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1 **Title**
2 Molecular determinants of enterotoxigenic *Escherichia coli* heat-stable toxin secretion and
3 delivery
4
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32 ## Abstract

33 Enterotoxigenic *Escherichia coli* (ETEC), a heterogeneous diarrheal pathovar defined by
34 production of heat-labile (LT) and/or heat-stable (ST) toxins, remain major causes of mortality
35 among children in developing regions, and cause substantial morbidity in individuals living in or
36 traveling to endemic areas. Studies demonstrating a major burden of ST-producing ETEC have
37 focused interest on ST toxoids for ETEC vaccines. We therefore examined fundamental aspects
38 of ETEC ST biology using ETEC H10407, which carries *estH* and *estP* genes encoding ST-H and
39 ST-P, respectively, in addition to *eltAB* genes responsible for LT. In this background, we found
40 that deletion of *estH* significantly diminished cGMP activation in target epithelia, while deletion
41 of *estP* had a surprisingly modest impact, and a dual *estH/estP* mutant was not appreciably
42 different than the *estH* mutant. Nevertheless, either ST-H or ST-P recombinant peptides
43 stimulated cGMP production. We also found that the TolC efflux protein was essential for both
44 toxin secretion and delivery, providing a potential avenue for efflux inhibitors in treatment of
45 acute diarrheal illness. In addition, we demonstrated that the EtpA adhesin is required for
46 optimal delivery of ST and that antibodies against either the adhesin or ST-H significantly
47 impaired toxin delivery and cGMP activation in target T84 cells. Finally, we used FLAG epitope
48 fusions to demonstrate that the ST-H pro-peptide sequence is secreted by the bacteria,
49 potentially providing additional targets for antibody neutralization. These studies collectively
50 extend our understanding of ETEC pathogenesis and potentially inform additional avenues to
51 mitigate disease by these common diarrheal pathogens.

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53
54 ## Introduction
55
56 Diarrheal illnesses in low income countries continue to cause substantial morbidity and remain
57 one of the leading causes of death in young children in developing countries under the age of
58 five years. Among the bacterial causes of diarrheal illness enteroxigenic *Escherichia coli* (ETEC)
59 are commonly linked to more severe forms of illness in young children(1). These organisms are
60 perennially the most common cause of diarrhea in those who travel to endemic areas where
61 sanitation is poor(2, 3), however they have been identified repeatedly as the etiology of
62 diarrheal outbreaks and sporadic cases of illness in industrialized countries including the U.S(4-
63 8).
64 Acute clinical presentations of ETEC infection may range from mild self-limited illness to severe
65 cholera-like diarrhea(9-11). In addition, ETEC and other diarrheal pathogens have been linked
66 to pernicious sequelae of malnutrition, growth stunting, and impaired cognitive(12)
67 development. Presently, there are no vaccines to protect against these common infections.
68 ETEC are a genetically(13) and serotypically (14) diverse pathovar of *E. coli* defined by the
69 production of heat-labile (LT) and/or heat stable (ST) enterotoxins which activate production of
70 host cyclic nucleotides to alter intestinal salt and water transport that culminate in net fluid
71 losses and diarrhea.
72 Heat-stable toxins are synthesized as 72 amino acid proteins consisting of a signal peptide, a
73 pro peptide and a carboxy terminal region of 18-19 amino acids, which forms the mature active
74 enterotoxin(15). Two enterotoxins cause disease in humans: STp (ST1a) 18 amino acids, and
75 STh (ST1b) 19 amino acids. Both mature toxins contain four cysteine residues which form two
76 intramolecular disulfide bonds. Their overall structure is shared with two homologous
77 mammalian peptides, guanylin and uroguanylin. Each of the bacterial and mammalian peptides
78 binds to guanylate cyclase C(16, 17), leading to increases in intracellular cGMP(18). Increases in

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79 this cyclic nucleotide result in activation of protein kinases which phosphorylate and activate
80 the cystic fibrosis transmembrane regulatory(CFTR) channel, and inhibit sodium-hydrogen ion
81 exchange(19). These effects lead to a net loss of salt and water into the intestinal lumen with
82 ensuing watery diarrhea.
83 Bacteria producing any of the toxins LT, STh, or STp have been linked to diarrheal illness in
84 humans(20-23), and recent studies suggest that ST-producing ETEC are commonly represented
85 among the pathogens that cause severe diarrheal illness among young children in low income
86 countries leading to substantial interest in the development of a vaccine that incorporates ST-
87 toxoids(24).
88 Enterotoxigenic *E. coli* strain H10407, originally isolated from a case of severe cholera-like
89 diarrheal illness in Bangladesh, is to date the most extensively characterized isolate of this
90 pathovar. Interestingly, this isolate encodes all three enterotoxins associated with ETEC
91 diarrheal illness in humans(25, 26), with the gene for STh on the largest 94,797 bp virulence
92 plasmid (NCBI Genbank acccession NC_017724.1), and the genes for both LT and STp on a
93 66681 bp plasmid(27). H10407 is frequently used as the challenge strain in controlled human
94 infection models to test candidate vaccines. Therefore, we set out to examine the relative
95 contribution of STh and STp to activation of cGMP in host epithelia by H10407, and the ability
96 of anti-ST and anti-adhesin antibodies to mitigate effective toxin delivery by the bacteria.
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99 **Materials and methods**

100 ## Bacterial strains and growth conditions

101 Bacterial strains used are listed in Table 1. For general purposes, ETEC bacteria were grown in
102 lysogeny broth at 37°C overnight from frozen glycerol stocks. To optimize synthesis and
103 secretion of ST toxin, bacteria were grown in Casamino Acids-yeast extract –sucrose medium
104 (CAYE-ST) (2% Casamino acids, 0.6% Yeast extract, 43mM NaCl, 38mM K_2HPO_4, 203mM
105 $MgSO_4$, 25mM $MnCl_2$, 18mM $FeCl_3$, 2% sucrose) at 37°C overnight from frozen glycerol stocks.
106

107 ## Construction of mutants lacking production and secretion of heat stable toxins

108 Lamda red recombinase mediated recombination(28) was used to disrupt genes encoding heat
109 stable toxins in H10407. In brief, we used the primers jf070212.1 and jf070212.2 to amplify the
110 kanamycin cassette from pKD13 with 36 bp overhang sequence from *estH* gene, and primers of
111 jf070212.3 and jf070212.4 to amplify kan cassatte from pKD13 with 36 bp overhang sequence
112 from *estP* gene. The PCR product was electroporated into competent H10407 containing helper
113 plasmid pKD46, and selected on LB plate with 50 µg/ml kanamycin. Kanamycin-resistant
114 colonies were screened by PCR with primers of jf071612.1 and jf071612.2 for the *estH* locus, and
115 primers of jf071612.3 and k2 for *estP*. The deletion mutants were further confirmed by toxin
116 multiplex PCR as previously described (29).
117 To construct complemented strains, we amplified the *estP* gene with primers jf072412.3 and
118 jf060614.1 for cloning into XhoI site of pBAD/mycHisA, yielding pSMD002; *estH* was amplified
119 with primers of jf072414.5 and jf072414.6 and cloned into the EcoRV site of pACYC184 to
120 produce pSMD001. We also cloned *estH* and *estP* genes individually and together in pETDuet-1
121 at NcoI & EcoRI sites and BglII & XhoI sites with primer pairs of jf060614.2 and jf060614.3,
122 jf060614.4 and jf060614.5, respectively. This resulted in plasmids pQL230 (*estP*), pQL231(*estH*),

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123 and pQL238 (*estP/estH*) (table 1). After confirmation by sequencing, the respective plasmids
124 were transformed into the deletion strains, and complementation confirmed by PCR.
125 To generate an ETEC *tolC* deletion mutant, primers jf110716.44 /45 were first used to amplify a
126 ΔtolC732:: Km^R fragment from JQ55031(30). Next, the regions flanking *tolC* in the H10407
127 genome were amplified with primer pairs jf110716.38/ jf110716.43 and jf110716.46/ jf110716.39
128 to amplify 1027 bp of upstream and 985 bp of downstream sequence, respectively. The three
129 resulting fragments were then fused by PCR with primer pairs jf110716.38/ jf110716.39, using
130 high-fidelity polymerase Phusion (Thermo Fisher Scientific), with denaturing for 2 min at 98°C,
131 followed by 30 cycles of 10 s at 98°C, 30 s at 65°C, and 1.5 min at 72°C, and a final extension for
132 10 min at 72°C. The resulting 3,325-bp amplicon was then introduced into H10407(pKD46) as
133 described above. Kanamycin resistant, Ampicillin sensitive colonies were then screened by
134 colony PCR using primer pair jf112016.50/51 flanking the entire amplicon for a 4269-bp product,
135 and primers jf101716.21/22 specific to *tolC* gene (603-bp product). Primers k2/jf112016.51
136 (2,024-bp product) were then used to confirm the *tolC* gene deletion and the Km^R cassette
137 integration in the H10407 genome. To complement the *tolC* mutant, the *tolC* gene was
138 amplified from H10407 genomic DNA using primers jf120716.59/jf120716.60, and the plasmid
139 vector backbone was amplified from plasmid pBAD/myc-His B using primers
140 jf120716.52/jf120716.53. The recombinant pYZ008 complementation plasmid was assembled
141 using an adaptation of circular polymerase extension cloning (CPEC) (31) (30 s denaturation at
142 98°C, followed by 20 cycles of 10 s at 98°C, 30 s at 55°C, and 1.5 min at 72°C, and a final
143 extension for 10 min at 72°C.). Following sequence verification, pYZ008 or the pBAD/myc-His B
144 vector control plasmids were then used to transform the Δ*tolC* mutant. Complementation was
145 confirmed by PCR with primers jf101716.21/22.

