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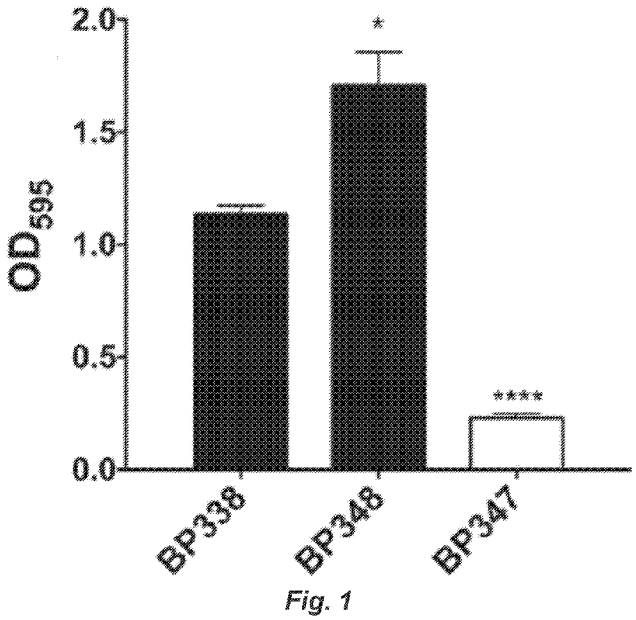

Fig. 1

(57) Abstract: Provided are compositions that include an effective amount of a peptide or polypeptide derived from a *Bordetella* ACT AC domain, optionally wherein the peptide or polypeptide is 80-100% identical to an amino acid sequence as set forth in SEQ ID NOs: 1 -5 and 44-53. Also provided are methods of using the same for preventing and/or treating a diseases, disorders, and conditions associated with the presence and/or development of biofilm; and for reducing the incidence of nosocomial infections; for inhibiting biofilm development and/or for reducing or eliminating biofilm present on medical, dental, and industrial surfaces.

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DESCRIPTION

COMPOSITIONS FOR INHIBITING FORMATION OF AND/OR DISRUPTING BACTERIAL BIOFILMS AND METHODS OF USE THEREFOR

CROSS-REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. Provisional Patent Application Serial No. 62/318,158, filed April 4, 2016, the disclosure of which is incorporated herein by reference in its entirety.

GRANT STATEMENT

This invention was made with government support under Grant Nos. AI018000 and AI007046 awarded by the National Institutes of Health. The government has certain rights in the invention.

TECHNICAL FIELD

The presently disclosed subject matter relates to compositions and methods for inhibiting the formation bacterial biofilms, disrupting bacterial biofilms, and methods of use therefor. In some embodiments, the compositions and methods described herein are used to prevent and/or treat diseases, disorders, and conditions associated with the formation and/or presence of bacterial biofilms, and in some embodiments the compositions and methods described herein are used to prevent the formation of and/or disrupt bacterial biofilms formed in various settings including but not limited to industrial settings and medical settings, thereby reducing or eliminating one or more detrimental effects associated with the presence of bacterial biofilms.

BACKGROUND

Biofilms are communities of surface-associated bacteria, encased in a matrix of polysaccharides, eDNA, and proteins. In nature, bacteria are more frequently found in biofilm structures than they are isolated as individual, free-floating organisms in settings other than the laboratory. These biofilms can lead to serious medical problems in humans, and it is estimated that more than two thirds of all infections of bacterial origin are associated with biofilm. In addition to its role in human infections, biofilm can cause substantial environmental and industrial problems, by clogging water pipelines, disrupting processes in sewage treatment plants, recycling plants, paper pulping plants, and even in oil pipelines. The

removal of biofilm is a serious issue across many fields, and although treatments are available to remove biofilm, which include abrasive mechanical disruption coupled with harsh chemical treatments, these are not feasible for all types of biofilm. A major goal of medical and industrial biofilm research is to prevent biofilm formation from occurring in the first place, and for this, broad-spectrum biofilm inhibitors are needed.

SUMMARY

This Summary lists several embodiments of the presently disclosed subject matter, and in many cases lists variations and permutations of these embodiments. This Summary is merely exemplary of the numerous and varied embodiments. Mention of one or more representative features of a given embodiment is likewise exemplary. Such an embodiment can typically exist with or without the feature(s) mentioned; likewise, those features can be applied to other embodiments of the presently disclosed subject matter, whether listed in this Summary or not. To avoid excessive repetition, this Summary does not list or suggest all possible combinations of such features.

