=4, 2 independent experiments; the smooth lines show the non-linear least square regression analysis of the binding isotherms;

one-way ANOVA (with overall p<0.001) Dunn's Multiple Comparison Test (A-B). (C) Consensus motif of BCN family of proteins. (D) Sequence alignment and consensus motif of BCN homologues used in this study.
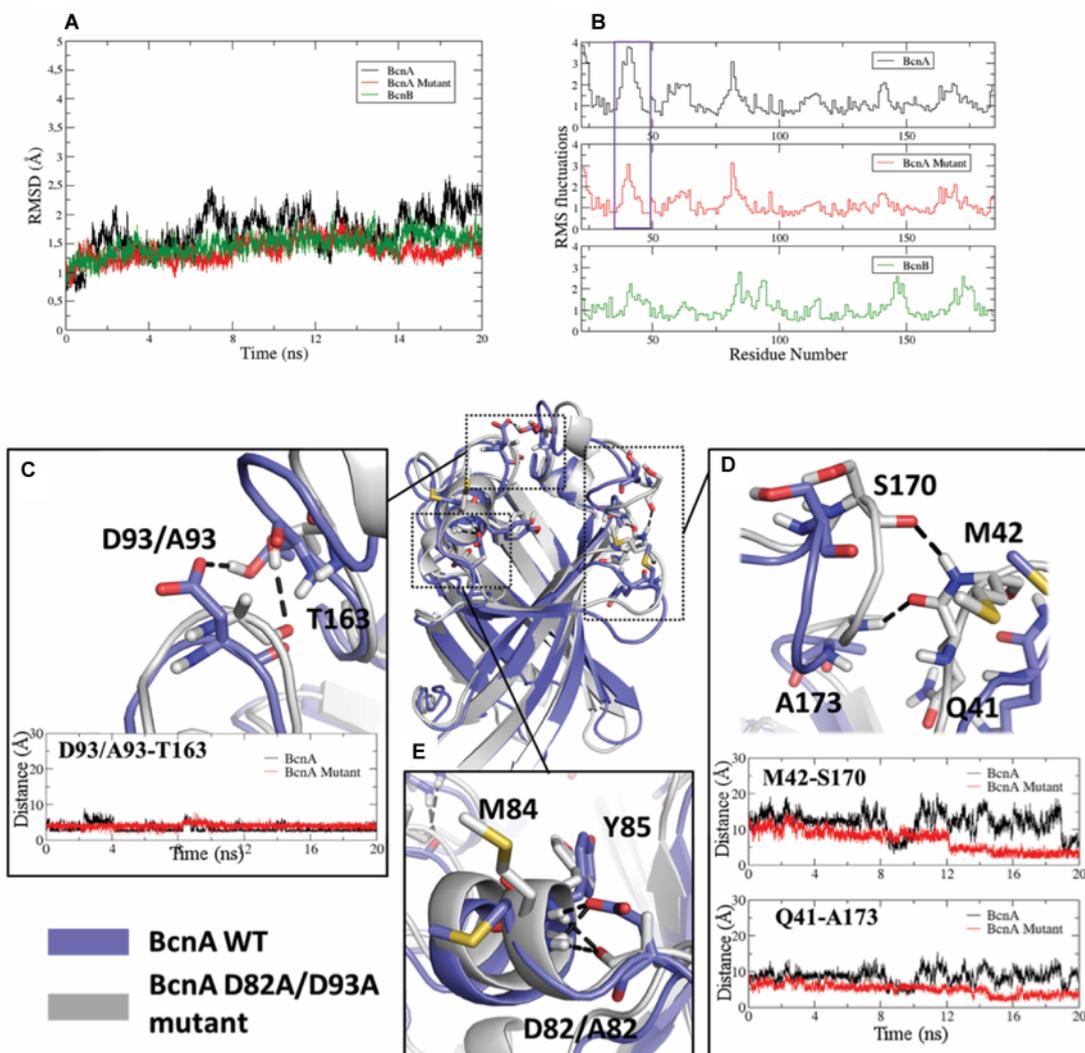
Fig. S6. (A-B) Overall MD simulation analysis of the BcnA, BcnA D82A-D93A mutant and BcnB structures. (A) Plot showing the RMSD of the position of the α-carbons of BcnA (black), BcnA D82A-D93A mutant (red) and BcnB (green) during the 20ns MD simulation. (B) Plot showing the RMS fluctuations of the position of all atoms during the 20ns MD simulation. The V38-E47 loop marked in the purple box remarks the higher flexibility observed in the case of the BcnA WT (black) compared to the BcnA D82A-D93A mutant (in red, lower fluctuations).