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146 **FLAG-STH fusions**

147 FLAG epitope fusions were constructed to introduce the 3X FLAG sequence between the signal
148 peptide of *estH* and the beginning of the propeptide encoding region (on plasmid pFLAG^3-STH)
149 or at the 3′ end of *estH* (on pSTH-FLAG^3). The 3X FLAG fragment was 1st constructed by
150 annealing complementary synthetic oligonucleotides jf092616.9 encoding + strand bases 1-43
151 and jf092616.10 representing– strand bases 66-19 of a 66 base pair sequence encoding the 3x
152 FLAG peptide (DYKDHDGDYKDHDIDYKDDDDK).
153 To generate plasmid pFLAG^3-STh where the 3xFLAG encoding sequence was inserted between
154 the STh signal peptide and the STh propeptide, primers jf101916.33/ jf092616.4 were first used
155 to amplify the nucleotides (1-63) of *estH* encoding the signal peptide. Next primers jf092616.1/
156 jf092616.2 were used to amplify the 3xFLAG fragment from the above synthetic
157 oligonucleotide 3XFLAG template flanked by nucleotide extensions representing nucleotides
158 44-60 and 64-99 of *estH*, while primers jf092616.3/ jf101916.34 were used to amplify the 3′ end
159 of *estH* from nucleotide 64 to the native stop codon. The three fragments were fused in a single
160 PCR reaction using primers jf101916.33/jf101916.34. Next, the vector backbone was amplified
161 using primers jf101916.31/ jf101916.32 from pFLAG-CTC (Sigma), followed by final assembly of
162 pFLAG^3-STh by CPEC. Similarly, to make plasmid pSTh-FLAG^3 primers jf092616.5/ jf101916.35
163 were used to amplify 3XFLAG with a 5′ nucleotide extension representing nucleotides 197-216
164 of *estH*, while primers jf101916.33/jf092616.8 were used to amplify the *estH* gene with a 5′
165 nucleotide extension corresponding to pFLAG-CTC. The resulting amplicons were then fused in
166 a single PCR reaction using primers jf101916.33/ jf101916.35, and assembled with the pFLAG-
167 CTC backbone by CPEC.

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169 ## Cloning, expression, and purification of recombinant ST proteins peptides

170 To construct a GST-STh or mutant GST-mSTh (A14Q) expression plasmid, synthetic
171 oligonucleotides were synthesized (IDT, Coralville, Iowa) which encompassed the region of the
172 *estH* gene corresponding to the mature peptide minus the native start codon, preceded by an
173 in-frame flexible linker sequence (32). The forward sequence (jf042715.1 for STh and jf042517.1
174 for mSTh), preceded by a *BamHI* overhang sequence (GATCC) and the reverse sequence
175 (jf042715.2 for STh and jf042517.2 for mSTh), preceded by an *EcoR*I overhang sequence
176 (AATTC) were mixed in 1:1 molar ratio, heated to 95° C for 5 minutes, and cooled to room
177 temperate. The annealed double stranded 91 base pair DNA fragments were then cloned
178 directly into the pGEX-4T1 vector digested with *BamH*I and *EcoR*I, yielding plasmids pCW002
179 and pYZ011, respectively. A similar strategy was to construct a GST-STp expression plasmid
180 using forward oligonucleotide jf031915.5 and the reverse sequence jf031915.6, resulting in
181 plasmid pCW003. *E. coli* TOP10(pCW002), TOP10(pCW003), and TOP10(pYZ011) were then
182 used to express recombinant GST-STh, GST-STp, or GST-mSTh, and the resulting fusion
183 proteins were purified by affinity chromatography. In brief, the bacterial cultures were grown in
184 Luria broth at 37°C to an OD_{600} between 0.6 and 0.7, and induced with 1 mM IPTG final
185 concentration for 1-3 hours. The cell pellets were re-suspended in 30 ml cold PBS containing 5
186 mM DTT, 1 protease inhibitor tablet (Roche), and 0.1 mg/ml lysozyme. Following sonication,
187 supernatants were clarified by centrifugation at 12,000 rpm for 20 minutes at 4°C, followed by
188 passage through a 0.45 µm filter. Filtered supernatants were then loaded onto columns
189 (Glutathione Sepharose High Performance, GE Healthcare) pre-equilibrated with phosphate
190 buffered saline (PBS), pH 7.3 (140 mM NaCl, 2.7 mM KCl,10 mM Na2 HPO4, 1.8 mM KH 2 PO4,
191 pH 7.3). After washing with 20 column volumes of PBS containing 1 mM dithiothreitol (DTT),

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192 GST fusion proteins were eluted in fresh buffer containing (100 mM Tris-HCl, pH 8.0 and 10 mM
193 reduced glutathione), and dialyzed against PBS.
194
195 To liberate native STh or mutant STh (mSTh) from its GST fusion partner, GST-fusion protein
196 was dialyzed in 50 mM NH_4HCO_3 (pH 8.5), containing 1 unit of thrombin (Sigma) per mg GST-
197 fusion protein at 4°C overnight, centrifuged at 4000 x g for 20 minutes a 10 kDa MWCO
198 centrifugal filter to collect the filtrate, and dried by vacuum centrifugation. Peptide
199 concentrations were then determined by measurement of the molar extinction coefficient at
200 280 nm (Take3, BioTek).
201 Plasmids pCW002, and pCW003 have been deposited in Addgene (https://www.addgene.org/)
202 under accession numbers 90225, and 90226, respectively.

203 transcriptional analysis of *estH, estP* and *tolC* genes

204 Confluent T84 cells were inflected with early-log phase H10407 bacteria (~10^9 cfu) for 30 or 90
205 min. Total RNA was isolated from adherent and planktonic (nonadherent) bacterial fractions
206 using an RNAspin Mini Isolation Kit (GE Life Sciences, 25050070) and treated with DNase I
207 (ThermoFisher, 18068015). PCR for the *arcA* housekeeping gene was used to confirm the
208 removal of DNA. Total RNA was reverse-transcribed (SuperScript VILO cDNA Synthesis Kit,
209 ThermoFisher, 11754250). RNA transcripts were quantified by real-time PCR (Fast SYBR Green
210 Master Mix, ThermoFisher, 4385612; ViiA 7 Real-Time PCR system, Applied Biosystems).
211 Primers specific to *arcA*, *estH*, *estP*, or *tolC* gene are listed in Table 2. All transcripts were
212 normalized to *arcA*, and presented as a ratio of transcripts in adherent bacteria relative to that
213 of planktonic bacteria.

214 Production and purification of anti-ST polyclonal antibody

215 To generate rabbit polyclonal antibody which recognizes ST-H, New Zealand white rabbits
216 were immunized (Rockland) with recombinant GST-ST-H. We used lyophilized *E. coli*

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217 AAEC191A (33), and an immobilized *E. coli* lysate column (Pierce 44938) to absorb *E. coli*-
218 reactive antibodies, followed by protein G column purification (HiTrap, GE 17-0404-01). IgG
219 GST-ST-H antibodies were affinity purified using GST-STh immobilized on nitrocellulose as
220 previously described and anti-GST-reactive antibodies were removed by cross-absorption
221 against GST coupled to Glutathione Agarose Resin (Gold Bio G-250-100, St. Louis).
222 Anti-STp antibody, was purified from rabbit antisera (provided by Weiping Zhang, Kansas State
223 University) by cross-absorption against an immobilized *E. coli* lysate column and then followed
224 by affinity purification against recombinant GST-STp) immobilized on nitrocellulose
225 membranes for affinity purification.

