

1 BIOLOGICAL SCIENCES: Biochemistry

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3 **Actin activates *Pseudomonas aeruginosa* ExoY nucleotidyl cyclase toxin**
4 **and ExoY-like effector domains from MARTX toxins**

5

6 **short title:** actin activate bacterial ExoY-like toxins

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24 bacterial virulence factors, nucleotidyl cyclase toxins, cGMP, cAMP, actin cytoskeleton.

25

26 **ABSTRACT**

27 *Pseudomonas aeruginosa* is a major cause of chronic infections in cystic fibrosis patients.
28 The nucleotidyl cyclase toxin ExoY is a virulence factors injected by the pathogen and
29 associated with severe damage to lung tissue. ExoY-like cyclases are also found in other
30 Gram-negative pathogens and shown to contribute to virulence, although they remained
31 poorly characterized. Here we demonstrate that filamentous actin (F-actin) is the hitherto
32 unknown co-factor that activates *P. aeruginosa* ExoY within host target cells. Highly
33 purified actin, when polymerized into filaments, potently stimulates (>10,000 fold) ExoY
34 activity. ExoY co-localizes *in vivo* with actin filaments in transfected cells and, *in vitro*, it
35 interferes with the regulation of actin assembly/disassembly-dynamics mediated by important
36 F-actin-binding proteins. We further show that actin also activates an ExoY-like adenylyate
37 cyclase from a *Vibrio* species. Our results thus highlight a new sub-class within the class II
38 adenylyl cyclase family, defined as actin-activated nucleotidyl cyclase (AA-NC) toxins.

39

40 INTRODUCTION

41 *Pseudomonas aeruginosa* is an opportunistic human pathogen that causes severe infections in
42 immune-compromised individuals and is a major cause of chronic infections in cystic fibrosis
43 patients. Equipped with a type III secretion system (T3SS), *P. aeruginosa* can inject effector
44 proteins directly into the host cells where they contribute to virulence of the pathogen [for
45 review see ^{1, 2}]. Four different T3SS delivered effectors have been characterized (exoenzyme
46 T, Y, U and S) , but new effectors were recently identified³. Exoenzyme Y (ExoY) is present
47 in 89% of clinical isolates⁴, and was originally identified as an adenylate cyclase in 1998 (ref.
48 ⁵) due to amino acid sequence homology with two well characterized class II adenylate
49 cyclase toxins, CyaA from *Bordetella pertussis* and edema factor (EF) from *Bacillus*
50 *anthracis*. Recent results revealed that substrate specificity of these enzymes expressed in
51 cell cultures is not restricted to ATP: EF and CyaA were shown to use UTP and CTP as
52 substrate⁶ while ExoY was shown to promote the intracellular accumulation of several cyclic
53 nucleotides^{7, 8} with a preference for cGMP and cUMP over cAMP and cCMP formation⁷.
54 The physiological effects of ExoY resulting from accumulation of these cyclic nucleotides
55 include the hyperphosphorylation of tau and the disruption of microtubules causing the
56 formation of gaps between endothelial cells and increased permeability of the endothelial
57 barrier^{8, 9, 10, 11}. Most recent results showed that ExoY presence correlates with long term
58 effects on recovery after lung injury from pneumonia¹².

59 Recent whole genome sequencing projects have identified ExoY nucleotidyl cyclase
60 modules among a variety of toxic Multifunctional Autoprocessing repeats-in-toxins
61 (MARTX) effector domains in multiple bacterial species of the *Vibrio genus*¹³ that represent
62 emerging human or animal pathogens. These ExoY-like domains can be essential for
63 virulence¹³. Elucidating the cellular activities and specificities of ExoY and ExoY-like toxins

64 may therefore help to develop new therapeutic strategies against the toxicity and virulence of
65 several pathogens.

66 Despite the progress in understanding downstream effects of ExoY activity,
67 fundamental information on ExoY is lacking: as other bacterial soluble related cyclases such
68 as CyaA and EF, ExoY is inactive in bacteria and is activated by an eukaryotic cofactor after
69 its delivery to the target cells⁵. While the other class II adenylate cyclase toxins like CyaA
70 and EF are strongly activated upon interaction with calmodulin^{14, 15}, calmodulin is unable to
71 stimulate ExoY enzymatic activity and the precise nature of the eukaryotic activator has
72 remained elusive up to now. Here, we describe the identification of actin as the cofactor that
73 activates *P. aeruginosa* ExoY and the ExoY-like module present in MARTX toxin of *V.*
74 *nigripulchritudo* in host cells. Our findings suggest that, within the class II adenylate cyclase
75 family¹⁶, a new class of nucleotidyl cyclase toxins share actin as a common host activator.

76

77 **RESULTS**

78 **An activator of ExoY is present in *Saccharomyces cerevisiae***

79 Arnoldo et al. have reported that overexpression of ExoY impairs yeast growth¹⁷, suggesting
80 that ExoY should be catalytically active in this organism and therefore that a cofactor
81 required for ExoY catalytic activity should be present in yeast. To test this hypothesis, we
82 prepared extracts from *Saccharomyces cerevisiae* BY4741 cells and measured adenylate
83 cyclase activity of recombinant ExoY carrying an N-terminal His-Flag tag (HF-ExoY) in the
84 presence of increasing amounts of yeast cell extract *in vitro*. Extract from HeLa cells were
85 used as control. We observed a dose dependent stimulation of ExoY activity by yeast cell
86 extract, to levels that were similar to those measured when using the extract from HeLa cells
87 (Fig. 1a). Thus, we decided to use *S. cerevisiae* as a convenient experimental system to

88 identify the putative yeast activator that was likely to be evolutionarily conserved in human
89 cells.

90

91 **Identification of actin as the main protein interacting with ExoY-TAP in *S. cerevisiae***

92 To identify putative ExoY-binding proteins in yeast, we expressed ExoY containing a C-
93 terminal epitope-tag (ExoY-TAP or ExoY-HA) in *S. cerevisiae* to isolate proteins co-
94 purifying with the affinity purified bait protein. To avoid toxic effects due to cyclic
95 nucleotide accumulation, we expressed a catalytically inactive variant of ExoY, ExoY^{K81M}
96 (in which the Lys81 was changed to Met⁵). Material obtained in a one-step purification using
97 IgGs covalently bound to magnetic beads was directly processed by tryptic digestion for
98 protein identification through LC-MS/MS. The raw data were then analyzed by MaxQuant
99 for protein identification and quantitative estimation of the specific enrichment of proteins in
100 the experimental sample (ExoY^{K81M}-TAP) as compared to the control (ExoY^{K81M}-HA).
101 While many abundant proteins were present in both samples to a comparable degree, as
102 estimated from the label free quantitation score (LFQ,¹⁸), ExoY was identified only in the
103 purification done with ExoY^{K81M}-TAP extract, as expected. A second protein that was about
104 1000 times more abundant in the ExoY^{K81M}-TAP purification than in the control was yeast
105 actin (Uniprot P60010, YFL039C, Act1), which showed an LFQ score close to the score of
106 the tagged ExoY (Fig. 1b). Other factors were identified specifically in the ExoY^{K81M}-TAP
107 purification, but with much lower LFQ scores (see Supplementary table 2). These results
108 suggest a specific interaction of ExoY^{K81M} with yeast Act1. Since actin is one of the most
109 highly conserved and abundant proteins in eukaryotic cells, it appeared to be a potential
110 candidate for activating ExoY ubiquitously in eukaryotic cells.

111

112

113 **ExoY interacts with mammalian actin *in vitro***

114 To verify the interaction between ExoY and mammalian actin *in vitro*, we performed Ni-
115 NTA agarose pulldowns. ExoY with a C-terminal Flag-His tag (ExoY-FH) and α -actin from
116 rabbit skeletal muscle (Cytoskeleton, Inc., designated here MA-99) were added at equimolar
117 concentrations to Ni-NTA agarose beads in batch. After 1 hr incubation the beads were
118 washed in Durapore columns and the retained proteins were eluted with imidazole. While
119 very little actin was bound unspecifically to the beads in the absence of ExoY, considerably
120 more actin was present in the eluate from the sample containing ExoY-FH, suggesting
121 specific binding between the two pure proteins (Fig. 2a).

122 Under our experimental conditions, which promote actin spontaneous polymerization
123 (300 mM NaCl, 2.5 μ M ATP, 660 nM actin), actin should exist in solution both in its
124 monomeric (G-actin) and filamentous (F-actin) states. To investigate more specifically
125 whether ExoY could bind to F-actin, we performed high-speed co-sedimentation assays: we
126 polymerized G-actin-ATP and incubated F-actin at steady state subsequently with ExoY.
127 Samples were then centrifuged at high speed to separate F-actin present in the pellet from G-
128 actin present in the supernatant. Fig. 2b shows that ExoY, which alone partitioned in the
129 supernatant fraction, was mostly found in the pellet fraction in the presence of F-actin,
130 indicating ExoY capacity to interact with F-actin.

131

132 **Actin stimulates ExoY nucleotidyl cyclase activity *in vitro***

133 We next tested whether purified actin could activate ExoY *in vitro*. Highly pure non-muscle
134 (cytoplasmic) actin isolated from human platelets (Cytoskeleton, Inc., designated here A-99)
135 strongly stimulated the adenylate cyclase activity of ExoY (HF-ExoY, having 6xHis and Flag
136 tags at the N-terminus), with a maximal activity reaching 120 μ mol of cAMP.min⁻¹.mg⁻¹.