(C-E) Superimposition of the BcnA wild type (WT, indigo) and BcnA D82A/D93A mutant (grey) minimized average structures from the last 5ns of MD simulation. (C) Interaction between D93/A93 and T163 respectively, along with a plot representing the interaction distance between the D93 carboxylate oxygen and T163 side chain OH group in the BcnA WT, and the A93 carbonyl oxygen and T163 side chain OH group in the BcnA D82A/D93A mutant (marked with black dotted lines) along the MD simulation time. (D) Hydrogen bonds (marked with black dotted lines) between the M42 NH group and S170 CO group, and the Q41 CO group and A173 NH group (BcnA WT in indigo, and BcnA D82A/D93A mutant in grey), together with the plots of the corresponding interaction distances along the MD simulation time. (E) Detailed views of the hydrogen bonds established between the NH groups from M84 and Y85 and the D82 carboxylate oxygen in the BcnA WT, and the same NH groups from M84 and Y85 and the carbonyl oxygen from A82 in the BcnA D82A/D93A mutant (marked with black dotted lines).
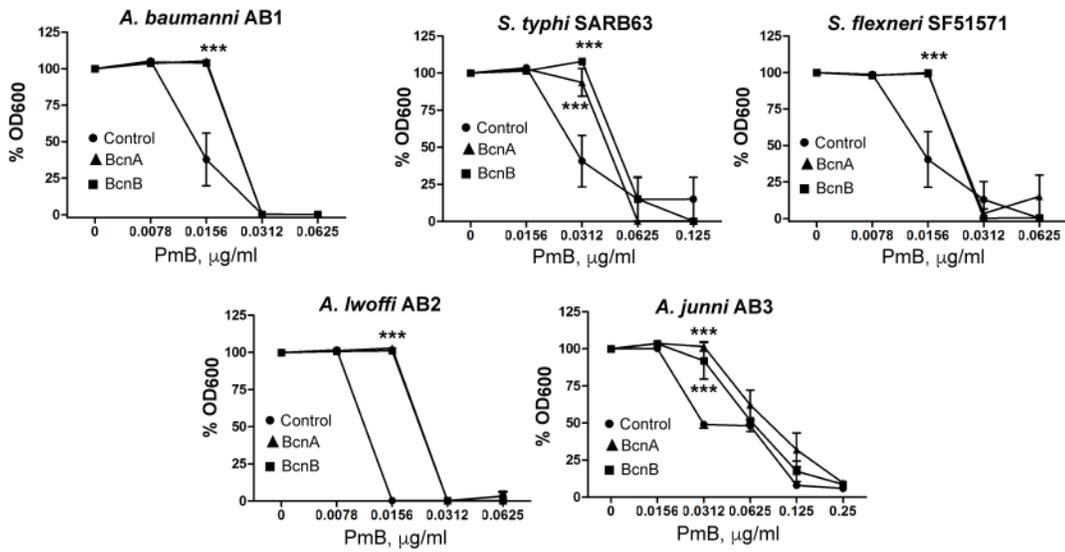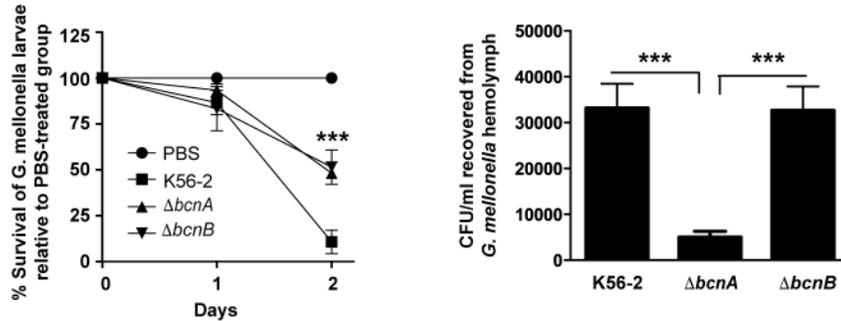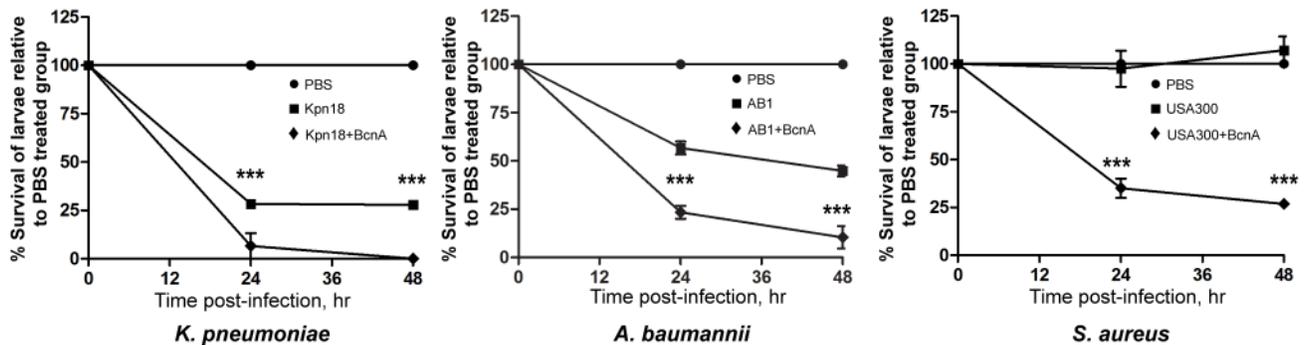