226 Immunoprecipitation and detection of heat-stable toxins in culture supernatants

227 Purified anti-STh polyclonal IgG was immobilized with AminoLink Plus Coupling Resin (Pierce
228 Direct IP kit Thermo Scientific, 26148). Clarified culture supernatants of overnight cultures of
229 ETEC were mixed with protease inhibitor cocktail (ThermoFisher 88666), and filtered through a
230 0.45 µm filter. Sixty ml of supernatant was then filtered through 10 kDa molecular weight cutoff
231 membrane (Amicon). Filtrates were then dried by vacuum centrifugation, and dialyzed in PBS
232 against a 1 kDa-cutoff membrane (Float-A-Lyzer G2, MWCO 0.5-1 kDa, Spectrum Labs).
233 Immunoprecipitations were conducted by incubation of filtrates with anti-STh immobilized
234 resin for 2 hours at room temperature followed by elution with 4.5% acetic acid. Eluates were
235 dried by under vacuum centrifugation, and reconstituted in PBS. Immunoprecipitated samples
236 or purified protein/peptide as controls were applied directly to 0.22 µm pore-size PVDF
237 membranes (Bio-Rad), and detected by anti-STh and/or anti-STp primary antibody and anti-
238 Rabbit-HRP conjugated secondary antibody using Clarity Western ECL Substrate (Bio-Rad).
239 FLAG-STh fusion peptides were prepared from strain jf2847 carrying pFLAG^3-STh, pSTh-FLAG^3,
240 or the blank vector pFLAG-CTC. Briefly, following overnight growth at 37°C in lysogeny broth

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241 containing ampicillin (100 μg/ml), cultures were diluted 1:100 in fresh media, grown to OD_{600} of
242 ~0.2, then induced with 1 mM IPTG for 7 h. 40 ml of clarified supernatant was mixed with 200 μl
243 of anti-FLAG (M2) affinity gel (#A2220, Sigma), then incubated overnight at 4°C with agitation.
244 After washing with TBS buffer, bound proteins were eluted with 0.1M glycine-HCl, pH 3.5,
245 separated by SDS-PAGE, transferred to nitrocellulose for subsequent immunoblotting, and
246 detected by anti-FLAG antibody (#F1804, Sigma).

247 ## Confocal immunofluorescence imaging and quantification

248 To examine the delivery of FLAG-tagged STh toxin to intestinal epithelial cells, *estP/estH*
249 mutant strains with or without plasmids encoding FLAG-tagged STh were grown overnight and
250 diluted 1:50 in CAYE-ST medium, grown to OD_{600} of ~0.2, then induced with 1 mM IPTG for 2 h.
251 The bacteria were added to T84 cells at a multiplicity of infection (MOI) of ~1:50, maintaining
252 the induction with 1 mM IPTG. After infection with the bacteria, T84 cells were washed with
253 PBS, fixed with 4% paraformaldehyde for 10 min at room temperature, permeabilized with
254 0.1% TRITON X-100 for 5 min, blocked with 1.5% BSA/PBS at 37°C for 1 h. Anti-O78 rabbit
255 polyclonal antisera (Penn State University) diluted 1:300 in PBS with 0.02% Tween-20 (PBST)
256 and 1.5% BSA was used to identify H10407 and monoclonal Anti-FLAG M2 antibody (#F1804,
257 Sigma) diluted 1:500 in PBST with 1.5% BSA to detect FLAG-tagged STh. Following incubation
258 overnight at 4°C, slides were washed 3x with PBS, and incubated for 1 hour at 37°C with goat-
259 anti rabbit IgG (H&L) AlexaFluor-488 conjugate (ThermoFisher A11070) and goat-anti mouse
260 IgG (H&L) conjugated to AlexaFluor 594 (ThermoFisher A11032) at a dilution of 1:500 in PBST
261 containing 1.5% BSA. After washing, DAPI (4′, 6-diamidino-2-phenylindole) was added at 1:
262 6,000. Confocal microscopy images were acquired using a Nikon C2+ Confocal Microscope
263 System. To quantitate binding of FLAG-epitope tagged STh molecules, fluorescence detection
264 was normalized to the DAPI signal using NIS-Elements DUO software (v4.4).

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265 ### In vitro assessment of toxin delivery

266 T84 (ATCC® CCL-248™) intestinal epithelial cells were maintained in DMEM/F12 (1:1) medium
267 containing FBS (5% [vol/vol]). T84 cell monolayers were grown in 96 well plates for 24-48 hours
268 at 37°C, 5% CO2 incubator, to > 90% confluency. Cultures of bacteria were grown overnight in
269 lysogeny broth from frozen glycerol stocks in CAYE-ST medium. Phosphodiesterase inhibitors
270 vardenafil hydrochloride trihydrate (#Y0001647), rolipram (#R6520), and cilostazol (#C0737)
271 (all from Sigma-Aldrich), were each added to target T84 cells at a final concentration of 16.7 or
272 50 μM and incubated with cells for one hour. Bacteria or toxin were added to T84 monolayers
273 seeded into 96-well plates and continued the treatment for the indicated duration. After
274 washing in PBS cyclic GMP (cGMP) levels were determined by enzyme immunoassay (EIA)
275 (Arbor Assays, K020-H1) using the acetylated protocol as directed by the manufacturer.
276 To examine the capacity of antibodies to neutralize ST delivery, antibody against ST-H and/or
277 EtpA was added directly to T84 cell monolayers at the indicated dilution at the time of
278 infection. After 1.5 hours, cells were washed by PBS, and lysed, and cGMP assays were
279 performed as described above.

280

281 ## Results

282 ### contribution of ST-H, ST-P and EAST1 to activation of cGMP in target epithelial cells

283 Understanding the individual contributions of ST and ST-like molecules of ETEC is relevant to
284 development and testing of toxin neutralization strategies. H10407 encodes three peptides
285 with the potential to activate cGMP in target intestinal epithelial cells: ST-H1, ST-P, and EAST1,
286 a heat stable toxin originally identified in enteroaggregative *E. coli* (34). ST-H (ST-1b) is
287 encoded by the *estH* gene on the large 94,797 bp p948 plasmid (NCBI Genbank acccession
288 NC_017724.1). The *astA* gene which encodes EAST1 peptide (35) is imbedded within a IS1414
289 insertion sequence(36) is also located on the p948 plasmid immediately downstream from the

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290 *etpBAC* adhesin locus(27, 37). ST-P (ST-1a) is encoded by the *estP* gene on a second 66 kb
291 virulence plasmid NCBI GenBank FN649417.1 in H10407.
292 We found that deletion of *estH* resulted in appreciable decreases in cGMP production at or near
293 background levels of cGMP production by uninfected cells, and that complementation of *estH*
294 in trans restored activation of this cyclic nucleotide (Figure 1A). In contrast, the *estP* mutant was
295 not appreciably different than the wild type strain, and the introduction of the *estP* mutation to
296 the *estH* strain did not yield further measurable decreases in cGMP production by target
297 epithelial cells. However, we found that the target T84 cells used in these assays did respond to
298 GST fusions to either ST-H or ST-P (Figure 1B) suggesting that these cells have the capacity to
299 respond to either peptide. Interestingly, we found that in contrast to *estH, estP* transcription
300 was significantly influenced by bacterial cell contact with transcription of the gene encoding
301 ST-P enhanced by bacterial adhesion, as was the *tolC* gene which encodes the putative export
302 channel for ST (Figure 1C). Deletion of *astA* gene encoding EAST on the p948 plasmid did not
303 impact bacterial activation of cellular cGMP under the conditions of the assay (Figure 1D).
304

305 ## TolC is required for effective ETEC secretion of ST1 toxins

306 The precise mechanism for secretion of heat stable toxins from ETEC strains associated with
307 disease in humans is presently uncertain. Prior studies of heat-stable toxin investigated the
308 secretion STb(ST-II)(38), or STp(STIa)(39) from laboratory strains of *E. coli* containing
309 recombinant expression plasmids. While both studies suggested that the outer membrane
310 protein TolC is involved in secreting these toxins from the recombinant *E. coli* background,
311 there is conflicting data regarding the involvement of the STb (STII) toxin in human disease(40,
312 41), and unlike the ST1 toxins, STb does not bind to guanylate cyclase C. Similarly, to our
313 knowledge, there has been no verification of the role of *tolC* in mediating the secretion of either

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314 of the ST1 toxins (STh and STp) from strains of ETEC isolated from humans. Therefore, to verify
315 the importance of the TolC in secretion of both STh (ST1b) and STp(ST1a) from ETEC which
316 cause human illness, we constructed an isogenic *tolC* mutant in the ETEC H10407 strain, and
317 tested the ability of the mutant bacteria to deliver ST to target epithelial cells.
318 We found that mutants lacking *tolC* were markedly deficient in their ability to deliver ST toxins
319 to epithelial monolayers as we observed only background levels of cGMP production following
320 infection with the *tolC* mutant strain, and complementation with *tolC in trans* restored the
321 ability of the bacteria to provoke a cGMP response in targeted cells (Figure 2).