137 Since in mammalian cells the transfection with a vector expressing ExoY led to accumulation
138 of cGMP to levels exceeding that of cAMP^{7, 8}, we also tested GTP as substrate and found
139 that, in agreement with the preferential accumulation of cGMP over cAMP observed *in vivo*,
140 the guanylate cyclase activity of HF-ExoY was approximately 8 times higher than the
141 adenylate cyclase activity in the presence of actin *in vitro* (Fig. 3a). The background activity
142 without actin was estimated to be about 1 nmol and 10 nmol.min⁻¹.mg⁻¹ for cGMP and
143 cAMP, respectively. Thus, the ExoY nucleotidyl cyclase activity was stimulated more than
144 10,000 fold by submicromolar concentrations of F-actin. Different mammalian actin isoforms
145 (A-99, a mixture of 85% β- and 15% γ-actin, or α-actin from rabbit skeletal muscle) led to a
146 similar activity for cGMP synthesis (Supplementary Fig. 2). All together these data indicate
147 that actin is a specific activator of ExoY in eukaryotic cells. Subsequent experiments were
148 performed using highly pure skeletal muscle α-actin purified in one of our laboratory
149 (designated MA-L).

150 To examine a possible dependence of ExoY activation on the different states of actin
151 (ATP- versus ADP-bound, monomeric versus polymeric forms), we measured ExoY cGMP
152 synthesis activity at different actin concentrations below or above the critical polymerizing
153 concentrations in different actin nucleotide states. Measurements were performed at various
154 concentrations of actin that was initially loaded with either Mg-ATP or Mg-ADP. A similar
155 maximal activity of 1000 - 1200 μmol of cGMP.min⁻¹.mg⁻¹ was obtained with both ATP-
156 bound and ADP-bound actin (Fig. 3b). In contrast, the actin concentrations required for half
157 maximal activation of ExoY (K_{1/2}) were dependent upon the bound nucleotides. The actin
158 concentration for half maximal ExoY activation with ATP-loaded actin was about 0.2 μM
159 (Fig. 3b). This is just above the critical concentration of 0.1 μM above which Mg-ATP-actin
160 spontaneously polymerizes in solution with salt¹⁹. Conversely, the actin concentrations
161 required for half-maximal ExoY activation with ADP-loaded actin was about 2.4 μM (Fig.

162 3b), a shifted value that correlates with the alternative critical concentration of 1.7 μM
163 obtained with Mg-ADP-actin. Altogether, these results suggest that the maximal activation of
164 ExoY by actin was correlated with F-actin formation.

165

166 **Activation of ExoY by actin is antagonized by latrunculin A or G-actin binding proteins**

167 We then examined whether proteins or molecules that are known to bind to G-actin and to
168 inhibit its polymerization or spontaneous nucleation, could affect the activation of ExoY by
169 actin. We examined the activation of ExoY by G-actin in the presence of the drug latrunculin
170 A or G-actin binding proteins such as profilin or thymosin- β 4 (T β 4), which are among the
171 main monomeric actin-binding proteins in vertebrate cells¹⁹. These three molecules are
172 known to inhibit actin polymerization or spontaneous nucleation by binding to distinct G-
173 actin interfaces. The small macrolide Latrunculin A from the Red Sea sponge *Negombata*
174 *magnifica*^{20, 21} inhibits actin self-assembly by binding ($K_D \approx 0.2 \mu\text{M}$) to a cleft located on the
175 pointed face of G-actin. The protein profilin binds ($K_D \approx 0.1 \mu\text{M}$) in contrast to the opposite
176 face of monomers, called barbed face, and favors *in vivo* the unidirectional elongation of the
177 most-dynamic barbed ends of filaments. *In vitro*, G-actin:profilin complexes inhibit actin
178 spontaneous nucleation and thus polymerization in absence of actin nuclei or filament seeds.
179 T β 4 is a small intrinsically disordered β -thymosin domain of 4 kDa that acts as a major G-
180 actin-sequestering polypeptide in cells^{22, 23}. Here, we used a chimeric β -thymosin domain
181 between T β 4 and ciboulot from *Drosophila* called Chimera 2 (CH2), as it exhibits a higher
182 affinity for G-actin than T β 4 ($K_D \sim 0.5 \mu\text{M}$ versus $2 \mu\text{M}$) while retaining its sequestering
183 activity^{22, 23}. Like T β 4, CH2 displays an extended binding interface on actin monomers by
184 interacting with both their barbed and pointed faces^{22, 23, 24}.

185 In ExoY activity measurements, actin monomers were saturated by the above
186 molecules to inhibit or significantly slow down the spontaneous nucleation or polymerization

187 of actin in solution. The inhibitory effect on actin assembly was verified in cosedimentation
188 assays (Supplementary Fig. 3). In these conditions, all molecules tested inhibited ExoY
189 activation by micromolar actin concentrations (Fig. 4), which normally induce maximal
190 activation of the toxin. CH2 reduced at least 9 fold (up to 15) the ExoY activity measured in
191 the presence of 1-3 μM of actin (Fig. 4). Latrunculin A reduced at least 7 fold (up to 13) and
192 profilin decreased at least 4 fold (up to 7) the ExoY activity at similar ranges of actin
193 concentrations (Fig. 4). Latrunculin A, profilin, and CH2 did not affect the low background
194 activity of ExoY in the absence of actin. These data thus indicated that filamentous-actin is
195 the preferred activator of ExoY.

196

197 **ExoY is an F-actin binding protein that can modify the intrinsic or regulated dynamics**
198 **of filaments by binding along filament sides**

199 We next examined whether ExoY affects the intrinsic or regulated dynamics of actin self-
200 assembly *in vitro* in assembly/disassembly assays with ExoY /ExoY^{K81M}. The kinetics of
201 polymerization or depolymerization were monitored by following the increase or decrease,
202 respectively, in pyrene-actin fluorescence intensity (pyrenyl-labeled actin subunits exhibit
203 higher fluorescence when incorporated in filaments than free in solution). In polymerization
204 kinetics, ExoY slightly accelerated the rate of G-actin-ADP-Mg (Fig. 5a) and G-actin-ATP-
205 Mg (Fig. 5b) self-assembly, confirming that ExoY can interact with actin without preventing
206 its self-assembly. Yet, this stimulation of G-actin-ATP/ADP polymerization by ExoY was
207 detectable only at high ExoY concentrations (in μM range). The dose-dependent acceleration
208 was independent of the ExoY adenylate cyclase activity since it was observed with the
209 inactive ExoY^{K81M} variant and also with the wild-type ExoY when only ADP was present.
210 We further examined whether the ExoY-stimulation of actin polymerization was achieved by
211 increasing elongation rates on barbed- or pointed-end, or by severing filaments, but found no

212 effects of the toxin on these processes (Supplementary Fig. 4). We were unable to isolate
213 stable G-actin/ExoY complexes in solution even at micromolar concentrations of both
214 proteins (and in presence of latrunculin A to prevent actin polymerization). Besides, the
215 ExoY-induced stimulation of actin polymerization was fully inhibited when actin was
216 saturated by profilin (Fig. 5b). These results confirm that ExoY is unlikely to stimulate actin
217 polymerization in host cells. Indeed, profilin-actin complexes form the major part of the
218 polymerization competent G-actin pool within eukaryotic cells^{19,24}.

219 To delineate the interaction of ExoY with F-actin, we performed dilution-induced
220 depolymerization assays monitoring filament disassembly from free barbed- and pointed
221 ends. As shown in Fig.5c, ExoY^{K81M} inhibited the spontaneous disassembly of F-actin
222 induced by dilution. This indicates that ExoY directly binds to filaments and thus stabilizes
223 actin inter-subunit contacts. The inhibition of filament disassembly by ExoY^{K81M} was also
224 observed when barbed ends were capped by gelsolin (Fig. 5c), thus excluding the possibility
225 that ExoY inhibited disassembly by binding to barbed ends. These results and the absence of
226 ExoY effects on the pointed-end elongation rate (Supplementary Fig. 4) indicate that ExoY
227 binds along the sides of filaments where it likely interacts with several adjacent actin
228 subunits thus stabilizing actin inter-subunit contacts and preventing spontaneous disassembly
229 of filaments.

230 The binding of ExoY to filamentous actin was then quantified by co-sedimentation
231 assays using F-actin steadily polymerized in the presence of ADP-BeF₃⁻ (that mimics the
232 filament transition state F-actin-ADP-Pi) in order to keep most actin firmly polymerized
233 despite the efficient ATP-hydrolyzing activity of ExoY. To quantify more rigorously the
234 ExoY bound to F-actin on SDS-PAGE gels in presence of high concentrations of F-actin (as
235 ExoY and actin have close molecular weights), we used an ExoY protein fused to the
236 Maltose-Binding-Protein (MBP), MBP-ExoY (note that MBP-ExoY has also a C-terminal

237 Strep-Tag). As shown in Fig. S5, MBP-ExoY or MBP-ExoY^{K81M}, which alone partitioned in
238 the supernatant fraction, both pelleted with F-actin in a dose-dependent manner, and were
239 almost completely found in the pellet fraction at concentrations above 25 μ M F-actin-ADP-
240 BeF₃⁻, i.e. much below the F-actin concentrations in cells²⁵. The estimated dissociation
241 constants (Kd) of MBP-ExoY or MBP-ExoY^{K81M} for F-actin-ADP-BeF₃⁻ ($0.8\pm 0.3\mu$ M and
242 $2.5\pm 0.4\mu$ M, respectively) (Supplementary Fig. 5), were in the same range as that of a number
243 of eukaryotic cytoskeletal proteins that bind along filaments^{26, 27, 28}.

244 Finally, we analyzed whether ExoY binding to F-actin could interfere with the
245 regulation of filament dynamics by eukaryotic cytoskeletal proteins that are known to bind
246 along the sides of filaments. We considered two key regulatory proteins that are ubiquitous
247 among eukaryotic cells: Arp2/3 complex and Actin-Depolymerizing Factor (ADF). The
248 Arp2/3 complex, upon its activation by VCA domains of the WASP family proteins, can
249 attach to the side of a pre-existing filament and catalyzes actin filament branching^{26, 29}.
250 ADF/cofilin proteins, present at micromolar concentrations in eukaryotic cells, bind
251 cooperatively and preferentially to F-actin-ADP subunits along filaments (Kd $\sim 0.1\mu$ M),
252 stimulating their disassembly and thus the turnover of actin filaments in cells^{19, 26}.