Fig. S7. Effects of *B. cenocepacia* BCNs on bacterial species *in vitro* and *in vivo*. (A) *in vitro* protection assays against PmB with 1.5 μM of BcnA or BcnB on *A. baumannii* AB1 (n=6 from 2 independent experiments), *Salmonella typhi* SARB63 (n=7 from 3 independent experiments), *Shigella flexneri* SF51571 (n=7 from 3 independent experiments), *Acinetobacter lwoffi* AB2 (n=5 from 2 independent experiments), and *Acinetobacter junii* AB3 (n=5 from 2 independent experiments). Mean ±SEM.* $p<0.05$, ** $p<0.01$ and *** $p<0.001$ determined by 2-way ANOVA (with overall $p<0.001$ for AB1, AB2, AB3 and SF51571, $p=0.001$ for SARB63) and Bonferroni post-hoc tests. (B) Survival of Δ*bcnA* and Δ*bcnB* in *Galleria mellonella* larvae over a 48-h infection. 10 larvae per group; the results are obtained from 3 independent experiments and shown as mean of % larval survival in each experiment ±SEM. *** $p<0.001$ determined by 2-way ANOVA (with overall $p<0.001$) and Bonferroni post-hoc tests. At the chosen sample size (n), the actual power of the assay to detect statistically significant effects at significance level (alpha) of 0.05, two-tailed is 90-95%. The right graph shows the bacterial recovery from hemolymph at 200 min postinfection; n=10 from 2 independent experiments shown as mean ±SEM. ***