The presently disclosed subject matter provides in some embodiments a composition for inhibiting bacterial biofilm development and/or for reducing or eliminating a bacterial biofilm present on a surface. In some embodiments, the composition comprises an effective amount of a peptide or polypeptide derived from Adenylate Cyclase Toxin (ACT) of *Bordetella* or a catalytic domain (AC domain) thereof, optionally wherein the peptide or polypeptide comprising an amino acid sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to an amino acid sequence selected from the group consisting of SEQ ID NOs: 1-5 and 44-53, optionally wherein the percent identity exists over the full length of one of SEQ ID NOs: 1-5 and 44-53. In some embodiments, the peptide or polypeptide comprises, consists essentially of, or consists of an amino acid sequence selected from the group consisting of SEQ ID NOs: 1-5 and 44-53.

In some embodiments, the composition is a pharmaceutical composition comprising or consisting essentially of the peptide or polypeptide and one or more pharmaceutically acceptable excipients and/or carriers. In some embodiments, the composition comprises a delivery vehicle, optionally wherein the peptide or

polypeptide is associated with, conjugated to, and/or encapsulated by a delivery vehicle. In some embodiments, the delivery vehicle comprises a liposome, a microparticle, or a nanoparticle, optionally wherein the liposome, microparticle, or nanoparticle is designed to be biodegradable in a subject. In some embodiments, the one or more pharmaceutically acceptable excipients and/or carriers are pharmaceutically acceptable for use in a human. In some embodiments, the pharmaceutical composition is formulated for oral administration, intravenous administration, intramuscular administration, intrathecal administration, cutaneous administration, topical administration, transdermal administration, systemic administration, subcutaneous administration, sublingual administration, buccal administration, ocular administration, otic administration, nasal administration, inhalation, nebulization, or any combination thereof.

In some embodiments, the bacterial biofilm comprises a strain of bacteria selected from the group consisting of *Bordetella spp.*, optionally *Bordetella pertussis* or *Bordetella bronchiseptica*; *Salmonella spp.*, optionally *Salmonella typhimurium*; *Pseudomonas sp.*, optionally *Pseudomonas aeruginosa*; coliform bacterial including *E. coli spp.*; *Listeria spp.*; *Neisseria spp.*; *Streptococcus spp.*; *Staphylococcus spp.*; *Yersinia spp.*; *Campylobacter spp.*; *Helicobacter spp.*; *Aeromonas spp.*; atypical Mycobacteria; and *Legionella spp.*

The presently disclosed subject matter provides in some embodiments a method for preventing and/or treating a disease, disorder, or condition associated with the presence and/or development of bacterial biofilm in a subject. In some embodiments, the method comprises administering to the subject a composition of the presently disclosed subject matter in an effective amount and via a route sufficient for preventing and/or reducing the severity of at least one symptom of the disease, disorder, or condition. In some embodiments, the disease, disorder, or condition is selected from the group consisting of whooping cough, cystic fibrosis, bacterial vaginosis, urinary tract infections, infections associated with catheter use, middle ear infections, formation of dental plaque, gingivitis, eye infections associated with contact lens use, endocarditis, and infections resulting from use of medical and/or dental implants such as but not limited to joint prostheses and heart valves.

The presently disclosed subject matter provides in some embodiments a method for reducing the incidence of nosocomial infection. In some embodiments, the method comprises contacting a surface present in a medical and/or dental facility with a composition of the presently disclosed subject matter in an amount sufficient to inhibit bacterial biofilm development and/or reduce or eliminate bacterial biofilm present on the surface, wherein the bacterial biofilm is associated with the incidence of nosocomial infection. In some embodiments, the surface is a door surface, a door handle surface, a sink surface, a toilet surface, a faucet surface, a furniture surface, optionally a bed surface, and a window surface.