253 We performed actin polymerization assays with G-actin saturated by profilin to
254 simulate a more physiological context^{19, 24, 30}. As shown in Fig. 5d, the acceleration of actin
255 polymerization by Arp2/3 (25-35 nM), activated by N-WASP VCA domain, was
256 significantly inhibited by high concentrations (≥ 100 nM) of ExoY^{K81M}. This inhibition
257 demonstrates that ExoY could antagonize the binding of the activated Arp2/3 complex along
258 filaments and hence VCA-Arp2/3 regulation. In dilution-induced F-actin-ADP disassembly
259 assays, ExoY^{K81M} (100 nM) were able to completely inhibit the acceleration of filament
260 disassembly promoted by ADF (4 μ M) (Fig. 5e). This inhibition suggests that ExoY (at

261 submolar ratio with respect to ADF) could prevent the cooperative binding of ADF along F-
262 actin-ADP and its disassembling activity.

263

264 **ExoY co-localizes with actin fibers in mouse NIH3T3 cells**

265 To our knowledge, the localization of ExoY in eukaryotic cells was not previously reported
266 and is of particular interest in light of its direct interaction and good affinity with naked F-
267 actin *in vitro*. To avoid potential toxic effects of ExoY when expressed in eukaryotic cells,
268 we examined the localization of the catalytically inactive variant ExoY^{K81M} fused to AcGFP,
269 a monomeric green fluorescent protein. The ExoY^{K81M}-AcGFP fusion protein was
270 constitutively expressed from pUM518 under the control of the P_{CMV} IE promoter in
271 transiently transfected NIH3T3 cells. Acti-stainTM 555 fluorescent phalloidin (Cytoskeleton,
272 Inc.) was used to visualize F-actin in cells. Unlike GFP alone, which showed a homogeneous
273 cytoplasmic and partially nuclear localization, the signal for ExoY^{K81M}-AcGFP partially co-
274 localized *in vivo* with F-actin filaments (Fig. 6).

275

276 **The ExoY-like virulence factor present in the *Vibrio nigripulchritudo* MARTX toxin is** 277 **also activated by actin**

278 Finally we examined whether actin could also activate other putative ExoY-like cyclases
279 that can be found in various MARTX toxins that are produced by several Gram-negative
280 pathogens including *Burkholderia* or several *Vibrio*¹³, *Providencia*, or *Proteus* species
281 (Fig. 7a). For this, we selected the ExoY-like module from the MARTX toxin encoded by
282 the virulence-associated plasmid pA_{SFnI} from *Vibrio nigripulchritudo*^{13, 31}, an emerging
283 marine pathogen infecting farmed shrimps. The multidomain MARTX toxin is processed
284 inside host cells by an inbuilt cysteine protein domain (CPD), into individual effector
285 domains³². The N- and C-terminus of the *V. nigripulchritudo* ExoY-like effector domain

286 were chosen based on sequence alignments of *P. aeruginosa* ExoY and ExoY-like
287 containing sequences from several *Vibrio* MARTX toxins (Supplementary Fig. 6), the
288 signature of CPD cleavage sites present in some *Vibrio* ExoY-like modules and HCA
289 secondary prediction analysis. The MARTX-ExoY protein corresponding to residues
290 Y3412 to L3872 of Uniprot reference F0V1C5 was termed here VnExoY-L. The protein
291 carrying a C-terminal Flag-His tag (VnExoY-L-FH) was purified and tested for its
292 adenylate cyclase activity in the presence and absence of actin. Fig. 7b shows that
293 VnExoY-L displayed a potent adenylate cyclase activity in the presence of actin, which
294 stimulates the enzymatic activity more than 10,000 fold. In contrast to *P. aeruginosa*
295 ExoY, VnExoY-L did not exhibit any cGMP synthesizing activity. We conclude that actin
296 may be a common activator of the various ExoY-like cyclase modules, even though these
297 differ in their substrate selectivity.

298

299 **DISCUSSION**

300 Actin is the target of a variety of bacterial toxins. These toxins can affect the polymerization
301 state of actin in different ways by introducing modifications, namely ADP-ribosylation at
302 different position or crosslinking (for reviews see ^{33, 34}). The provoked rearrangements have a
303 profound effect on the cytoskeleton of the host cells and affect their response to bacterial
304 invasion.

305 Here, we show that actin is a potent activator of a group of bacterial toxins that are
306 homologous to the *P. aeruginosa* ExoY effector and that display nucleotidyl cyclase
307 activities with different substrate selectivity.

308 We identified actin as a potential candidate for *P. aeruginosa* ExoY activation
309 through its enriched presence among the proteins that co-purified with TAP-tagged ExoY
310 expressed in *S. cerevisiae*. Actin is among the most abundant proteins in eukaryotic cells

311 with a large number of known interaction partners. It is also frequently retrieved un-
312 specifically in “pull-down” experiments and it is ranked among the top contaminants in the
313 so-called “CRAPome”, Contaminant Repository for Affinity Purification³⁵. In our case,
314 however, we found that the interaction between ExoY and actin is highly specific and
315 conserved between yeast and different mammalian actin isoforms. We further showed that
316 ExoY is an F-actin binding protein (Fig. 5) and we demonstrated that F-actin is a potent
317 activator of ExoY, able to stimulate its adenylate and guanylate cyclase activity more than
318 10,000 fold. In accordance with this view, we found that ExoY activation by actin was
319 strongly antagonized by different G-actin binding proteins, such as profilin, or a Tβ4-
320 derivative protein with similar activity as Tβ4 (CH2), or by latrunculin A that prevents actin
321 polymerization (Fig. 4). Profilin and latrunculin A interact with non-overlapping, opposite
322 binding sites on G-actin, which are mostly buried by actin:actin contacts in filaments.
323 Profilin may partially overlap and compete with ExoY binding sites as it inhibits as well
324 ExoY-mediated stimulation of actin self-assembly (Fig. 5b). In contrast, the antagonizing
325 effect of the small molecule latrunculin A is likely due to its inhibition of actin
326 polymerization rather than to a steric hindrance of the ExoY binding-sites. In the presence of
327 saturating concentrations (> 2-5 μM) of F-actin, the very low basal enzymatic activity of
328 ExoY was strongly stimulated to reach specific activities of about 120 μmol.min⁻¹.mg⁻¹ and
329 900 μmol.min⁻¹.mg⁻¹ for cAMP and cGMP synthesis, respectively. The higher guanylate
330 cyclase activity as compared to the adenylate cyclase one is in agreement with the
331 preferential accumulation of cGMP over cAMP observed *in vivo*^{7, 8}. The corresponding kcat
332 for cGMP synthesis is approaching 1000 s⁻¹ and therefore within the same order of
333 magnitude as the catalytic rates measured for cAMP synthesis for the related cyclase toxins
334 CyaA or EF, when activated by calmodulin, their common eukaryotic activator^{36, 37}.

335 While ExoY represents to our knowledge the first example of a bacterial toxin that is
336 activated by F-actin, G-actin has been shown before to activate a bacterial toxin secreted by
337 the T3SS namely YopO/YpkA, a multidomain protein produced by pathogenic *Yersinia*
338 species (*Y. enterocolitica* and *Y. pseudotuberculosis*, respectively), and involved in the
339 disruption of the actin cytoskeleton³⁸. YopO directly binds to an actin monomer and
340 sterically blocks actin polymerization while, conversely, the bound actin induces
341 autophosphorylation and activation of the YopO N-terminal serine/threonine kinase
342 domain³⁹. In this dimeric protein complex, the bound actin then serves as bait to recruit
343 various host actin-regulating proteins that are then phosphorylated by YopO⁴⁰. The
344 mechanisms of activation of ExoY and YopO by F- and G-actin, respectively, are therefore
345 likely different.

346 ExoY binds along naked filaments with a sub-micromolar affinity (Kd of about
347 $0.8 \pm 0.3 \mu\text{M}$, Supplementary Fig. 5) that should allow efficient competition *in vivo* with many
348 eukaryotic cytoskeletal side-binding proteins that also bind along filaments with sub- to low
349 micromolar affinities^{26, 27, 28}. In agreement with this, we found that, *in vitro*, ExoY could
350 antagonize the regulation of actin self-assembly dynamics by VCA-activated Arp2/3
351 complex (Fig. 5d) and/or the Actin-Depolymerizing Factor (ADF) (Fig. 5e): the enhanced
352 turnover of actin filaments by ADF was inhibited by low and substoichiometric
353 concentrations of ExoY. Considering the ExoY affinity for F-actin and the very high F-actin
354 concentrations in eukaryotic cells (in non-muscle cells, G-and F-actin concentrations can
355 reach 150 and 500 μM , respectively²⁵), ExoY is expected to be fully bound to filaments
356 within host cells, and this was indeed directly confirmed *in vivo* by the co-localization of
357 ExoY-GFP with actin filaments in transfected NIH3T3 cells (Fig. 6). As many F-actin
358 binding proteins, ExoY can also weakly interact with actin monomers as indicated by the fact
359 that (i) ExoY is weakly activated (up to 5-10 % of maximal activity) in a dose dependent

360 manner by actin bound to the polymerization-inhibiting drug latrunculin A (Figs. 3b and 4)
361 and that (ii) ExoY weakly stimulated G-actin-ATP/-ADP polymerization in absence of
362 profilin (Fig. 5a,b).

363 While most of the ExoY related effects in infected cells likely depend on its catalytic
364 activity, the catalytically inactive ExoY^{K81M} mutant has been observed to induce temporary
365 actin redistribution to the cell margins¹⁰ as well as minimal intercellular gap formation¹¹ in
366 endothelial cells. These effects may be linked to a residual nucleotide cyclase activity of
367 ExoY^{K81M} and/or to its direct binding to actin polymers in host cells. We showed that *in vitro*
368 (Fig. 5e), ExoY can antagonize the cooperative activity of ADF at sub-molar ratios of ExoY
369 with respect to the regulatory cytoskeletal side binding protein. Although ExoY is likely
370 present only at low concentrations in infected host cells, its binding along actin filaments
371 could possibly contribute to the dysregulation of actin cytoskeleton dynamics *in vivo*. It will
372 thus be interesting to examine in more detail the actin cytoskeleton dynamics of host cells
373 upon infection with bacteria expressing catalytically inactive ExoY or ExoY-like proteins
374 alone or together with other toxins affecting actin cytoskeleton regulation (ExoS, ExoT from
375 *P. aeruginosa*, Actin Cross-linking (ACD) or Rho-GTPase Inactivation Domain (RID) from
376 various MARTX toxins of the *Vibrio* genus¹³).