p<0.001 from one-way ANOVA test (overall p<0.001) and Bonferroni's post-hoc test. At the chosen sample size (n), the actual power of the assay to detect statistically significant effects at significance level (alpha) of 0.05, two-tailed is >99%. * p<0.05, *** p<0.001. (C) *in vivo* protection assay using *Galleria mellonella* infections. Each larva was injected with 10 µl of suspensions of different bacteria in PBS with or without BcnA. The survival was monitored over time and compared to control group injected with sterile PBS. Each group included 10 larvae. Data are from 3 independent experiments. * p<0.05, ** p<0.01 and *** p<0.001 determined by 2-way ANOVA (with overall p<0.001) and Bonferroni post-hoc tests compared to the respective infection control.

SUPPLEMENTAL RESULTS

Antibiotic capture by bacterial lipocalins uncovers an extracellular mechanism of intrinsic antibiotic

resistance

Omar M. El-Halfawy[a,b], Javier Klett[c], Rebecca J. Ingram[d], Slade A. Loutet[e], Michael E. P. Murphy[e],

Sonsoles Martín-Santamaría[c], and Miguel A. Valvano[a,d,1]

X-ray crystallography and SEC-MALS. BCNs generally consist of an extended, eight-stranded, antiparallel β-barrel with a lipocalin fold, able to host lipophilic ligands inside a lipophilic tunnel, together with a characteristic α-helix (1). The crystal structure of BcnA was solved to 1.4 Å resolution in space group $P6_1$ (Table S1). The asymmetric unit contained two molecules of BcnA. Recombinant BcnA lacked the predicted signal peptide (first 26 amino acids) of the full-length protein. In both chains, the model contained N-terminal histidyl and methionyl residues encoded from the expression plasmid, residues D27 through T185 of BcnA; only the terminal amino acid (K186) was not modeled. There are few contacts between the two protein chains in the asymmetric unit. The PDBePISA server (2) predicted that the molecules in the asymmetric unit are monomers in solution. The oligomeric solution state of BcnA was assessed using SEC-MALS. Recombinant BcnA, after cleavage of the 6X-His tag, has a predicted molecular mass of 17.4 kDa. The measured mass of BcnA in solution was 18 kDa (Fig. S4D). Based on these results we concluded that BcnA is a monomeric protein (Fig. 4A). Within the interior cavity of BcnA, continuous density was observed with a shape similar to that observed for isoprene-based molecules identified in homologous BCN structures (PDB IDs 1Y0G, 3Q34, 2X32, 2X34, 1WUB). We have modeled this density as octaprenyl pyrophosphate (OTP) (Fig. S4A) as observed in other BCN proteins after recombinant expression in *E. coli* (3, 4). Unambiguous density for the isoprene chain of OTP is observed in the BcnA structure; however, little density is present corresponding to the pyrophosphate portion of OTP. The isoprene tail of OTP makes numerous hydrophobic contacts with amino acid side chains located inside the BcnA barrel. The binding of these lipophilic ligands to the BCN homologues is likely due to recombinant expression in *E. coli*; hence these molecules might not be predictive of the functions of these BCN homologues.

The crystal structure of BcnB was solved to 1.6 Å resolution in space group $P2_1$ (Table S1) with four molecules of BcnB in the asymmetric unit. Recombinant BcnB lacked a predicted signal peptide (first 22 amino acids) of the full-length protein. For chains B, C, and D, residues S24 to Q192 were modeled, while for chain A residues A25 to Q192 were modeled. All chains lack the C-terminal amino acid (Q193). PDBePISA predicted a stable dimer reconstructed through crystallographic symmetry that buries ~2840 Å^2 of surface area, 15% of the solvent accessible surface area of each protomer. The mass of BcnB in solution was measured to be 38.1 kDa (Fig. S4E) by SEC-MALS. Recombinant BcnB, after cleavage of the 6X-His tag, has a predicted molecular mass of 19.2 kDa, indicating that BcnB is dimeric (Fig. 4B). Again, an interior of the cavity of BcnB contains continuous density that was modeled as OTP whose isoprene tail also makes numerous hydrophobic contacts with amino acid side chains located inside the BcnB barrel (Fig. S4B). In this case, the density supports both the isoprene chain and the pyrophosphate portions of OTP.

Structural alignments by the DALI server (5) indicated that both BcnA and BcnB were most similar to *E. coli* YceI (PDB ID 1Y0G). For BcnA, 156 residues were aligned (24% sequence identity) with a root mean squared deviation (rmsd) of 1.5 Å. For BcnB, 165 residues were aligned (33% sequence identity) with a

rmsd of 1.7 Å. BcnA and BcnB also shared structural similarity (Z-score > 3.0) to eukaryotic lipocalins such as porcine odorant binding protein (PDB ID 1DZM) and plasma retinol-binding protein (PDB ID 1KT6), as well as avidin (PDB ID 2A5C). These results confirm that despite low sequence identity, BcnA and BcnB are members of the BCN family and both possess a lipocalin fold. A structural alignment of BcnA and BcnB (Fig. 4C) shows that the overall folds are very similar (148 residues aligned with an rmsd value of 1.66 Å) despite only sharing 22% sequence identity. The largest structural differences in the proteins are in two of loops at the open ends of the β-barrels. In BcnB these form a longer tunnel with amino acid residues extending past the OTP pyrophosphate (Fig. S4B), while in BcnA the OTP pyrophosphate extends past the end of the protein barrel. BcnB residues H42, W49, S84 (*via* a water-bridge), and K86 provide a hydrogen bonding network for the OTP pyrophosphate (Fig. S4C), while BcnA does not provide such a network.

Extended computational studies of BcnA, BcnB, and complexes with the studied ligands. We conducted structure-function analyses of BcnA to propose a plausible binding mode for antibiotics. The stability of the BcnA and BcnB models was tested by running 20 ns of molecular dynamics (MD) simulations with AMBER 12 (http://ambermd.org/). RMSD deviations and RMS fluctuations (Fig. S9a-b) show that the two systems reach stability along the MD simulation.