322 Optimal delivery of ST requires the EtpA adhesin.

323 We have previously demonstrated that intimate interaction of ETEC with intestinal epithelial
324 cells is essential for efficient delivery of heat labile toxin to intestinal epithelial cells(42).
325 Moreover, delivery of LT requires the concerted action of several ETEC adhesins with different
326 receptor specificities. To examine the dependence of ST delivery on bacterial adhesion we
327 compared cGMP activation of target intestinal epithelial cells by wild type ETEC to a mutant
328 strain lacking EtpA, a plasmid-encoded adhesin, expressed by a diverse population of ETEC(43).
329 These studies demonstrated that cGMP activation in target epithelia by wild type bacteria was
330 significantly accelerated relative to the *etpA* mutant (Figure 3) suggesting that efficient delivery
331 of these small peptides also requires effective bacterial-host interactions.

332 Anti-toxin and anti-adhesin antibodies mitigate delivery of heat-stable toxins

333 ETEC delivery of heat labile toxin can be effectively blocked by antibodies directed against
334 either LT or the EtpA adhesin molecule(44). These data suggest that anti-adhesin and anti-
335 toxin strategies could provide complementary approaches to vaccine development. The *etpA*
336 gene, like those encoding LT, and ST1 molecules appears to be highly conserved within the

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337 ETEC pathovar(43). Therefore, we examined the ability of antibodies against the EtpA adhesin
338 to inhibit activation of cGMP in target intestinal cells.
339 Antibodies directed against either the EtpA adhesin molecule or STh significantly inhibited the
340 delivery of heat-stable toxins to target cells (Figure 4). Although we were not able to
341 demonstrate that the combination of these antibodies was synergistic, these data add
342 additional support to the concept that EtpA could be useful as a target to engender coverage
343 against a wide variety of ETEC isolates that produce heat-stable and/or heat-labile
344 enterotoxins(43, 45).

345 Delivery and secretion of epitope-tagged ST-H

346 Understanding the nature of ST secretion and its delivery to epithelial receptors could be
347 relevant to informed development of ST toxoid molecules. ST-H is synthesized as a 72 amino
348 acid molecule that includes a 19 amino acid signal peptide, followed by a 34 amino acid pro-
349 peptide, and a mature ST molecule of 19 amino acids. Although most attempts to develop ST
350 toxoids have targeted the mature peptides, there are earlier, but conflicting data regarding the
351 precise form of ST that is secreted into the extracellular milieu (15, 46), with some data
352 suggesting that the pro-peptide may be exported with subsequent processing to the mature
353 peptide outside the bacteria (47). To investigate the potential secretion of the pro-peptide, we
354 engineered 3x-FLAG-epitope fusions to the amino terminal end of the pro-peptide region
355 ($FLAG^3$-pro-ST-H) of ST-H and compared the export, and delivery of the resulting peptides to
356 carboxy-terminal fusions to the mature peptide (pro-ST-H-$FLAG^3$, schematic, figure 5).
357 We were able to recover either the amino-terminal or carboxy-terminal FLAG-tagged
358 molecules from culture supernatants of *estH/estP* mutant ETEC transformed with the pST-
359 $FLAG^3$ or p$FLAG^3$-STH plasmids, respectively (figure 5a). Both molecules appeared to yield
360 functional ST mature peptides as either plasmid was sufficient to complement the ability of
361 *estH/estP* to activate cGMP in target epithelial cells (figure 5b). Likewise, we were able to

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362 demonstrate binding of FLAG-epitope tagged molecules to target epithelial cells following
363 infection with either of the complemented strains (figure 5c, d). Collectively, these data appear
364 to reaffirm earlier observations of STA3 (ST-H)(47) suggesting that both the mature form (19
365 amino acids) of the peptide and the extended Pro-ST-H (53 amino acids) may be found outside
366 the bacteria.
367
368

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369 **Discussion**

370 Because of the significant global burden of diarrheal illness caused by ETEC, these pathogens
371 have been a target for vaccine development since they were first identified as a cause of severe
372 diarrheal illness more than four decades ago. Currently, there is no vaccine available that
373 affords broad-based protection against ETEC, in part due the substantial antigenic diversity
374 within the pathovar, and limited mechanistic insight into immunologic correlates of protection
375 that appears to follow early infections among young children endemic areas. Moreover, many
376 features of the molecular pathogenesis of these common pathogens have not been explored in
377 sufficient detail to inform vaccine development.

378 Because ST-producing ETEC comprise a large proportion of strains associated with
379 symptomatic diarrheal illness(48), we investigated the molecular contributions of the known
380 heat-stable toxins, their proposed secretion apparatus, and bacterial adhesion to toxin delivery.
381 The present studies suggest that efficient delivery of ST toxins is a complex process that
382 requires the ability to directly engage host cells and at least transient adhesion afforded by
383 EtpA and other adhesins(49). Moreover, as antibodies directed at either the EtpA adhesin or ST
384 effectively impaired toxin delivery, our studies provide further support for development of a
385 vaccine platform that combines ST-toxoid(24) and anti-adhesin approaches.

386 Interestingly, clinical studies have suggested that either ST-H originally identified in humans
387 and ST-P can cause diarrheal illness in humans(23). In keeping with these observations, we
388 found no difference in the ability of ST-H or ST-P peptides to elicit cGMP activation(18) in
389 target epithelia. Although deletion of the gene encoding ST-H resulted in lower cGMP
390 activation in infected monolayers than when the ST-P gene was deleted from the ETEC H10407
391 strain, we cannot exclude the possibility of a compensatory increase in ST-H secretion in the
392 absence of a potentially competing peptide. Indeed, deletion of the *tolC* gene resulted in

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393 complete abrogation of cGMP activation in target epithelial cells, similar to deletion of both of
394 the ST1 toxin genes from H10407.
395 Earlier studies also noted that many ETEC strains including H10407 bear copies of an insertion
396 sequence(36) that encompass the *astA* gene encoding EAST1(35), a cGMP activating peptide
397 structurally similar to ST1 that was originally identified as a heat-stable enterotoxin in
398 enteroaggregative *E. coli(34).* However, we saw no appreciable decrease in cGMP in target
399 epithelial cells following infection with the *astA* mutant, compared to wild type ETEC. We
400 cannot rule out the possibility of EAST1 expression from additional copies of *astA* not residing
401 on the 92 MDa virulence plasmid, or that EAST1 is not optimally expressed in *vitro*. However,
402 the complete absence of a cGMP response in cells infected with the ST-1a/ST-1b deletion
403 mutant might alternatively suggest that EAST1 does not contribute to ETEC virulence and that
404 further toxoid vaccine development can simply focus on engendering neutralizing antibody
405 responses to the established ST1 and LT enterotoxins.
406 The reaffirmation of TolC as a key mechanism for export of ST1 toxins could be relevant to
407 management of diarrheal illness. The rapid emergence of multi-drug resistance in the
408 Enterobacteriaciae that is in part dependent on drug efflux through TolC has stimulated
409 interest in efflux inhibitors to enhance the potency of available antimicrobial agents(50-52).
410 Theoretically, these inhibitors could offer novel therapeutic agents for treatment of ETEC.
411 Our studies also revisit the concept that the larger pro-peptide form of ST-H may be exported
412 by the bacteria, and the data presented here are consistent with prior observations suggesting
413 that some processing of the pro-peptide occurs outside the bacteria(15, 47). Further study will
414 be needed however to determine whether the pro-peptide sequence contributes to
415 immunologic recognition of ST following infection and whether these larger molecules might
416 be exploited in the development of improved toxoids to neutralize ST.

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418
419 ## Figure legends
420

421 **Figure 1. relative contribution of STh, STp, and EAST to cGMP production in target epithelia.**

422 **A.** cGMP activation of T84 target epithelial cells following infection with wild type *estH*,
423 complemented *estH* mutant *estH*(pQL231), *estP*, the complemented *estP* mutant
424 *estP*(pQL230), and the *estH/estP* dual deletion mutant. ø=uninfected cells. **B.** cGMP production
425 following the addition of GST, GST-STh, or GST-STp fusions. Numbers on the x-axis represent
426 final concentration of protein in µg/ml. ø=untreated cells. **C.** transcription of genes encoding ST
427 toxins ST-H (*estH*), ST-P (*estP*) and TolC. Data are normalized relative to the *arcA* housekeeping
428 gene, and represent the ratio of transcripts in attached to planktonic bacteria at 30 and 90
429 minutes after infection of monolayers. Whisker plots represent the range of data obtained over
430 six replicates from two independent experiments. Horizontal lines represent mean values. **D.**
431 Activation of cGMP in T-84 cells after addition of wild type H10407, the *astA* mutant or the dual
432 *estH/estP* mutant. Data for each group represent mean of n=3 replicates ± SEM.