377 ExoY-like modules are frequently found among the effector domains of
378 Multifunctional Autoprocessing RTX (MARTX) toxins in multiple bacterial species of the
379 *Vibrio* genus¹³, which represent emerging human or animal pathogens. In addition, ExoY-
380 like proteins can be found in various other Gram-negative pathogenic bacteria from the genus
381 *Providencia*, *Burkholderia* or *Proteus* (Fig. 7a). Here we showed that VnExoY-L, a rather
382 distantly related ExoY-like module from *V. nigrispulchritudo* (38% sequence similarity with
383 *P. aeruginosa* ExoY and 28% with *B. anthracis* EF or *B. pertussis* CyaA, Fig. 7a and
384 Supplementary Table 3), was also strongly stimulated (over a 10,000 fold) by actin and

385 efficiently synthesized cAMP but not cGMP. The lack of guanylate cyclase activity is in
386 agreement with the results obtained with the *V. vulnificus* ExoY-like module¹³, a close
387 homolog of VnExoY-L (>75% sequence similarity, Fig. 7a and Supplementary Table 3), and
388 may thus reflect a more general difference regarding the nucleotide substrate specificities
389 between the *P. aeruginosa* ExoY and other ExoY-like proteins found in MARTX toxins like
390 those of the *Vibrio* genus (Fig. 7a and Supplementary Fig. 6).

391 Actin may thus represent a common eukaryotic activator for a sub-group (Fig. 7a) of
392 the class II adenylyl cyclase toxin family (described in ¹⁶). This newly defined, actin-
393 activated nucleotidyl cyclase (AA-NC) sub-family is also characterized by wider nucleotide
394 substrate specificity than the original class II members, the adenylate cyclase toxins CyaA
395 from *B. pertussis* and EF from *B. anthracis*. As calmodulin, the common cofactor of CyaA
396 and EF, actin is an abundant and highly conserved protein specific to eukaryotic cells. It
397 appears, therefore, to be a suitable molecular signal to indicate the arrival of the ExoY toxin
398 in the eukaryotic environment of the host target cells, where it should display its cyclic
399 nucleotide synthesizing activity.

400 Future studies should address the mechanism of activation of ExoY and ExoY-like
401 proteins by actin through structural analysis. This could eventually open several interesting
402 prospects in particular regarding the development of small molecules able to specifically
403 inhibit the activation of these toxins by actin, as a therapeutic approach against bacterial
404 infections, as well as the structural basis of the differential substrate selectivity of these AA-
405 NCs.

406 Interestingly, Beckert et al.⁷ have reported notable differences in accumulation of
407 various cNMPs in different cell lines exposed to *P. aeruginosa* ExoY, in particular, with
408 respect to the mode of delivery of the toxin (transfection versus infection). It will be
409 interesting to examine whether the relative efficacy in synthesizing different cNMPs depends

410 solely on availability of substrates or whether the actin dynamics and turnover in cells may
411 also play a role.

412

413

414 **MATERIAL AND METHODS**

415 **Strains, plasmids and growth conditions**

416 Strains, plasmids and growth conditions are described in table S1.

417

418 **Purification of ExoY, actin from rabbit skeletal muscle, and actin-binding proteins**

419 ExoY-FH and VnExoY-L-FH were purified by nickel affinity chromatography under
420 denaturing conditions (in the presence of 8M urea) from the non-soluble protein fraction
421 obtained from 1 liter cultures of *E. coli* BLR (pUM460) or (pUM522), respectively.

422 Proteins were expressed from the λP_L promoter controlled by the temperature sensitive cI
423 repressor (cI857), which was induced by shifting the temperature from 30°C to 42°C.

424 Proteins were renatured by dialysis into 25 mM Tris pH8.0, 250 mM NaCl, 10 % glycerol,
425 1 mM DTT for ExoY-FH and 50 mM Tris pH 8.0, 200 mM NaCl, 10 % glycerol, 1 mM
426 DTT for VnExoY-FH. HF-ExoY was purified from the soluble fraction obtained from 0.5
427 liter cultures of MG1655 (pUM447) that were grown at 30°C.

428 The fusion constructs of ExoY/ExoY^{K81M} with an N-terminal maltose-binding
429 protein (MBP), designed as follows: (His-Tag)-(MBP)-(PreScission-site)-
430 (ExoY/ExoY^{K81M})-(Strep-tagII) and referred in the text as MBP-ExoY/ExoY^{K81M}-ST, or
431 their truncated forms (ExoY/ExoY^{K81M}-ST) were purified under non-denaturing
432 conditions successively from HisTrap, StrepTrap, and Superdex 200 16*60 columns using
433 standard protocols. The HisTag-MBP fusion was either cleaved or not using PreScission

434 protease prior to the StrepTrap purification step. Proteins were stored in 25 mM Tris-HCl
435 pH 8.8, 150 mM KCl, 3 mM NH₄SO₄, 0.5% Glycerol, 1 mM DTT.

436 Rabbit skeletal muscle alpha-actin was purified in our laboratory (referred as MA-
437 L) using several cycles of polymerization and depolymerization as previously described⁴¹
438 and stored in G-buffer (5 mM Tris pH 7.8, 0.1 mM CaCl₂, 0.2 mM ATP, 1 mM DTT).
439 The purity of MA-L was estimated to be >95% according to analysis on denaturing
440 SDS-PAGE gels. Functionality was controlled by several cycles of
441 polymerization/depolymerization and by verifying that the measured actin concentrations
442 of our samples fit the known critical concentration values of a fully functional and highly
443 pure actin. ADP-actin was prepared by treatment of ATP-G-actin with hexokinase and
444 glucose⁴².

445 Recombinant profilin I from mouse, chimera 2 of Thymosin-β₄ and Drosophila
446 ciboulot first β-Thymosin domain (CH2), full-length human gelsolin, VCA domain of
447 human neural Wiskott-Aldrich syndrome protein (N-WASP), the Arp2/3 complex from
448 bovine brain, or Spectrin-actin seeds from *human* erythrocytes were purified as described
449 previously^{22, 43, 44, 45}. The P529-P1013 FH2-WH2 construct of human INF2 formin
450 (UniProt accession: Q27J81) was purified similarly as ExoY-ST.

451

452 **Affinity Purification**

453 *S. cerevisiae* cells expressing ExoY_{MUT}-TAP from pUM497 or ExoY_{MUT}-HA from pUM498
454 as mock control were grown in 2 L YPGal at 30 °C. One step purification using
455 Dynabeads® magnetic beads conjugated to IgG were performed according to⁴⁶. One half of
456 the methanol/chloroform precipitated protein was analyzed by PAGE followed by staining
457 with Bio-Safe Coomassie G-250 (BIO-RAD) followed by silver staining (Pierce). The
458 second half was directly digested by trypsin and analyzed by LC-MS/MS analysis at the

459 proteomic facility of the Paris Descartes University (3P5) according to the details specified
460 below. The raw data were analyzed by MaxQuant 1.3.0.5. software ⁴⁷ for protein
461 identification and quantitative estimation of the specific enrichment of proteins in the
462 experimental sample as compared to the control.

463

464 **LC-MS/MS analysis**

465 Proteomics analyses were realized at the 3P5 proteomics facility, Université Paris
466 Descartes, Sorbonne Paris Cité, Institut Cochin, Paris as previously described⁴⁸. Briefly:

467 LC-MS protein analysis: peptides from Trypsin-digested extracts were concentrated,
468 washed and analyzed using a reverse phase C18 column on an u3000 nanoHPLC
469 hyphenated to a Linear Trap Quadrupole-Orbitrap mass spectrometer (all from Thermo).
470 LTQ MS/MS CID spectra were acquired from up to 20 most abundant ions detected in the
471 Orbitrap MS scan.

472 Protein identification: Proteome discoverer 1.3 (Thermo) with Mascot (matrixscience⁴⁹)
473 was used for protein identification. Separate analyses were compared using the
474 MyPROMS software⁵⁰.

475

476 **Interaction between ExoY and actin**

477 12.5 µg of ExoY-FH, skeletal muscle actin from rabbit (AKL99, Cytoskeleton, Inc.,
478 designated in the text by MA-99), or both proteins in 450 µl of binding buffer [50 mM Na-
479 phosphate pH 8.0, 300 mM NaCl, 20 mM imidazole, 0.01 % triton X-100, complete EDTA-
480 free protease inhibitor cocktail (Roche)] were allowed to bind in batch to 5 µl Ni-NTA
481 agarose (Quiagen) for 1 h at 4 °C rotating in Durapore filter units (Millipore). Unbound
482 material was removed by centrifugation at 1,000 g for 1 min, after which the beads were
483 washed three times with 450 µl of binding buffer and once with binding buffer supplemented

484 with 40 mM imidazole. Elution of bound proteins was performed by adding 50 μ l of binding
485 buffer supplemented with 0.5 M imidazole followed by incubation on ice for 10 min after
486 which the eluate was collected by centrifugation at 3,000 g for 1 min. A second elution was
487 carried out using 20 μ l of elution buffer. A 12 μ l aliquot of the combined eluates was
488 analyzed by SDS-PAGE.