The presently disclosed subject matter provides in some embodiments a method of inhibiting bacterial biofilm development and/or for reducing or eliminating a bacterial biofilm present on a surface, the method comprising contacting the surface or the biofilm present thereon with an effective amount of a composition of the presently disclosed subject matter, whereby bacterial biofilm development on the surface is inhibited and/or existing bacterial biofilm on the on the surface is reduced or eliminated. In some embodiments, the bacterial biofilm comprises a strain of bacteria selected from the group consisting of *Bordetella spp.*, optionally *Bordetella pertussis* or *Bordetella bronchiseptica*; *Salmonella spp.*, optionally *Salmonella typhimurium*; *Pseudomonas sp.*, optionally *Pseudomonas aeruginosa*; coliform bacterial including *E. coli spp.*; *Listeria spp.*; *Neisseria spp.*; *Streptococcus spp.*; *Staphylococcus spp.*; *Yersinia spp.*; *Campylobacter spp.*; *Helicobacter spp.*; *Aeromonas spp.*; atypical Mycobacteria; and *Legionella spp.*

In some embodiments, the surface is a part of a device selected from the group consisting of a medical device, a dental device, and an industrial device. In some embodiments, the medical device is selected from the group consisting of a surgical tool, an implant, a catheter, a stent, a ventilator tubing, and a bone or joint implant, optionally a hip, knee, ankle, wrist, elbow, or shoulder prosthesis. In some embodiments, the implant is a cardiac implant. In some embodiments, the industrial device is selected from the group consisting of a pipe, a tube, a valve, an air-cooled tower, a warm water system, a coolant circuit, a silo, a fermenter, a colander, a piece of furniture, and a sink. In some embodiments, the industrial

device is part of device used for water treatment, sewage treatment, petroleum manufacturing and/or storage, or recycling.

In some embodiments, the surface is a cellular surface, a tissue surface, and/or an organ surface present within a subject. In some embodiments, the contacting comprises administering a pharmaceutical composition comprising the peptide or polypeptide to the subject in an amount and via a route of administration whereby the peptide or polypeptide contacts the surface or the biofilm present thereon and inhibits bacterial biofilm development on the surface and/or reduces or eliminates the existing bacterial biofilm present thereon. In some embodiments, the surface is a nasal surface and/or a lung surface and the pharmaceutical composition is configured for inhalation and/or insufflation by the subject. In some embodiments, the composition comprises a delivery vehicle, optionally wherein the peptide or polypeptide is associated with, conjugated to, and/or encapsulated by a delivery vehicle in the pharmaceutical composition. In some embodiments, the delivery vehicle comprises a liposome, a microparticle, or a nanoparticle, optionally wherein the liposome, microparticle, or nanoparticle is designed to be biodegradable in the subject. In some embodiments, the method further comprises contacting the surface with one or more additional compositions that inhibit bacterial biofilm development and/or reduces or eliminates bacterial biofilm present on the surface.

Various aspects and embodiments of the presently disclosed subject matter are described in further detail below.

These and other aspects and embodiments which will be apparent to those of skill in the art upon reading the specification provide the art with tools and agents useful for preventing and/or treating biofilms.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is bar graph showing that BP348, a *B. pertussis* strain lacking ACT, made more biofilm than wild-type (WT) BP338 *B. pertussis*. Strains were grown in 96-well microtiter plates and biofilm formation was assessed using the crystal violet assay at 96 hours. Bvg(-) BP347 serves as a negative control. Data expressed as the mean ± two (2) standard deviations, compiled from 3 experiments run in triplicate. $*p < 0.05$ and $****p < 0.0001$ compared to wild-type BP338.

Figures 2A and 2B are growth curves of bacterial strains grown in SSM shaking 10 mL culture (Figure 2A) and SSM static 100 µL culture in 96 well plates (Figure 2B). OD_{600} measurements were recorded over 24 hours for shaking cultures and 96 hours for static 100 µL cultures.

Figure 3 is a bar graph showing that ACT inhibited biofilm in a concentration-dependent manner. wild-type BP338 biofilm formation in the presence of increasing concentrations of recombinant purified ACT (ng/ml) was assessed at 96 hours. Biofilm formation was measured by crystal violet assay. Bvg(-) BP347 served as negative control. Data expressed as the mean ± two (2) standard deviations, compiled from 5 experiments run in triplicate. ***$p < 0.001$, ****$p < 0.0001$ compared to wild-type BP338 without ACT.

Figures 4A and 4B are growth time course plots for *B. pertussis* strains. In Figure 4A, *B. pertussis* strains were grown in 5 mL cultures with increasing concentrations of urea added to ensure urea had no effect on bacterial growth. Data are expressed as the mean ± two (2) standard deviations, compiled from 3 experiments run in triplicate. In Figure 4B, *B. pertussis* strain BP338 was grown in static 100 µL cultures in the presence and absence of ACT or AC domain in 96 well plates. OD_{600} measurements were recorded every 24 hours.