433
434

435 **Figure 2 role of *tolC* in ST1 toxin secretion and delivery to target epithelial cells.**

436 **A.** Immunoblot detection of ST1 toxins in culture supernatants of the wild type H10407 strain,
437 *tolC* mutant, or *estH/estP* dual deletion mutant following immunoprecipitation of culture
438 supernatants with affinity-purified anti-STh antibodies (IP α-STh). BSA and GST-STh fusion are
439 shown as negative and positive controls, respectively. **B.** cGMP production by T84 target
440 epithelial cells following infection with wild type (wt) ETEC strain H10407 (ST1a, ST1b), the *tolC*
441 mutant, the vector complemented mutant (pBAD-Myc-HisB) or the *tolC*-complemented
442 mutant (pZY008).

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444 **Figure 3 The EtpA adhesin is required for optimal delivery of heat stable toxins**

445 Shown are comparisons of cGMP activation in target T84 intestinal epithelial cells following
446 infection with wild type ETEC H10407, or the *etpA* mutant (n=4 replicates). The *estH/estP* dual
447 deletion mutant and uninfected monolayers (n=3). Dashed lines in each group connect
448 geometric mean values obtained at 30, and 60 minutes following the addition of bacteria. *
449 represents $p<0.05$ obtained by Mann-Whitney, (two tailed) comparisons.

450
451

452 **Figure 4. antitoxin and anti-adhesin antibodies inhibit heat-stable toxin delivery**
453 Shown are monolayers infected with the dual heat-stable toxin mutant (*estH/estP*), wild type
454 H10407, or uninfected control monolayers (ø). Dotted horizontal lines for each group represent
455 geometric means. (*=0.028) by Mann Whitney two-tailed nonparametric comparisons.

456
457

458 **Figure 5. secretion and delivery of FLAG-epitope tagged ST-H.**
459 Schematic at top depicts the location of the region encoding the 3xFLAG peptide relative to the
460 *estH* gene. The arrow at top shows the location of the predicted signal peptide cleavage site
461 which is retained in both constructs. **a.** anti-FLAG and anti-STH immunoblots of FLAG^3-STH
462 and STH- FLAG^3 peptides recovered by anti-FLAG immunoprecipitation from culture
463 supernatants of the ETEC *estH/estP* mutant bearing the pFLAG^3-STH and pSTH- FLAG^3. **b.**
464 complementation of *estP/estH* mutant strain with pSTH- FLAG^3 or pFLAG^3-STH restores
465 toxicity upon infection of T84 cells. **c.** quantitation of FLAG^3 -tagged ST delivered to epithelial
466 cells by *estH/estP* bacteria complemented with pSTH- FLAG^3 or pFLAG^3-STH. Values represent
467 fluorescence intensity per field. **d.** Confocal immunofluorescence images of bacteria expressing
468 pST-FLAG^3 (anti-O78, yellow) attached to T84 cells (nuclei, blue) and the distribution of FLAG
469 epitope-tagged toxin (cyan).

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471 # tables

472 ## table 1

table 1 bacterial strains and plasmids used in these studies		
Strain or plasmid	Description	Source or reference(s)
Strains		
H10407	Wild-type ETEC strain O78:H11; CFA/1; LT, ST-H, ST-P ; EtpA	(27, 37, 53)
jf2656	H10407 derivative with isogenic deletion of *estP*	This study
jf2649	H10407 derivative with isogenic deletion of *estH*	This study
jf2847	H10407 derivative with isogenic deletion of *estP* and *estH*	This study
jf3038	*deletion of astA* in on the large p948 virulence plasmid of H10407; Km^R	this study
jf3081	jf2649 complemented with pQL231	this study
JW5503-1	*E. coli* K-12 in-frame ΔtolC732:: Km^R	(30)
jf4652	H010407 derivative with tolC::Km^R mutation	this study
jf4709	jf4652 with pBAD/mycHisB	this study
jf4712	jf4652 complemented with pYZ008	this study
jf4644	jf2847 with pFLAG-CTC, Cm^R Km^R Amp^R	this study
jf4648	jf2847 with pFLAG-STh, Cm^R Km^R Amp^R	this study
jf4651	jf2847 with pSTh-FLAG, Cm^R Km^R Amp^R	this study
AAEC191A	afimbriate *E. coli* K-12 derivative	(33)
Plasmids		
pBAD/mycHisA	pBR322ori; PBAD; Amp^R; *araC;* C-terminal *myc* epitope tag. Arabinose inducible expression plasmid	Invitrogen
pBAD/mycHisB	pBR322ori; PBAD; Amp^R; *araC;* C-terminal *myc* epitope tag. Arabinose inducible expression plasmid	Invitrogen
pKD13	oriRSKγ Tn5 neomycin phosphotransferase (Km^R), FRT, ß-lactamase (Amp^R)	(28)
pKD46	lambda red recombinase helper plasmid	(28)
pSMD002	*estP* cloned into *XhoI* site of pBAD/myc-HisA in frame with myc-His tags	this study
pACYC184	p15Aori, Cm^R, Tc^R	(54)
pSMD001	*estH* cloned into *EcoRV* site of pACYC184	this study
pGPS4	oriRSKγ Cm^R, Tc^R	NEB
pETDuet-1	pBR322ori; *lacI*; Amp^R;IPTG inducible expression plasmid	Novagen
pQL230	estP cloned into BglII & XhoI sites of pETDeut-1	
pQL231	estH cloned into *NcoI* & *EcoRI* sites of pETDeut-1	
pQL238	estP cloned into *BglII* & *XhoI* sites and *estH* cloned into *NcoI* & *EcoRI* sites of pETDeut-1	
pGEX-4T1	*lacI^q*, Amp^R, expression plasmid for N-terminal GST fusions	GE Healthcare Life Sciences
pCW002[a]	5047 bp GST-STh expression plasmid, Amp^R	this study
pCW003[b]	*estP* cloned into BamHI & EcoRI sites of pGEX	this study
pYZ011[c]	5047 bp GST-mSTh expression plasmid for mSTh (A14Q), Amp^R	this study
pYZ008	H10407 *tolC* cloned into pBAD/mycHisB	this study
pFLAG-CTC	5.3 Kb plasmid, expressing C-terminal FLAG fusion proteins, Amp^R	Sigma
pFLAG^3-STh	5609 bp Flag-Pro-STh expression plasmid, Amp^R	this study
pSTh-FLAG^3	5612 bp Pro-STh-Flag expression plasmid, Amp^R	this study

Km^R:kanamycin resistance; Tc^R : tetracycline resistance; Cm^R: chloramphenicol resistance; Amp^R:ampicillin resistance; NEB: New England Biolabs;
[a]addgene accession number 90225: https://www.addgene.org/90225/
[b]addgene accession number 90226: https://www.addgene.org/90226/
[c]addgene accession number 110570: https://www.addgene.org/110570/

473

474 ## Table 2: primers used in these studies

Primer	Sequence (5'-3')	Description
jf070212.1	ATGAAAAAATCAATATTATTTATTTTTCTTTCTGTAG TGTAGGCTGGAGCTGCTTCG	Forward primer for amplifying estH flanking from pKD13
jf070212.2	TTAATAGCACCCGGTACAAGCAGGATTACAACACA	Reverse primer for amplifying estH flanking from