489 F-actin co-sedimentation assays (Fig. 2b) were performed using α -actin (MA-99)
490 according to the instructions of Cytoskeleton, Inc supplied with the “Actin binding Protein
491 Biochem KitTM Muscle actin). 20 μ l of a 48 μ M actin (MA-99) solution in G'-buffer (5
492 mM Tris pH 8.0, 0.2 mM CaCl₂, 0.5 mM DTT, 0.2 mM ATP, 5% glycerol) were thawed
493 on ice, then added to 50 μ l 50 mM Bis-tris propane (BTP) pH 9.5 and allowed to sit on ice
494 for 40 min before polymerization was induced according to the protocol. 30 μ l of
495 polymerized F-actin stock solution were combined with 20 μ l of a solution containing 12
496 μ g ExoY-FH in 50 mM BTP pH 9.5, 270 mM NaCl, 2 mM DTT and 1x polymerization
497 buffer from which non-soluble aggregates had been removed previously by centrifugation
498 at 54,000 rpm in a TL55 rotor (Beckmann) at 18 °C for 1 h. This mixture as well as
499 controls containing only actin or only ExoY and the corresponding buffers present in the
500 experiment were incubated at room temperature for 30 min and centrifuged at 54,000 rpm
501 for 90 min at 18 °C. Aliquotes of supernatant and resuspended pellet fraction
502 corresponding to 15 % of the total samples were analyzed by SDS-PAGE.

503 To measure the equilibrium dissociation constant (K_d) of the ExoY:F-actin complex by co-
504 sedimentation assays we used MBP-ExoY/ExoY^{K81M}-ST and muscle α -actin (MA-L)
505 (Supplementary Fig. 5). MBP-ExoY/ExoY^{K81M}-ST should provide a reliable estimate of
506 ExoY affinity for F-actin because these constructs perform similarly as ExoY/ExoY^{K81M}-ST
507 in depolymerization assays. MBP-ExoY/ExoY^{K81M}-ST allowed separating and quantifying

508 unambiguously by densitometry the fraction of the bound toxin at 88.9 kDa from actin at 42
509 kDa on SDS-PAGE gels, while ExoY-ST (M.W.of 43 kDa) was migrating too close to actin
510 (M.W. of 42 kDa). No bundling activity was observed for ExoY in low-speed pelleting
511 assays with F-actin. 1.5 μ M of MBP-ExoY/ExoY^{K81M}-ST was mixed for 1h with increasing
512 amounts of F-actin-ADP-BeF₃ (0 to 25 μ M Mg-ADP-actin) at steady state in F1 buffer
513 containing 5 mM ADP, 6 mM NaF, 0.6 mM BeCl₂. The supernatant/unpolymerized (S) and
514 pellet/polymerized (P) fractions were separated by an ultracentrifugation of 40 min at
515 200 000*g, resolved by 15% SDS-PAGE and detected by coomassie Blue staining. The
516 ExoY-bound fraction was quantified by densitometry using the ImageJ software and plotted
517 *versus* F-actin concentration. The following equation was used to fit the data, in which [E0]
518 is the initial concentration of ExoY, [F0] the total concentration of F-actin in each
519 measurement, and K_d the equilibrium dissociation constant. The fraction R of ExoY bound to
520 F-actin is as follows:

$$R = \frac{K_d + [E0] + [F0] - \sqrt{(K_d + [E0] + [F0])^2 - 4 * [E0] * [F0]}}{2 * [E0]}$$

521

522 **Quantification of cAMP or cGMP synthesis *in vitro***

523 cAMP and cGMP synthesis were measured in 50 μ l reactions containing 50 mM
524 Tris pH 8.0, 7.5 mM MgCl₂, 0.5 mg/ml BSA, 200 mM NaCl, 1 mM DTT, 2 mM ATP or
525 GTP spiked with 0.1 μ Ci of [α -³³P] ATP or [α -³³P] GTP, respectively, ExoY and
526 indicated amounts of HeLa/*S. cerevisiae* cell extracts or purified actin (collectively termed
527 activator). Reactions for Figs. 1A and 3A contained in addition 0.02% triton X-100, 0.1
528 mM CaCl₂ and were lacking NaCl. Reactions were performed at 30 °C and were started by
529 adding nucleotide substrate after a 5-10 minutes preincubation of ExoY plus activator.
530 Under the conditions used, reactions were time linear for at least 20 minutes. Reactions

531 were stopped by the addition of 450 μ l stop solution (20 mM HEPES pH 7.5, 20 mM
532 EDTA, 0.5 mM cAMP) and the mixtures were filtered on Al₂O₃ columns, which included
533 3 washes with 1 ml 20 mM HEPES pH 7.5 each to separate nucleotide substrates that were
534 retained in the columns from cyclic nucleotides present in the filtrates. Filtrates were
535 collected in 20 ml scintillation vials. 16 ml scintillation liquid (HiSafe3, Perkin Elmer)
536 were added before measuring ³³P in a TriCarb scintillation counter (Perkin Elmer). All
537 reactions were performed in duplicates. Differences between cpm values of most
538 duplicates were around or less than 10%. Standard deviations between duplicates are
539 indicated by error bars.

540 Muscle actin 99% pure (designated MA-99) from rabbit skeletal muscle
541 (Reference AKL99), or 99% pure non-muscle actin from human platelets (Reference
542 APHL99, designated A-99) was obtained from Cytoskeleton, Inc. Alternatively, we used
543 actin from rabbit skeletal muscle prepared in one of our laboratories (designated MA-L)
544 according to the procedure described above. For activity assays, all actin solutions were
545 diluted in G-buffer supplemented with BSA at 0.1 mg/ml.

546 Preliminary experiments to optimize reaction conditions showed that ExoY-FH
547 was most active at pH values between 8 and 9 and in Tris as compared to HEPES or Na-
548 phosphate and shows a broad optimal NaCl concentration (between 100 and 300 mM
549 NaCl).

550 Extracts from HeLa cells for activation of ExoY were prepared as follows: Cells
551 grown in Dulbecco's modified Eagle's medium (DMEM) + 10% fetal bovine serum were
552 harvested after reaching 75% of confluence as follows: One wash with PBS was followed
553 by incubation in 10 ml PBS containing 0.01M EDTA for 5 min at 37 °C before detaching
554 the cells by gentle tapping of the flasks. Cells were collected by centrifugation and washed
555 3 times in PBS. The cell pellet was resuspended in 2 ml of lysis buffer [50 ml Tris pH 7.5,

556 300 mM NaCl, 0.5 % NP50, complete EDTA-free protease inhibitor cocktail (Roche)] per
557 ml of cell pellet volume, after which the cells were snap frozen in liquid nitrogen and
558 stored at -80 °C or processed immediately. Frozen cells were allowed to thaw on ice,
559 rotated at 4 °C for 20 min and centrifuged for 1 h at 18,000 rpm in a SS34 rotor (Sorval).
560 The supernatant was centrifuged at 100,000 rpm at 4 °C for 1 h in a TLA-110 rotor
561 (Beckmann). The resulting supernatant was filtered through a 0.45 µM durapore PVDF
562 filter unit (Millipore) and dialyzed against Tris/Triton/Glycerol buffer (TTG-buffer)
563 containing 25 mM Tris pH 8.0, 0.1 % Triton X-100, 10 % glycerol, 1 mM DTT, 0.4 mM
564 PMSF). Insoluble material was removed by centrifugation at 18,000 rpm in a SS34 rotor
565 at 4 °C for 20 min. HeLa cell extract prepared according to this protocol contained
566 approximately 10 mg/ml of protein and was stored in aliquots at -20 °C.

567 Extracts from *S. cerevisiae* were prepared from 100 ml cultures grown in YPD to
568 an OD600 between 0.5 and 2, at which cells were harvested by centrifugation, washed
569 once with water, and resuspended in 300 µl yeast lysis buffer [50 mM Tris pH 7.4, 50 mM
570 KCl, 1 mM DTT, complete EDTA-free protease inhibitor cocktail (Roche)]. Cells were
571 subsequently vortexed for a total of 5 min (5 times 1 min to prevent overheating) at 4 °C.
572 Debris were removed by centrifugation at 16,000 rpm for 15 min at 4 °C. The resulting
573 extract contained about 4 mg/ml protein and was stored at -20 °C after the addition of
574 glycerol to a final concentration of 10 %.

575 Cycles of freezing and thawing did not seem to affect the activity of the cofactor
576 necessary for ExoY activation in extracts from HeLa cells or *S. cerevisiae*.

577 Mg-ATP-actin was prepared from MA-L as follows: 90 µl of MA-L at 22.22 µM
578 were added to 10 µl 10 x ME buffer (420 µM MgCl₂, 4 mM EGTA) and incubated for 10
579 min at RT and put on ice.

580 A 34 μM solution of Mg-ADP-actin was prepared as follows: Ca-ATP-actin (MA-
581 L) was converted to Mg-ATP-actin by adding 100x concentrated ME-buffer and
582 incubating for 10 min at RT to achieve a final concentration of 40 μM MgCl_2 and 0.2 mM
583 EDTA. 15 U/ml of Hexokinase (Roche) was added together with glucose to a
584 concentration of 5 mM followed by 30 min incubation on ice. ApppppA was then added to
585 10 μM and the mixture was incubated for 5 min on ice. ADP and TCEP was added to 0.2
586 mM and 2 mM, respectively. Mg-ADP-actin was diluted in G-ADP-buffer (5 mM Tris pH
587 7.8, 0.2 mM ADP, 2 mM TCEP, 30 μM MgCl_2). 15 μl of the diluted actin solutions (Mg-
588 ATP-actin or Mg-ADP-actin) were combined with 30 μl of a mixture containing 1 ng
589 ExoY in reaction buffer to achieve final reaction buffer conditions of 50 mM Tris pH 8.0,
590 200 mM NaCl, 7.5 mM MgCl_2 , 2 mM DTT and 0.5 mg/ml BSA.