Docking of antibiotics in addition to Nile Red and α-tocopherol was performed in the BcnA X-Ray structure, and binding poses were predicted. In addition, flexibility of the protein was also taken into account by allowing some residues to be flexible during the docking calculations. Docking results are shown for BcnA in Fig. 4D-E and Fig. S4F-I. Interestingly, two distinct binding modes were predicted for BcnA: antibiotics were predicted to bind at the rim of the lipocalin pocket whereas more lipophylic molecules such as Nile Red bind deeper inside the lipophylic tunnel.

All tested antibiotics shared ionic interactions with several polar residues, mainly K40, T46, D82, Q88, Y85, D93, and E165 (Fig. 4D and Fig. S4F-I). In addition to ionic interactions, other interactions were observed between the aromatic moieties present in PmB, rifampicin, norfloxacin, and ceftazidime, and lipophylic residues such as I171, Y85, M42, M84, V89, W94 and W166. Gentamicin does not possess any aromatic moieties, matching with its weak binding to BcnA. However, the inability to bind BcnA cannot be correlated exclusively to the absence of aromatic moieties; other factors such as entropic effects from solvation and/or conformational factors may be involved.

Docking calculations of Nile Red and the fat-soluble α-tocopherol led to the prediction of the ligand deeply binding inside the lipophylic tunnel, establishing lipophylic interactions. In case of Nile Red, we predicted two alternative binding modes in BcnA, one with the diethylamino group pointing towards the entrance of the pocket, and other one with the diethylamino group pointing towards the interior of the protein. In the case of α-tocopherol, it was found to be mainly docked with the alkyl chain buried into the pocket and the cyclic head placed towards the entrance of the pocket (Fig. 4E). The OH group from the chromanol head was not found to establish any preferred polar interaction over the different predicted poses.

BcnA model and docking calculations suggested D82 and D93 as important residues for the structure and function of BcnA. We found that in the wild type BcnA, the D82 carboxylate group establishes stable hydrogen bonds with the backbone NH groups of M84 and Y85 from helix D82-A89, and D93 carboxylate group establishes a stable hydrogen bond with the side chain OH group of T163. Prompted by this finding, we modeled the BcnA D82A-D93A mutant from the crystallographic structure, and submitted to MD simulations. The stability of the BcnA D82A-D93A double mutant model was tested by running 20 ns of MD simulation with AMBER 12. RMSD deviations and RMS fluctuations (Fig. S6A-B) show that the system reached stability along the MD simulation. We could only observe a slight lower fluctuation in the case of BcnA D82A-D93A mutant: a 3Å of RMS fluctuation is reached in the region of the V38-E47 loop in the case of the D82A-D93A mutant while, in the case of the WT BcnA, a 4 Å of RMS fluctuation is

observed for the same loop, in accordance with the B-factors extracted from the crystallography structures. By contrast, in the case of the MD simulation of the BcnB, the equivalent F38-R50 loop showed a 2Å of RMS fluctuation. To study the reason that causes this lower fluctuation in the BcnA mutant model, we inspected the MD simulations in detail. A higher mobility of the atoms belonging to the V38-E47 loop was found in the case of the wild type, as deduced from the RMS fluctuations of each residue (Fig. S6A-B). We observed that, in the case of the BcnA D82A-D93A mutant, the V38-E47 loop created new interactions between the backbone of M42 and S170, and Q41 and A173, maybe thus explaining this lower fluctuation.