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	AATTCCGGGGATCCGTCGACC	pKD13
jf070212.3	ATGAAAAAGCTAATGTTGGCAATTTTTATTTCTGTA GTGTAGGCTGGAGCTGCTTC	Forward primer for amplifying estP flanking from pKD13
jf070212.4	TTAATAACATCCAGCACAGGCAGGATTACAACAAA GATTCCGGGGATCCGTCGACC	Reverse primer for amplifying estP flanking from pKD13
jf071612.1	GGCGCACACAAATATAAAG	368 bp amplicon in H10407-*estH* downstream primer
jf071612.2	AGCGGAGAGTATAGTATGA	estH Upstream
jf071612.3	AAAACCAGATAGCCAGAC	168bp upstream from estP on H10407 plasmid p666
k2	CGG TGC CCT GAA TGA ACT GC	primer binding Km^R cassette for confirming estP and tolC deletion
jf072312.1	ATGAAAAAATCAATATTATTTATTTTTCTTTCTGTAC CCTGTTATCCCTAGATTT	Forward primer for amplifying estH from pGPS4
jf072312.2	TTAATAGCACCCGGTACAAGCAGGATTACAACACA ATAACGGTCCTAAGGTAGC	Reverse primer for amplifying estH from pGPS4
jf072412.3	TGGATCCGAGCTCGAGATGAAAAAGCTAATGTTG	pBAD_Myc-hisA_XHOI_ST-P Forward primer for in-fusion cloning
jf060614.1	AGCTGCAGATCTCGAG TTAATAACATCCAGCACA	for ST-P cloning to pBAD/mycHisA, pair to jf072412.3
jf072412.5	GGCCTCTTGCGGGATATCTAAATGAAAAAATCAATA TTA	pACYC184_ECORV_TAA_ST-H forward primer for in-fusion cloning
jf072412.6	GTCGGAATGGACGATATCTTAATAGCACCCGGTAC A	pACYC184_ECORV_TAA_ST-H reverse primer for in-fusion cloning
jf060614.2	AGGAGATATACCATGGATGAAAAAATCAATATTA	forward primer for ST-H in NcoI&EcoRI sites on pETDuet-1 vector
jf060614.3	CGCCGAGCTCGAATTCTTAATAGCACCCGGTACA	reverse primer for ST-H in NcoI&EcoRI sites on pETDuet-1 vector
jf060614.4	TACATATGGCAGATCTATGAAAAAGCTAATGTTG	forward primer for ST-P in BglII&XhoI sites on pETDuet-1 vector
jf060614.5	CTTTACCAGACTCGAGTTAATAACATCCAGCACA	reverse primer for ST-P in BglII&XhoI sites on pETDuet-1 vector
jf042715.1	GATCCGATCCCCGGGTACCGAGCTCGAATAGTAGC AATTACTGCTGTGAATTGTGTTGTAATCCTGCTTGT ACCGGGTGCTATTGAG	5' Mature estH gene with BamHI, linker, gene, stop codon
jf042715.2	AATTCTCAATAGCACCCGGTACAAGCAGGATTACAA CACAATTCACAGCAGTAATTGCTACTATTCGAGCTC GGTACCCGGGGATCG	3' Mature estH gene with Linker, gene, stop codon, EcoRI
jf042517.1	GATCCGATCCCCGGGTACCGAGCTCGAATAGTAGC AATTACTGCTGTGAATTGTGTTGTAATCCTcaaTGTA CCGGGTGCTATTGAG	5' mutant estH gene with BamHI, linker, gene, stop codon
jf042517.2	AATTCTCAATAGCACCCGGTACAttgAGGATTACAAC ACAATTCACAGCAGTAATTGCTACTATTCGAGCTCG GTACCCGGGGATCG	3' mutant estH gene with Linker, gene, stop codon, EcoRI
jf031915.5	GATCCGATCCCCGGGTACCGAGCTCGAACACATTTT ACTGCTGTGAACTTTGTTGTAATCCTGCCTGTGCTG GATGTTATTGAG	5' Mature estP gene with BamHI, linker, gene
jf031915.6	AATTCTCAATAACATCCAGCACAGGCAGGATTACAA CAAAGTTCACAGCAGTAAAATGTGTTCGAGCTCGG TACCCGGGGATCG	3' Mature estP gene with Linker, gene, EcoRI
jf021915.1	CGCTTACAGACAAGCTGTG	Reverse sequencing primer that binds 70 bp downstream of the pGEX cloning site
jf021915.2	CCAGCAAGTATATAGCATGG	Forward sequencing primer that binds 117 bp upstream of pGEX cloning site
jf110716.38	CGGGCGCAGTCTGTTCTATTG	Forward primer for amplifying 1027 bp left flank segment of H10407 *tolC*
jf110716.43	TCATTTGCATTCCTTGTGGTGAAGCAGTATTTAGCG C	Reverse primer for amplifying 1027 bp left flank segment of H10407 *tolC*
jf110716.44	AAAGGGTTATGTGTAGGCTGGAGCTGCTTCG	Reverse primer for amplifying Km^R cassette from strain JQ5503-1
jf110716.45	CTTCACCACAAGGAATGCAAATGATTCCGGGGATCC	Forward primer for amplifying Km^R cassette from strain JQ5503-1
jf110716.39	CTTTTCAACCTGGGCGAGGG	Reverse primer for amplifying 985 bp right flank segment of H10407 *tolC*
jf110716.46	CCTACACATAACCCTTTCCGTAACTGATGACGACGA	Forward primer for amplifying 985 bp right flank

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	CGGGGCTTCGG	segment of H10407 *tolC*
jf101716.21	CGATCGTGATGCTGCCTTTG	603 bp amplicon in H10407-*tolC* forward primer
jf101716.22	AGCGACAGGTTGCGTTTTTC	603 bp amplicon in H10407-*tolC* downstream primer
jf112016.50	ATTTGCCATTGCTCACCAATAAAC	forward primer binding 2 kb upstream of H10407 *tolC*
jf112016.51	CTTGCAGACTGTTAAACTGGTCG	reverse primer binding 2 kb downstream of H10407 *tolC*
jf120716.52	GAACAAAAACTCATCTCAGAAGAGGATCTGAATAGCG	Forward primer to amplify 4036 bp of pBAD/myc-His B
jf120716.53	GGTTAATTCCTCCTGTTAGCCCAAAAAACGG	Reverse primer to amplify 4036 bp of pBAD/myc-His B
jf120716.59	GGCTAACAGGAGGAATTAACCATGCAAATGAAGAAATTGCTCCCCATTCT	Forward primer H10407 *tolC* gene
jf120716.60	CTCTTCTGAGATGAGTTTTTGTTCTCAGTTACGGAAAGGGTTATGACCGTTACT	Reverse primer H10407 *tolC* gene
jf092616.1	TTTCACCTTTCGCTCAGGATTACAAAGACCAC	forward bp 44-60 of *estH*-5'3XFLAG
jf092616.2	GAAGACCCTGCTGGTTTAGCCTTGTCATCGTC	reverse bp 83-64 of *estH*-3'3XFLAG
jf092616.3	GCTAAACCAGCAGGGTCTTCAAAAGAAAAAATTACA	Forward primer bp 64-99 of *estH*
jf092616.4	ATCCTGAGCGAAAGGTGAAAAAGATAATACAGAAAGA	reverse primer bp 63-27 of *estH*
jf092616.5	CTGCTTGTACCGGGTGCTATGATTACAAAGAC	Forward primer bp 197-216 of *estH*-5'3XFLAG
jf092616.8	ATAGCACCCGGTACAAGCAGGATTACA	Reverse primer representing bp 216-190 of estH
jf092616.9	GATTACAAAGACCACGATGGTGACTATAAAGACCATGATATCG	bp 1-43 and 66 through 19 of the 3X FLAG encoding sequence.
jf092616.10	CTTGTCATCGTCGTCTTTATAATCGATATCATGGTCTTTATAGTCACC	
jf101916.31	CAGATCTGGTACCCGGGAATTCT	For amplifying pFLAG-CTC vector backbone
jf101916.32	TGAAGATCGATCTCTCGATCGAGTGA	
jf101916.33	ATTCCCGGGTACCAGATCTGATGAAAAAATCAATATTATTTATTTTTCTTTCTGTATT	forward primer beginning with pFLAG-CTC-sequence followed by (*estH* sequence bp 1-38)
jf101916.34	GATCGAGAGATCGATCTTCATTAATAGCACCCGGTACAAGCAGG	reverse primer beginning with pFLAG-CTC sequence followed by 3'estH native reverse sequence (bp 219-196).
jf101916.35	GATCGAGAGATCGATCTTCATTACTTGTCATCGTCGTCTTTATAATCGATATCATG	reverse primer beginning with pFLAG-CTC-sequence followed by bases 69-34 of the 3xFLAG sequence.
jf092313.5	TCTTTCCCCTCTTTTAGTCAG	*estP* RT-PCR forward primer
jf092313.6	ACAGGCAGGATTACAACAAAG	*estP* RT-PCR reverse primer
jf092313.7	TACAAGCAGGATTACAACAC	*estH* RT-PCR forward primer
jf092313.8	AGTGGTCCTGAAAGCATG	*estH* RT-PCR reverse primer
jf092210.1	ATCAATCTGCCGGGTAAGAACGGT	*arcA* RT-PCR forward primer
jf092210.2	TCCAGATCACCGCAGAAGCGATAA	*arcA* RT-PCR reverse primer