Figure 5 is a bar graph showing that ACT inhibited *B. bronchiseptica* biofilm in a concentration-dependent manner. wild-type RB50 biofilm formation in the presence of increasing concentrations of recombinant purified ACT (10, 100, or 1000 ng/ml) for 96 hours. Biofilm formation was measured by crystal violet assay. Bvg(-) RB54 strain served as negative control. Data expressed as the mean ± two (2) standard deviations, compiled from 3 experiments run in triplicate. **$p < 0.01$ and ***$p < 0.001$ compared to wild-type without ACT.

Figure 6 is a series of schema of ACT truncated and enzymatically inactive mutant proteins.

Figure 7 is a bar graph showing that the AC domain inhibited biofilm in a concentration-dependent manner. BP338 biofilm formation in the presence of increasing concentrations of recombinant purified ACT (10, 100, or 1000 ng/ml) for 96 hours. Biofilm formation was measured by crystal violet assay. Bvg(-) strain served as negative control. Data expressed as the mean ± two (2) standard deviations, compiled from 3 experiments run in triplicate. *$p < 0.05$; **$p < 0.01$;

***$p < 0.001$; ****$p < 0.0001$ compared to wild-type without ACT.

Figure 8 is a bar graph showing that the AC domain was necessary and sufficient for biofilm inhibition, although the catalytic activity of ACT was not required. ACT, iACT or other ACT mutant proteins were added to wild-type BP338 and biofilm formation was measured by crystal violet assay at 96 hours. AC domain was added at 10 ng/ml and additional ACT proteins including ΔH, ΔHR1, ΔR, and ΔAC (*see* Figure 6) were all added to a final concentration of 100 ng/ml. Data are expressed as the mean ± two (2) standard deviations, compiled from 3 experiments run in triplicate. ***P<0.0005 compared to wild-type BP338 without ACT.

Figure 9 is is a bar graph showing that the the AC domain was necessary and sufficient for *B. bronchiseptica* biofilm inhibition, although the catalytic activity of ACT was not required. ACT, iACT, or other ACT-truncated mutant proteins were added to RB50 and biofilm formation was measured by crystal violet assay at 96 hours. AC domain was added at 10 ng/ml and additional ACT proteins including ΔH, ΔHR1, ΔR, and ΔAC were all added to a final concentration of 100 ng/ml. Bvg(-) RB54 strain served as negative control. Data are expressed as the mean ± two (2) standard deviations, compiled from 3 experiments run in triplicate. **p <0.01 compared to wild-type RB50.

Figure 10 shows an image of a western blot of BP338 ΔAC, which provided confirmation of deletion of AC domain and presence of truncated ACT peptide. Bacterial strains were grown 48 hours on BG plates at 37ºC, transferred to 10 mL shaking SSM cultures, and grown for 24 hours. At 24 hours samples were taken and the OD_{600} of each sample was matched. Samples were boiled 5 minutes and 30 µl of the sample was loaded per well. 1 µg of ACT was loaded. A western blot was performed using a polyclonal ACT antibody (*see* Lee *et al.*, 1999).

Figure 11 is a bar graph showing that BP338 lacking the AC domain (BP338 ΔAC) made more biofilm than the parental wild-type strain. Strains were grown in 96 well microtiter plates and biofilm formation was assessed using the crystal violet assay at 96 hours. Mean values represented by bars, error bars represent standard deviations. A Bvg(-) strain served as a negative control. Data are expressed as the mean ± two (2) standard deviations, compiled from 3 experiments run in triplicate. **$p < 0.01$ and ****$p < 0.0001$ compared to wild-type.

Figures 12A-12C show SEM images showing that AC domain inhibits biofilm formation on glass coverslips. *B. pertussis* was grown in 24-well plates with inverted glass coverslips so that biofilm formation could occur at the air liquid interface. At 96 hours, the coverslips were fixed in 4% paraformaldehyde and prepared for SEM imaging using a Zeiss Sigma VP HD field emission scanning electron microscope at the University of Virginia Microscopy Core. Representative images were chosen from four experimental replicates. Figure 12A is an image of wild-type BP338 *B. pertussis* (15,000 X); Figure 12B is an image of Bvg(-) BP347 *B. pertussis* (5000 X); and Figure 12C is an image of wild-type BP338 + 10 ng/ml AC domain (5000 X).