591 Studies on the effect of profilin or latrunculin were performed with actin (MA-L)
592 polymerized to steady state. For studies on the effect of profilin: (1) control reactions : 5
593 μl 5x concentrated ME-buffer (225 μM MgCl_2 , 2 mM EGTA) were added to 20 μl actin at
594 16.7 μM (diluted in G-buffer) yielding a concentration of 45 μM MgCl_2 and 0.4 mM
595 EGTA (25 ml Mg-ATP-actin (MA) and incubated for 10 min at room temperature. 25 μl
596 fresh 2X polymerization buffer (F2: 300 mM KCl, 40 mM MgCl_2 , 10 mM ATP, and 10
597 mM DTT) was added and samples were allowed to sit at room temperature for 2 h to
598 allow polymerization to proceed to steady state. Varying amounts of the so-prepared F-
599 actin were combined with F1-buffer (F1: 150 mM KCl, 20 mM MgCl_2 , 5 mM ATP, and 5
600 mM DTT) to a total volume of 15 μl and added to 30 μl of a mixture containing 1 ng
601 ExoY in buffer to achieve final reaction buffer conditions of 50 mM Tris pH 8.0, 200 mM
602 NaCl, 7.5 mM MgCl_2 , 2 mM DTT and 0.5 mg/ml BSA. After 30 min preincubation at 30
603 $^\circ\text{C}$, reactions were started by the addition of GTP (2 mM, spiked with [α - ^{33}P] GTP) and

604 allowed to proceed for 10 min. (2) Reactions containing profilin: profilin was dialyzed
605 against G-buffer to remove the KCl present in the storage buffer before adding 5 μ l at
606 142.7 μ M directly to undiluted actin (6 μ l MA at 55.88 μ M in G-buffer), incubated at
607 room temperature for 10 min and diluted by adding 14 μ l G-buffer. Actin was not
608 converted into Mg-ATP-actin. 25 μ l fresh F2 buffer was added and 11.2, 7.5, or 5.6 μ l of
609 this mixture were combined with G-buffer to a total volume of 15 μ l and used
610 immediately in activity assays. At final actin concentrations of 1.5, 1 and 0.75 μ M,
611 profilin was present at 3, 2, and 1.5 μ M, respectively.

612 Latrunculin A was purchased from tebu-bio (produced by Focus Biomolecules).
613 Studies with latrunculin were done similarly to those on profilin except that higher
614 concentrations of actin (between 5.25 and 1.0 μ M final MA-L) were used, ME buffer (10
615 fold concentrated) was added to control reactions before polymerization but was added as
616 well to reactions containing latrunculin after combining latrunculin and actin and 10 min
617 incubation at room temperature. Samples containing latrunculin were incubated 2 hours at
618 room temperature as the corresponding controls. The mixture of latrunculin and actin was
619 diluted to different concentrations in G-buffer containing latrunculin to ensure a finale
620 concentration of 10 μ M in all reactions. Control reactions lacking latrunculin contained
621 DMSO at concentrations equivalent to that introduced with latrunculin.

622 Sedimentation-assays to verify effectiveness of profilin or latrunculin in preventing
623 polymerization were performed at 2 or 1 μ M actin (MA-L) in the exact same way as for
624 measuring activity except that GTP was not spiked with [α -³³P] GTP.

625

626 **Pyren-actin polymerization and depolymerization assays**

627 Actin polymerization or depolymerization were monitored at 25°C by the increase
628 or decrease in fluorescence, respectively, of 3-10% (polymerization) or 50%
629 (depolymerization) pyrenyl-labeled actin ($\lambda_{exc} = 340$ nm, $\lambda_{em} = 407$ nm). Actin-Ca-ATP
630 in G-buffer was converted just prior to the experiments into G-actin-Mg-ATP by adding
631 1/100 (vol./vol.) of (2 mM MgCl₂, 20 mM EGTA). Polymerization or depolymerization
632 were induced in a final F1-buffer containing (0.1 M KCl, 8 mM MgCl₂, 50 mM Tris-HCl
633 pH 7.8, 9 mM TCEP, 1 mM DTT, 0.3 mM NH₄SO₄), 10 to 15 mM ATP or ADP, and 3 to
634 5 mM GTP, unless indicated otherwise in figure legends. Fluorescence measurements
635 were carried out in a Safas Xenius model FLX (Safas, Monaco) spectrophotometer, using
636 a multiple sampler device. Dilution-induced depolymerization assays were performed by
637 quickly diluting 4 to 68 μ L of 9 to 13 μ M 50% pyrenyl-labeled F-actin at steady state into
638 a final volume of 160 μ L containing F1 buffer, ATP and the proteins of interest.

639

640 **Localization of ExoY-AcGFP and actin in mouse NIH3T3 cells**

641 NIH3T3 cells were grown in Dulbecco's modified Eagle's medium with high glucose (4.5 g/l)
642 and 10% fetal bovine serum (FCS, PAA). Cells were plated on coverslips and transiently
643 transfected by standard calcium phosphate precipitation the following day. Two days after
644 the transfection, the cells were washed twice with cold PBS and fixed by 4% formaldehyde
645 for 10 min at room temperature. Cells were then washed with PBS at room temperature,
646 permeabilized with 0.5% Triton X100 in PBS and washed again with PBS. 200 μ L of 100 nM
647 Acti-stainTM 555 fluorescent phalloidin (PHDH1, Cytoskeleton, Inc.) were added and the
648 coverslip was incubated at room temperature for 30 min. Cover slips were mounted on glass
649 slides using Fluoroshield mounting medium with DAPI (ab104139, Abcam). Cells were
650 visualized with a confocal scanning microscope (LEICA SPE).

651

652

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660

661 REFERENCES

662

- 663 1. Engel J, Balachandran P. Role of *Pseudomonas aeruginosa* type III effectors in
664 disease. *Curr Opin Microbiol* **12**, 61-66 (2009).
665
- 666 2. Hauser AR. The type III secretion system of *Pseudomonas aeruginosa*: infection
667 by injection. *Nat Rev Microbiol* **7**, 654-665 (2009).
668
- 669 3. Burstein D, *et al.* Novel type III effectors in *Pseudomonas aeruginosa*. *MBio* **6**,
670 e00161 (2015).
671
- 672 4. Feltman H, Schulert G, Khan S, Jain M, Peterson L, Hauser AR. Prevalence of
673 type III secretion genes in clinical and environmental isolates of *Pseudomonas*
674 *aeruginosa*. *Microbiology* **147**, 2659-2669 (2001).
675
- 676 5. Yahr TL, Vallis AJ, Hancock MK, Barbieri JT, Frank DW. ExoY, an adenylate
677 cyclase secreted by the *Pseudomonas aeruginosa* type III system. *Proc Natl Acad*
678 *Sci U S A* **95**, 13899-13904 (1998).
679
- 680 6. Gottle M, *et al.* Cytidylyl and uridylyl cyclase activity of bacillus anthracis edema
681 factor and *Bordetella pertussis* CyaA. *Biochemistry* **49**, 5494-5503 (2010).
682
- 683 7. Beckert U, *et al.* ExoY from *Pseudomonas aeruginosa* is a nucleotidyl cyclase with
684 preference for cGMP and cUMP formation. *Biochem Biophys Res Commun* **450**,
685 870-874 (2014).
686
- 687 8. Ochoa CD, Alexeyev M, Pastukh V, Balczon R, Stevens T. *Pseudomonas*
688 *aeruginosa* exotoxin Y is a promiscuous cyclase that increases endothelial tau
689 phosphorylation and permeability. *J Biol Chem* **287**, 25407-25418 (2012).
690

- 691 9. Balczon R, *et al.* Pseudomonas aeruginosa exotoxin Y-mediated tau
692 hyperphosphorylation impairs microtubule assembly in pulmonary microvascular
693 endothelial cells. *PLoS One* **8**, e74343 (2013).
694
- 695 10. Cowell BA, Evans DJ, Fleiszig SM. Actin cytoskeleton disruption by ExoY and its
696 effects on Pseudomonas aeruginosa invasion. *FEMS Microbiol Lett* **250**, 71-76
697 (2005).
698
- 699 11. Sayner SL, Frank DW, King J, Chen H, VandeWaa J, Stevens T. Paradoxical
700 cAMP-induced lung endothelial hyperpermeability revealed by Pseudomonas
701 aeruginosa ExoY. *Circ Res* **95**, 196-203 (2004).
702
- 703 12. Stevens TC, *et al.* The Pseudomonas aeruginosa exoenzyme Y impairs endothelial
704 cell proliferation and vascular repair following lung injury. *Am J Physiol Lung Cell*
705 *Mol Physiol*, (2014).
706
- 707 13. Ziolo KJ, Jeong HG, Kwak JS, Yang S, Lavker RM, Satchell KJ. Vibrio vulnificus
708 biotype 3 multifunctional autoprocessing RTX toxin is an adenylate cyclase toxin
709 essential for virulence in mice. *Infect Immun* **82**, 2148-2157 (2014).
710
- 711 14. Leppla SH. Anthrax toxin edema factor: a bacterial adenylate cyclase that
712 increases cyclic AMP concentrations of eukaryotic cells. *Proc Natl Acad Sci U S A*
713 **79**, 3162-3166 (1982).
714
- 715 15. Berkowitz SA, Goldhammer AR, Hewlett EL, Wolff J. Activation of prokaryotic
716 adenylate cyclase by calmodulin. *Ann N Y Acad Sci* **356**, 360 (1980).
717
- 718 16. Barzu O, Danchin A. Adenylyl cyclases: a heterogeneous class of ATP-utilizing
719 enzymes. *Prog Nucleic Acid Res Mol Biol* **49**, 241-283 (1994).
720
- 721 17. Arnoldo A, *et al.* Identification of small molecule inhibitors of Pseudomonas
722 aeruginosa exoenzyme S using a yeast phenotypic screen. *PLoS Genet* **4**, e1000005
723 (2008).
724
- 725 18. Lubber CA, *et al.* Quantitative proteomics reveals subset-specific viral recognition
726 in dendritic cells. *Immunity* **32**, 279-289 (2010).
727
- 728 19. Pollard TD, Blanchoin L, Mullins RD. Molecular mechanisms controlling actin
729 filament dynamics in nonmuscle cells. *Annu Rev Biophys Biomol Struct* **29**, 545-
730 576 (2000).
731
- 732 20. Coue M, Brenner SL, Spector I, Korn ED. Inhibition of actin polymerization by
733 latrunculin A. *FEBS Lett* **213**, 316-318 (1987).
734
- 735 21. Yarmola EG, Somasundaram T, Boring TA, Spector I, Bubb MR. Actin-
736 latrunculin A structure and function. Differential modulation of actin-binding
737 protein function by latrunculin A. *J Biol Chem* **275**, 28120-28127 (2000).
738