In the D82A-D93A mutant, the absent carboxylate group of D82 is replaced by the backbone CO group from A82, allowing the hydrogen bonding to NH groups from M84 and Y85. This new situation might affect the overall BcnA structure and, very likely, the binding of antibiotics since D82 has been identified by our docking calculations as an important residue for antibiotic anchorage. Furthermore, in the case of D93 mutation, the absence of the hydrogen bond between the carboxylate group and the T163 side chain is not replaced by other equivalent interaction, allowing the approach of T163-I171 loop towards the V38-E47 loop, leading to two novel hydrogen bonds: one between the M42 NH group and the S170 CO group, and another one between the Q41 CO group and the A173 NH group (Fig. S6C-E). Together, this relatively different arrangement for the BcnA D82A-D93A mutant led to a reduced flexibility of the V38-E47 loop along the MD simulation time (Fig. S6C-E). The higher mobility of this V38-E47 loop could resemble the required movement of a similar loop in other member of the lipocalin family (the lipocalin type prostaglandin D synthase), where the conformational change of the Y107-S114 loop allows the change between the open/closed conformers (6). Nevertheless, the higher flexibility observed for the wild type structure could account for a better ability to bind ligands. Taking always into account the limitation of working with computational simulations, these observed changes might point out to a structural role for D82 and D93, in addition to their putative functional role in the binding of the antibiotics. To further investigate the role of these two residues, site-directed mutants with alanine replacements of these residues were prepared. D82 and to a less extent D93 were demonstrated to be important for the binding interaction of ligands (exemplified by Nile Red) to BcnA (Fig. S5A-B). Docking of Nile Red and α-tocopherol into the BcnA D82A-D93A mutant (by following the general docking protocol with the minimized average structure from the ns 2.5 to the ns 5 of the MD simulation) did not lead to any binding pose inside the lipophilic tunnel. This agrees with the experimental results obtained with the BcnA D82A-D93A mutant protein.

Since BCNs are highly conserved among bacteria sharing the characteristic lipocalin tertiary structure, we determined the consensus motif of this protein family (Fig. S5C) (7).The D93 residue of BcnA of *B. cenocepacia* was highly conserved. Aspartate residues at distance from D93 comparable to the distance to D82 in *B. cenocepacia* BcnA were found in the motif (7). Together, this suggests that D82 and D93 may have a structural role to maintain the 3D structure and the opening of the lipophilic tunnel of BcnA, and also they may be proposed as key residues to interact with antibiotics, thus playing an essential role in the resistance mechanism mediated through BCNs. This mode of interaction between BCN and antibiotics could be common among this large family of conserved bacterial proteins.

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Table S1. X-ray crystallography data collection and refinement statistics

Parameter	BcnA	BcnB
Data Collection*		
Resolution Range (Å)	35.9 - 1.4 (1.43 – 1.4)	37.3 - 1.6 (1.63 – 1.6)
Space Group	$P6_1$	$P2_1$
Unit cell dimension (Å)	$a = 93.97$, $b = 93.97$, $c = 76.66$, $\gamma = 120^o$	$a = 51.2$, $b = 101.2$, $c = 63.5$, $\beta = 97.2^o$
Unique Reflections	75,559 (4,004)	82,019 (3,495)
Completeness (%)	99.9 (99.4)	97.3 (78.7)
CC1/2	0.999 (0.829)	0.997 (0.892)
Average $I/\sigma I$	17.1 (3.0)	11.3 (3.3)
Redundancy	10.5 (5.7)	3.8 (3.1)
R_{merge}	0.061 (0.492)	0.056 (0.265)
Refinement		
R_{work} (R_{free})	0.156 (0.183)	0.158 (0.192)
Number of water molecules	274	482
Overall B-factor (Å^2)	28.4	17.1
r.m.s.d.		
Bond lengths (Å)	0.01	0.025
Bond angles (°)	1.46	2.44
Ramachandran Plot (%)		
In most favorable	96.0	96.9
In disallowed	0.3	0.8

*Data collection statistics in parentheses represent the highest resolution shells. Coordinates for the X-ray crystal structures of BcnA and BcnB have been deposited to the Protein Data Bank with Accession Codes 5IXH and 5IXG, respectively.