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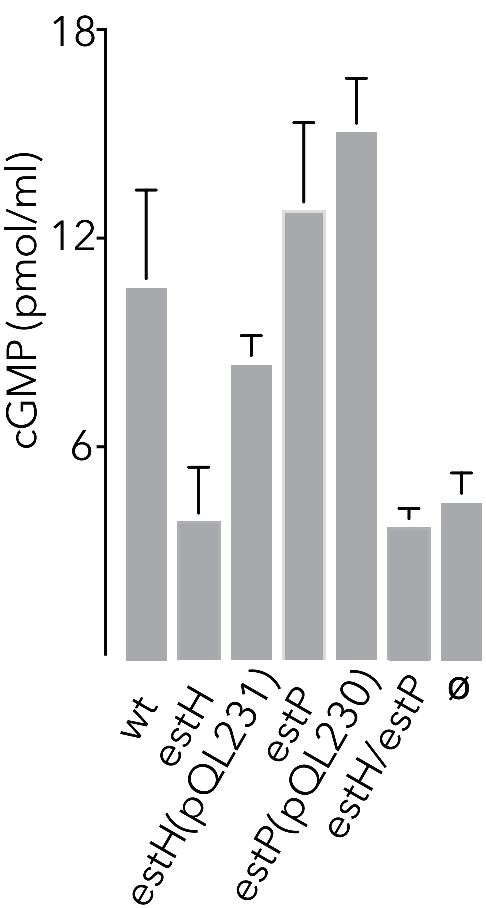
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estH
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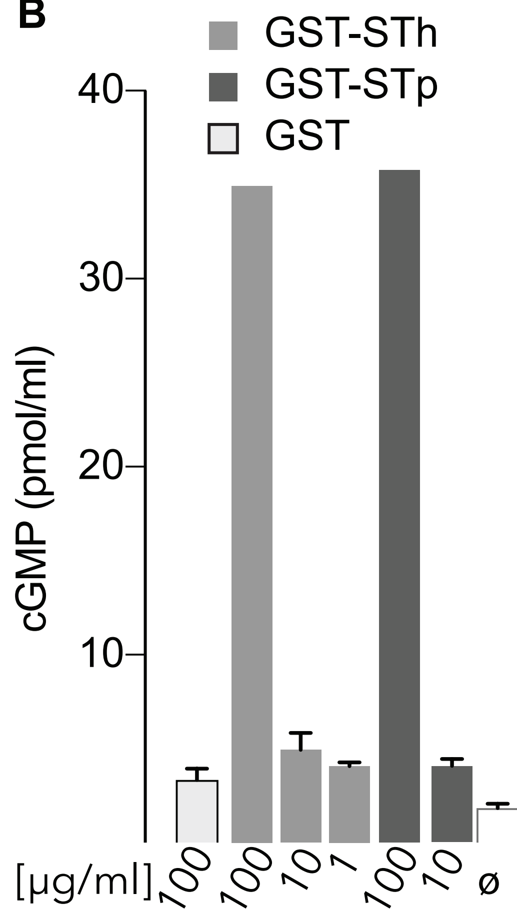
estP
estP(pQL230)
estH/estP
ø


B
GST-STh
GST-STp
GST
cGMP (pmol/ml)
40
30
20
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[µg/ml]
100
100
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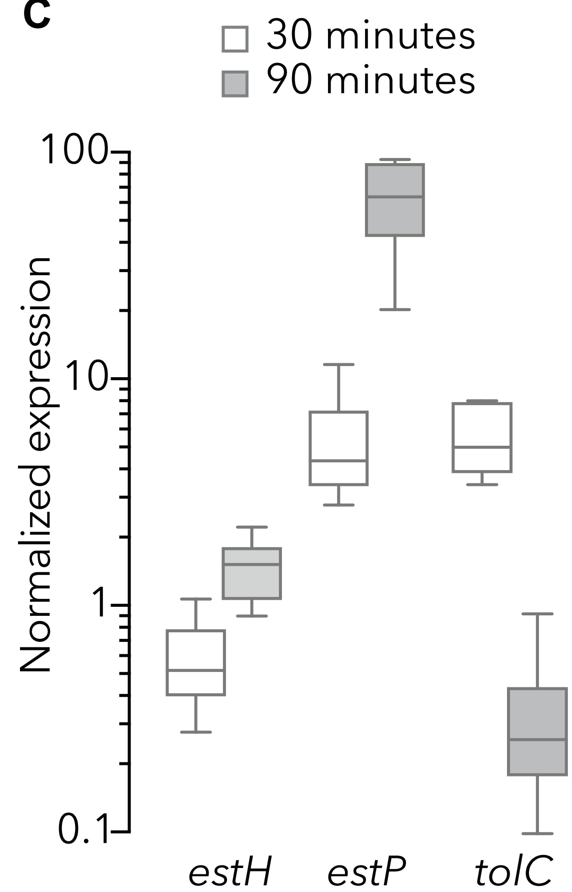
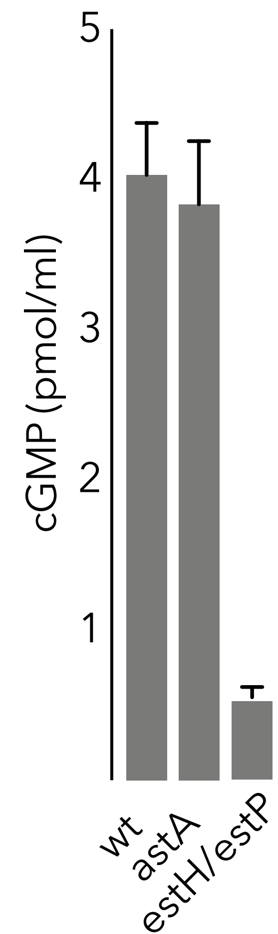

C
30 minutes
90 minutes
Normalized expression
100
10
1
0.1
estH
estP
tolC


D
cGMP (pmol/ml)
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4
3
2
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wt
astA
estH/estP

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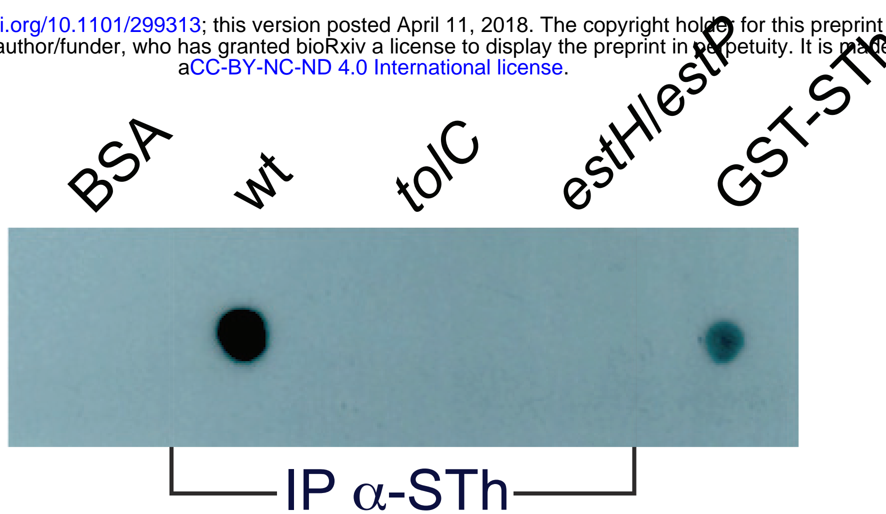

B

30
20
10
0
cGMP(pmol/ml)

PDE-I
+
-

WT
Δ*tolC*
Δ*tolC* (pBAD-Myc-His B)
Δ*tolC* (pYZ008)