- 739 22. Didry D, *et al.* How a single residue in individual beta-thymosin/WH2 domains
740 controls their functions in actin assembly. *Embo J* **31**, 1000-1013 (2012).
741
- 742 23. Husson C, *et al.* Multifunctionality of the beta-thymosin/WH2 module: G-actin
743 sequestration, actin filament growth, nucleation, and severing. *Ann N Y Acad Sci*
744 **1194**, 44-52 (2010).
745
- 746 24. Xue B, Leyrat C, Grimes JM, Robinson RC. Structural basis of thymosin-
747 beta4/profilin exchange leading to actin filament polymerization. *Proc Natl Acad*
748 *Sci U S A* **111**, E4596-4605 (2014).
749
- 750 25. Koestler SA, Rottner K, Lai F, Block J, Vincenz M, Small JV. F- and G-actin
751 concentrations in lamellipodia of moving cells. *PLoS One* **4**, e4810 (2009).
752
- 753 26. dos Remedios CG, *et al.* Actin binding proteins: regulation of cytoskeletal
754 microfilaments. *Physiol Rev* **83**, 433-473 (2003).
755
- 756 27. Olshina MA, *et al.* Plasmodium falciparum coronin organizes arrays of parallel
757 actin filaments potentially guiding directional motility in invasive malaria
758 parasites. *Malar J* **14**, 280 (2015).
759
- 760 28. Bugyi B, Didry D, Carlier MF. How tropomyosin regulates lamellipodial actin-
761 based motility: a combined biochemical and reconstituted motility approach.
762 *EMBO J* **29**, 14-26 (2010).
763
- 764 29. Ti SC, Jurgenson CT, Nolen BJ, Pollard TD. Structural and biochemical
765 characterization of two binding sites for nucleation-promoting factor WASp-VCA
766 on Arp2/3 complex. *Proc Natl Acad Sci U S A* **108**, E463-471 (2011).
767
- 768 30. Rotty JD, *et al.* Profilin-1 serves as a gatekeeper for actin assembly by Arp2/3-
769 dependent and -independent pathways. *Dev Cell* **32**, 54-67 (2015).
770
- 771 31. Goudenege D, *et al.* Comparative genomics of pathogenic lineages of *Vibrio*
772 *nigripulchritudo* identifies virulence-associated traits. *Isme J* **7**, 1985-1996 (2013).
773
- 774 32. Shen A, *et al.* Mechanistic and structural insights into the proteolytic activation of
775 *Vibrio cholerae* MARTX toxin. *Nat Chem Biol* **5**, 469-478 (2009).
776
- 777 33. Aktories K, Lang AE, Schwan C, Mannherz HG. Actin as target for modification
778 by bacterial protein toxins. *Febs J* **278**, 4526-4543 (2011).
779
- 780 34. Haglund CM, Welch MD. Pathogens and polymers: microbe-host interactions
781 illuminate the cytoskeleton. *J Cell Biol* **195**, 7-17 (2011).
782
- 783 35. Mellacheruvu D, *et al.* The CRAPome: a contaminant repository for affinity
784 purification-mass spectrometry data. *Nat Methods* **10**, 730-736 (2013).
785
- 786 36. Drum CL, *et al.* Structural basis for the activation of anthrax adenylyl cyclase
787 exotoxin by calmodulin. *Nature* **415**, 396-402 (2002).

- 788
789 37. Karst JC, Sotomayor Perez AC, Guijarro JI, Raynal B, Chenal A, Ladant D.
790 Calmodulin-induced conformational and hydrodynamic changes in the catalytic
791 domain of Bordetella pertussis adenylate cyclase toxin. *Biochemistry* **49**, 318-328
792 (2010).
793
794 38. Juris SJ, Rudolph AE, Huddler D, Orth K, Dixon JE. A distinctive role for the
795 Yersinia protein kinase: actin binding, kinase activation, and cytoskeleton
796 disruption. *Proc Natl Acad Sci U S A* **97**, 9431-9436 (2000).
797
798 39. Trasak C, *et al.* Yersinia protein kinase YopO is activated by a novel G-actin
799 binding process. *J Biol Chem* **282**, 2268-2277 (2007).
800
801 40. Lee WL, Grimes JM, Robinson RC. Yersinia effector YopO uses actin as bait to
802 phosphorylate proteins that regulate actin polymerization. *Nat Struct Mol Biol* **22**,
803 248-255 (2015).
804
805 41. Spudich JA, Watt S. The regulation of rabbit skeletal muscle contraction. I.
806 Biochemical studies of the interaction of the tropomyosin-troponin complex with
807 actin and the proteolytic fragments of myosin. *J Biol Chem* **246**, 4866-4871
808 (1971).
809
810 42. Mizuno H, Higashida C, Yuan Y, Ishizaki T, Narumiya S, Watanabe N. Rotational
811 movement of the formin mDial along the double helical strand of an actin
812 filament. *Science* **331**, 80-83 (2014).
813
814 43. Casella JF, Maack DJ, Lin S. Purification and initial characterization of a protein
815 from skeletal muscle that caps the barbed ends of actin filaments. *J Biol Chem* **261**,
816 10915-10921 (1986).
817
818 44. Gaucher JF, Mauge C, Didry D, Guichard B, Renault L, Carlier MF. Interactions
819 of isolated C-terminal fragments of neural Wiskott-Aldrich syndrome protein (N-
820 WASP) with actin and Arp2/3 complex. *J Biol Chem* **287**, 34646-34659 (2012).
821
822 45. Le Clainche C, Carlier MF. Actin-based motility assay. *Curr Protoc Cell Biol*
823 **Chapter 12**, Unit 12 17 (2004).
824
825 46. Defenouillere Q, *et al.* Cdc48-associated complex bound to 60S particles is
826 required for the clearance of aberrant translation products. *Proc Natl Acad Sci U S*
827 *A* **110**, 5046-5051 (2013).
828
829 47. Cox J, Mann M. MaxQuant enables high peptide identification rates,
830 individualized p.p.b.-range mass accuracies and proteome-wide protein
831 quantification. *Nat Biotechnol* **26**, 1367-1372 (2008).
832
833 48. Vallabhaneni KC, *et al.* Extracellular vesicles from bone marrow mesenchymal
834 stem/stromal cells transport tumor regulatory microRNA, proteins, and
835 metabolites. *Oncotarget* **6**, 4953-4967 (2015).
836

- 837 49. Perkins DN, Pappin DJ, Creasy DM, Cottrell JS. Probability-based protein
838 identification by searching sequence databases using mass spectrometry data.
839 *Electrophoresis* **20**, 3551-3567 (1999).
840
- 841 50. Pouillet P, Carpentier S, Barillot E. myProMS, a web server for management and
842 validation of mass spectrometry-based proteomic data. *Proteomics* **7**, 2553-2556
843 (2007).
844
- 845 51. Chhabra ES, Higgs HN. INF2 Is a WASP homology 2 motif-containing formin
846 that severs actin filaments and accelerates both polymerization and
847 depolymerization. *J Biol Chem* **281**, 26754-26767 (2006).
848
- 849 52. Dereeper A, *et al.* Phylogeny.fr: robust phylogenetic analysis for the non-
850 specialist. *Nucleic Acids Res* **36**, W465-469 (2008).
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854 **FIGURE LEGENDS**

855

856 **Figure 1. Presence of an activator of ExoY in *Saccharomyces cerevisiae*. (a) Activation**

857 **of HF-ExoY by extracts from HeLa cells or *S. cerevisiae*.** 50 μ l reactions containing 1 μ g

858 ExoY were started by the addition of the substrate ATP and stopped after 30 min incubation

859 at 30 °C and the amount of synthesized cAMP was measured. **(b) Specific association of**

860 **yeast Act1 to ExoY^{K81M}.** Log₂ transformed LFQ scores for the proteins identified in the

861 fraction that copurified with ExoY^{K81M}-TAP (y axis) were represented as a function of the

862 scores obtained for the control purification (ExoY^{K81M}-HA, x axis). Black circles are the

863 result of two or more superimposed grey circles. For clarity, only the 100 proteins with

864 highest LFQ scores in the TAP purification are shown; 45 of these factors, including ExoY,

865 were not identified in the control purification and are represented on the y axis alone. The

866 dashed line was computed by linear regression for the 55 proteins having LFQ values in both

867 experiments and indicates the trend for common contaminants in the affinity purification.

868

869 **Figure 2. Interaction between ExoY and skeletal muscle actin from rabbit (MA-99). (a)**

870 12.5 μ g of actin (MA-99) (A), ExoY-FH (E), or each actin and ExoY-FH (EA) were allowed

871 to bind to 5 μ l Ni-NTA agarose in 450 μ l binding buffer. Aliquots of imidazole-eluted

872 fractions were separated on 4-12 % NuPAGE® Bis-Tris gels (Invitrogen) in NuPAGE®

873 MES SDS running buffer and the gel was stained with Bio-Safe™ Coomassie stain. Lanes

874 A* and E* show the corresponding input for actin and ExoY alone, respectively.

875 Corresponding amounts were loaded to allow direct comparability. Aliquots correspond to

876 15% of the total sample volume. The band of lower molecular weight was confirmed to be

877 actin by western blots with anti-actin C4 antibodies. **(b) Co-sedimentation of ExoY with**

878 **skeletal muscle F-actin.** Supernatant (S) and pellet (P) fractions were separated on 4-12 %

879 NuPAGE® Bis-Tris gels (Invitrogen) in NuPAGE® MES SDS running buffer and the gel
880 was stained as above. Reaction (1): actin only, (2): ExoY-FH only, (3) actin plus ExoY-FH.