Table S2. Strains and Plasmids

Strain or plasmid	Relevant characteristics[a]	Source and/or reference
Strains		
Burkholderia cenocepacia		
K56-2	ET12 clone related to J2315, CF clinical Isolate	[b]BCRRC, (1)
OME19	K56-2 pSCrhaB2; Tp^R	(2)
OME37	K56-2 pOE12; BCAL3310 with C-terminus FLAG tag; Tet^R	This study
OME40	K56-2 pOE13; BCAL3311 with C-terminus FLAG tag; Tet^R	This study
OME60	K56-2, $P_{BCAL3310}$::pGSVTp-*luxCDABE*; Tp^R	This study
OME61	K56-2, $P_{BCAL3312-3311}$::pGSVTp-*luxCDABE*; Tp^R	This study
OME62	K56-2, ΔBCAL3311	This study
OME65	K56-2, ΔBCAL3310	This study
OME66	K56-2 pDA17; Tet^R	This study
OME71	OME62 pSCrhaB2; Tp^R	This study
OME72	OME62 pOE33 (BCAL3311); Tp^R	This study
OME73	OME62 pOE34 (PA0423); Tp^R	This study
OME74	OME62 pOE35 (PA4340); Tp^R	This study
OME75	OME62 pOE36 (PA4345); Tp^R	This study
OME76	OME62 pOE37 (Rv1890c); Tp^R	This study
OME77	OME62 pOE38 (SAUSA300_2620); Tp^R	This study
Escherichia coli		
DH5α	F$^-$ φ80*lacZ* M15 *endA1 recA1 supE44 hsdR*17(r_K^- m_K^+)*deoR thi-1 nupG supE44 gyrA96relA1* Δ(*lacZYA-argF*)*U169*, λ–	Laboratory stock
GT115	F$^-$ *mcrA*Δ(*mrr-hsdRMS-mcrBC*) φ80Δ*lacZ*ΔM15 Δ*lacX74 recA1 rpsL* (StrA) *endA1*Δ*dcm uidA*(ΔMluI)::*pir-116* Δ*sbcC-sbcD*	Invivogen, San Diego, CA
BL21	F$^-$ *dcm ompT hsdS*(r_B^- m_B^-) *gal*	Novagen
Pseudomonas aeruginosa		
PAO1	Non‑CF clinical isolate	(3)
Q502	CF clinical isolate	(4)
Salmonella typhi		
SARB63		(5)
Shigella flexneri		
SF51571	Serotype 1a, antigenic formula 1:4	Laboratory stock
***Acinetobacter* species**		
A. baumannii (AB1)	Clinical isolate	LHSC^c
A. lwoffi (AB2)	Clinical isolate	LHSC^c
A. junii (AB3)	Clinical isolate	LHSC^c

Klebsiella pneumoniae

| Kpn18 | Clinical isolate | Laboratory stock |

Staphylococcus aureus

| USA300 | Community acquired MRSA | Martin McGavin |

Plasmids

pRK2013	$ori_{colE1,}$ RK2 derivative, Kan^R, mob^+, tra^+	(6)
pGSVTp-*lux*	Mobilizable suicide vector containing lux operon, derivative from pGSV3-*lux*(7); OriT; Tp^R	(8)
pSCrhaB2	$ori_{pBBR1}rhaR$, *rhaS*, P_{rhaB}Tp$^R mob^+$	(9)
pDAI-SceI-SacB	ori_{pBBR1}, Tet^R, P_{dhfr}, mob^+, expressing I-SceI, SacB	(10)
pGPI-SceI	$ori_{R6K,}$ ΩTp^R, mob^+, including an I-SceI restriction site	(11)
pDA17	ori_{pBBR1}, Tet^R, mob$^+$, P$_{dhfr}$, FLAG epitope	D. Aubert, unpublished
pOE12	pDA17, BCAL3310, C-terminus FLAG, Tet^R	This study
pOE13	pDA17, BCAL3311, C-terminus FLAG, Tet^R	This study
pOE15	BCAL3310 without signal peptide encoding sequence cloned in pET28a(+)	(2)
pOE16	BCAL3311 without signal peptide encoding sequence cloned in pET28a(+)	(2)
pOE22	$P_{BCAL3310}$::*luxCDABE* transcriptional fusion in pGSVTp-*lux*, Tp^R	This study
pOE23	$P_{BCAL3312-3311}$::*luxCDABE* transcriptional fusion in pGSVTp-*lux*, Tp^R	This study
pOE25	pGPI-SceI with fragments flanking BCAL3310, Tp^R	This study
pOE26	pGPI-SceI with fragments flanking BCAL3311, Tp^R	This study
pOE33	pSCrhaB2, BCAL3311, Tp^R	This study
pOE34	pSCrhaB2, PA0423, Tp^R	This study
pOE35	pSCrhaB2, PA4340, Tp^R	This study
pOE36	pSCrhaB2, PA4345, Tp^R	This study
pOE37	pSCrhaB2, Rv1890c, Tp^R	This study
pOE38	pSCrhaB2, SAUSA300_2620, Tp^R	This study
pOE39	pOE16, D82A	This study
pOE41	pOE16, D93A	This study
pOE46	pOE39, D93A	This study

[a]Tp^R, trimethoprim resistance, Kan^R, kanamycin resistance, Tet^R, tetracycline resistance.
[b]BCRRC, *B. cepacia* Research and Referral Repository for Canadian CF Clinics.
[c]LHSC, London Health Science Centre, London, Ontario, Canada.

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