Strain

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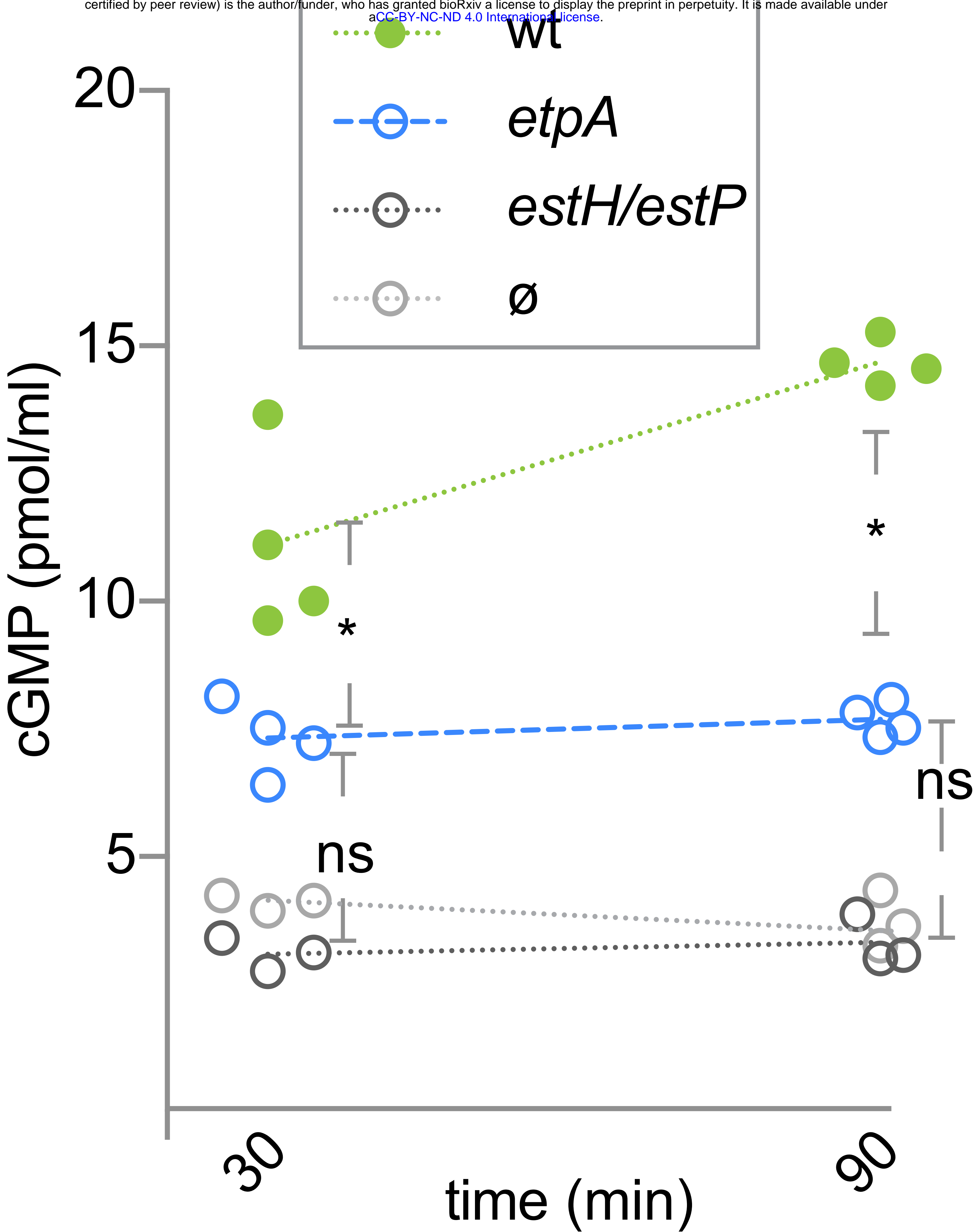

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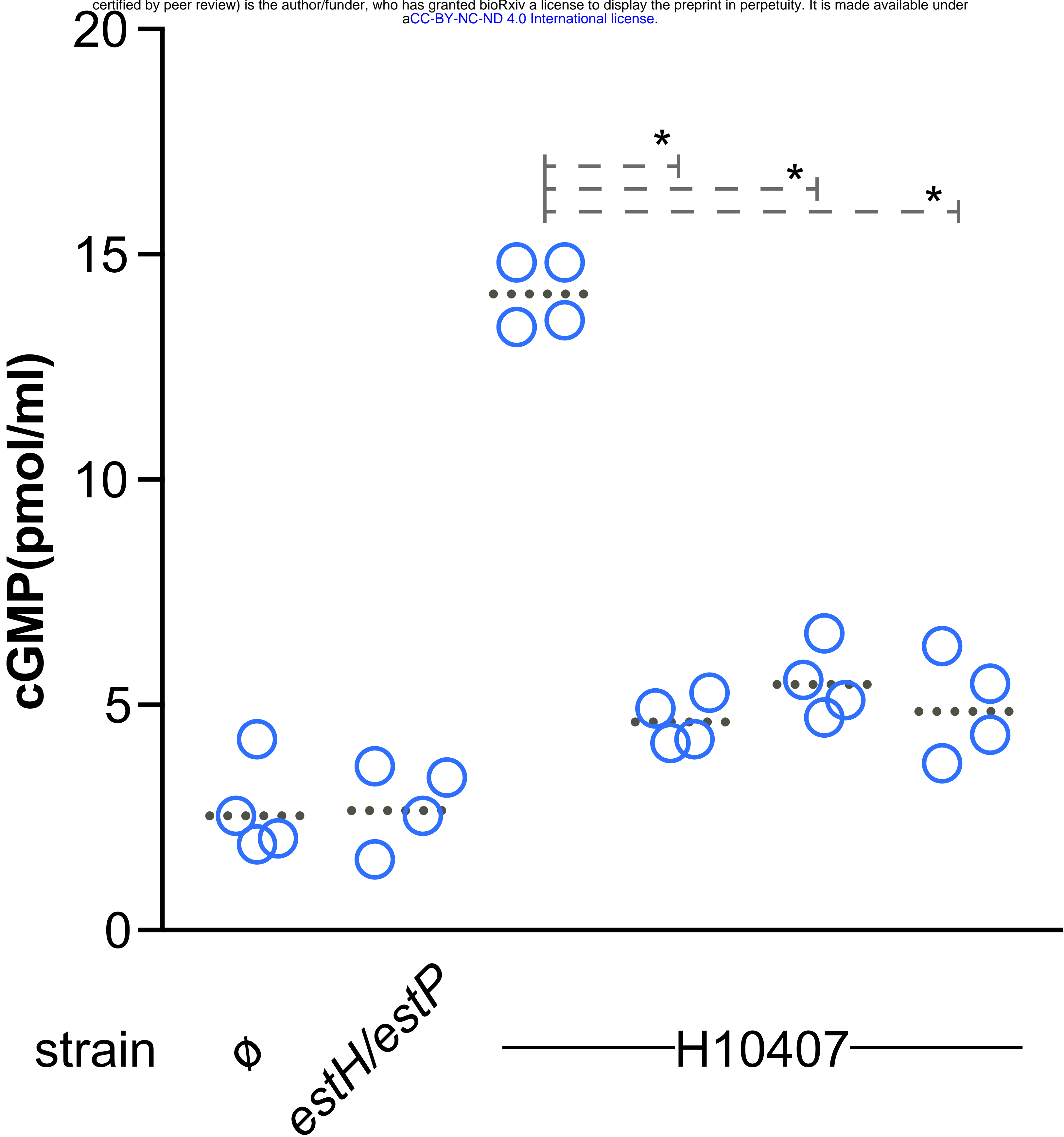
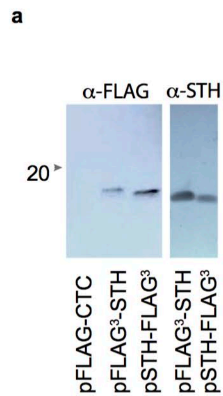
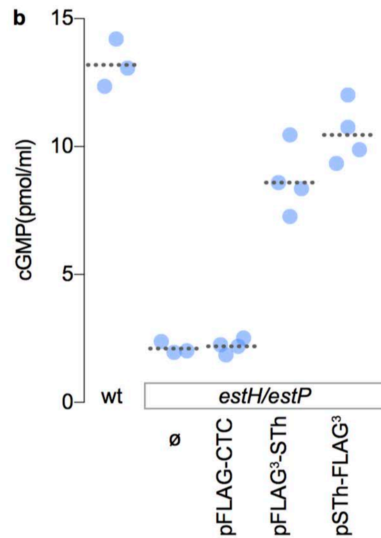
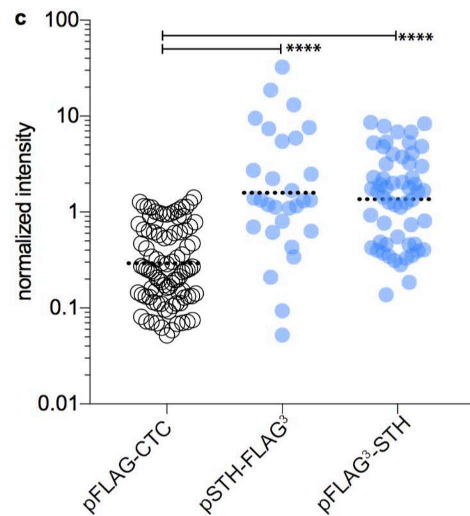


antibody						
αSTh	-	-	-	+	-	+
αEtpA	-	-	-	-	+	+







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αO78

αFLAG

DAPI

merge

STh-FLAG^3

merge

FLAG^{3}STh