881

882 **Figure 3. ExoY is efficiently activated by actin *in vitro*. (a) Preferential synthesis of**
883 **cGMP as compared to cAMP by ExoY activated by actin (A-99).**

884 Reactions containing HF-ExoY at 0.5 nM (1 ng) and actin at concentrations indicated were
885 started by the addition of 2 mM ATP or GTP substrate and incubated for 10 min at 30 °C. (b)

886 **Dependence of ExoY activation on the nucleotide and polymerization state of actin.**

887 Muscle α -actin (MA-L) was converted to Mg-ATP-actin or Mg-ADP-actin and used at the
888 concentration indicated to activate ExoY-FH. Mg-ATP-actin or Mg-ADP-actin polymerize
889 above 100 and 1700 nM, respectively. Activities with the polymerization-inhibiting drug
890 latrunculin A and Mg-ATP-actin (according to Figure 4) were plotted as comparison.

891

892 **Figure 4. Effect of latrunculin A and G-actin binding proteins on the activation of**

893 **ExoY.** Reactions containing actin (+/- inhibitor) and ExoY-FH were preincubated for 10 min

894 at 30 °C and started by the addition of 2 mM GTP and continued for 10 min. **Latrunculin A:**

895 latrunculin A (at a final concentration of 10 μ M) was added to actin and preincubated for 10

896 min at room temperature before conversion to Mg-ATP-actin and processing as for the

897 control. **Profilin:** Profilin was added to Ca-ATP-G-actin at a 2:1 ratio. Control reactions

898 lacking latrunculin or profilin contained G-actin (MA-L) that was converted to Mg-ATP-G-

899 actin and allowed to polymerize to steady state conditions before dilution to the indicated

900 concentrations. **Chimera 2 (CH2)** for a final concentration of 5 μ M was added to muscle G-

901 actin (MA-L) under conditions preventing salt-induced polymerization in G-buffer and

902 preincubated for 10 min at 30 °C. None of the molecules tested affected the low basal ExoY

903 activity in the absence of actin.

904

905 **Figure 5. ExoY is an F-actin binding protein whose binding along the sides of filaments**
906 **can interfere with the intrinsic or regulated dynamics of filaments. (a) ExoY-ST**
907 **mediated acceleration of G-actin-ADP-Mg self-assembly rate.** 8 μM G-actin-Mg-ADP
908 (10% pyrenyl labeled) was polymerized in ExoY-ST absence or presence at the indicated
909 concentrations (nM). **(b) ExoY^{K81M}-ST mediated acceleration of G-actin-ATP-Mg self-**
910 **assembly rate and its inhibition by profilin.** 4 μM G-actin-Mg-ATP (3% pyrenyl labeled)
911 was polymerized in the absence (continuous lines) or presence (dashed lines) of profilin (at
912 10 μM) with 0 to 2000 nM ExoY^{K81M}-ST. Efficient polymerization acceleration by a genuine
913 G-actin nucleator, namely the dimeric FH2 domain of INF2 formin⁵¹ (in green, 50 nM) is
914 shown for comparison. **(c) Effects of ExoY^{K81M}-ST binding to filaments on their**
915 **spontaneous disassembly kinetics from free barbed (continuous lines) or pointed**
916 **(dotted lines) ends.** From free barbed-ends: F-actin (2 μM , 50% pyrenyl) at steady state was
917 diluted to 50 nM in presence of 0 to 250 nM ExoY^{K81M}-ST. Disassembly from pointed-ends:
918 filaments with their barbed-ends capped by Gelsolin (10 μM actin, 33.3 nM Gelsolin) at
919 steady state were diluted to 300 nM in presence of 0 to 250 nM ExoY^{K81M}-ST. Different
920 fluorescence intensity levels were used between the two depolymerization assays. **(d) Effect**
921 **of ExoY^{K81M}-ST on the acceleration of filament formation induced by VCA-activated**
922 **Arp2/3 complex.** 3 μM G-actin-Mg-ATP (3% pyrenyl labeled) was polymerized with 7.5
923 μM profilin, 0.2 μM NWASP-VCA, in absence (black) or presence of 35 nM Arp2/3 and 0
924 to 600 nM ExoY^{K81M}-ST. **(e) Effect of ExoY/ExoY^{K81M}-ST on the acceleration of F-actin-**
925 **ADP disassembly promoted by ADF.** F-actin-ADP (9 μM) at steady state was diluted to 4
926 μM and preincubated for 2 min with 0 to 630 nM ExoY-ST (w.t., continuous lines) or
927 ExoY^{K81M}-ST (dotted lines) prior to depolymerization assays without ADF (0 nM
928 ExoY/ExoY^{K81M}, black) or with 4 μM ADF.

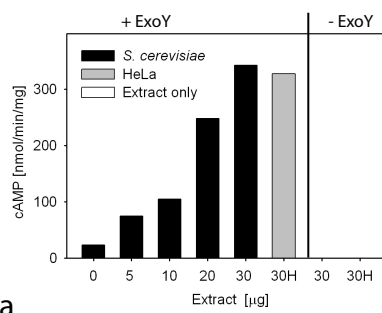
929

930 **Figure 6. ExoY colocalizes with actin fibers in mouse NIH3T3 cells.** Cells were
931 transiently transfected with pUM518 expressing ExoY^{K81M}-AcGFP (upper panel) or a control
932 plasmid (pEGFP-C1) (lower panel) and stained with Acti-stainTM 555 fluorescent phalloidin
933 and DAPI. (A) nuclei stained with DAPI, (B) distribution of actin fibers stained with
934 phalloidin, (C) distribution of ExoY^{K81M}-AcGFP, and (D) merge of images (B) and (C).

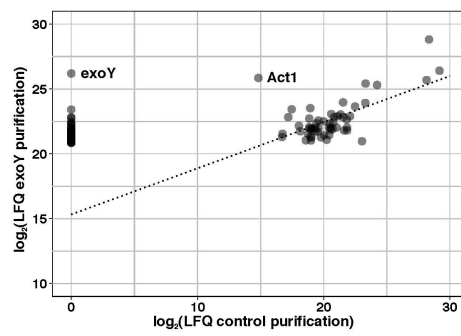
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936 **Figure 7. Phylogenetic tree of the bacterial ExoY-like nucleotidyl cyclase toxin**
937 **subfamily (a) and activation of VnExoY-L catalyzed synthesis of cAMP by actin (b).**

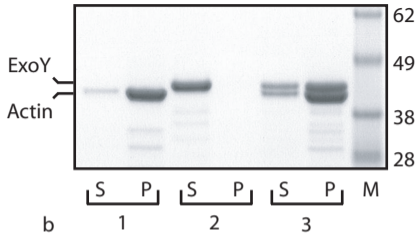
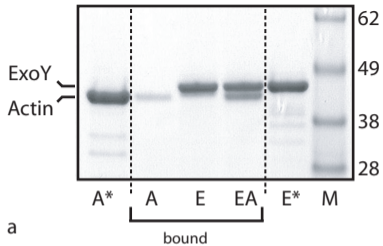
938 (a) The amino-acid sequences of *Pseudomonas aeruginosa* ExoY and various ExoY-
939 related effector domains/toxins found in several emerging Gram-negative bacterial
940 pathogens were aligned as shown in Supplementary Fig. 6 and clustered on phylogram
941 branches on the basis of the similarity of their amino acid sequences using the
942 Phylogeny.fr platform⁵². Calmodulin-activated EF and Cya Adenylate Cyclase Domains
943 (ACD) were used as an out group more distantly related to ExoY-like nucleotidyl cyclase
944 toxins to root the phylogeny. NCBI accessions of bacterial protein sequences are given in
945 Supplementary Table 3. Pairwise sequence similarities (%) with *P. aeruginosa* ExoY or *V.*
946 *nigripulchritudo* ExoY-like (VnExoY-L) are given in green and blue, respectively (to see
947 supplementary Table 3 for more details). Similarity values without parenthesis indicate the
948 ExoY-like sequences that are the most significantly related to actin-activated ExoY or
949 VnExoY-L nucleotidyl cyclases. (b) **Activation of VnExoY-L catalyzed synthesis of**
950 **cAMP by actin (MA-L).** Reactions containing VnExoY-L at 3.7 nM (10 ng) and actin at
951 concentrations indicated were started by the addition of 2 mM ATP substrate and
952 incubated for 30 min at 30 °C. The background activity without actin was estimated to be
953 1 nmol of cAMP.min⁻¹.mg⁻¹.

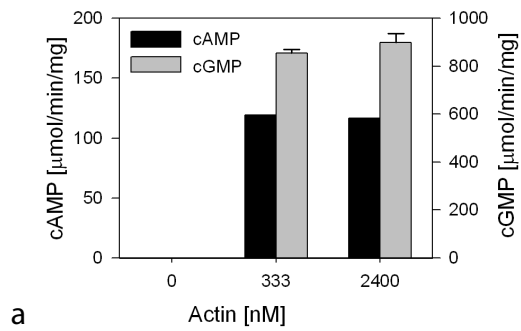


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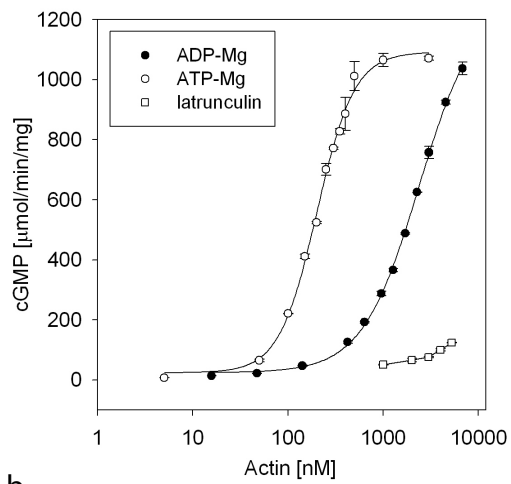


b





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b