Figures 13A and 13B are graphs showing that exogenous AC domain inhibited bacterial aggregation and disrupted preformed biofilm, respectively. In Figure 13A, *B. pertussis* strains were grown as 5 ml shaking cultures, in the presence or absence of 100 ng/ml AC domain. At 24 hours, samples were removed from the culture and the Aggregation Index was determined. Mean values are represented by bars, error bars represent standard deviations. Data expressed as the mean ± two (2) standard deviations, compiled from 3 experiments run in triplicate. In Figure 13B, BP338 biofilm formation (solid circles; black line) was measured every 24 hours via the crystal violet assay. AC domain was added at time zero (squares; gray line) or was added at 72 hours (open circles; dashed line) and biofilm was measured every 24 hours. Mean values represented by lines and error bars represent standard deviations. Data compiled from 5 experiments run in triplicate. $^{**}p < 0.001$ compared to BP338.

Figure 14 is a bar graph showing that CaM and anti-ACT antibodies blocked ACT inhibitory effects. Calmodulin (1 μM) was incubated with ACT (100 ng/ml, 565 pM) or AC domain (10 ng/ml, 0.233 pM) for 15 minutes before adding the combination to BP338 cultures. ACT and AC domain alone were also incubated for 15 minutes prior to addition to bacterial cultures. Monoclonal antibody 7CE4B1 directed against the AC domain (described in Lee *et al.*, 1999 but produced by the instant co-inventors; 2.4 mg/ml) was incubated with ACT or AC domain for 15 minutes before adding the combination to cultures in 96 well microtiter plates. Mixtures as indicated were added to bacterial cultures and biofilm formation was measured at 96 hours by crystal violet assay. Data are

expressed as the mean ± two (2) standard deviations, compiled from 3 experiments run in triplicate. *p < 0.05 compared to wild-type. +: component added; –: component not added.

Figure 15 is an image of a western blot of purified proteins used in SPR experiments. 10 µg of each protein were separated by 7.5% SDS-PAGE gel. Coomassie staining was used to visualize the purities of the proteins used in SPR experiments..

Figures 16A-16C are plots showing SPR kinetic binding analysis of the interaction between FHA and the AC domain of ACT. The recombinant AC domain at indicated concentrations was injected in parallel ("one-shot kinetics") over the sensor chip coated with purified FHA (Figure 16A) or FHA44 (Figure 16B) proteins at a flow rate of 30 µL/min for both association and dissociation phases of the sensogram. The kinetic data were fitted globally by using a 1:1 Langmuir model to obtain association [$k_a = 2.9 \pm 0.4 \times 10^3\ M^{-1}s^{-1}$] and dissociation [$k_d = 1.9 \pm 0.2 \times 10^{-2}\ s^{-1}$] rate constants of the interaction. The equilibrium dissociation constant, K_D, was determined as k_d/k_a and found to be 650 nM between AC domain and FHA. No binding was observed between AC domain and FHA44. The fitted curves are superimposed as lines on top of the binding curves. Representative data from a single experiment are shown. Figure 16C shows that CaM blocked the AC domain – FHA interaction. The AC domain (10 µM) and the freshly-prepared complexes of the AC domain with CaM mixed in molar ratios of 10:1, 1:1, and 1:10 AC domain:CaM were injected in parallel over the SPR sensor chip coated with FHA at flow rate of 30 µ//min. Inhibition of binding of the AC/CaM 1:10 complex to FHA is represented by a decrease of SPR signal response. No binding of CaM alone was observed to FHA. Results are representative data from three independent experiments.

Figure 17 is a bar graph showing that the AC domain and ACT inhibited MCD antibody binding to FHA. In an ELISA binding assay, plates were coated with FHA and an anti-MCD antibody was used as a detection reagent. ACT, AC domain, and $ACT_{\Delta AC}$, added at a range of mg/ml concentrations, were added prior to the addition of the anti-MCD antibody to determine if AC domain binding interfered with MCD antibody detection. In addition to these conditions, 10 µM CaM was incubated with the various concentrations of ACT or AC domain for 15

minutes prior to addition of ACT to the wells. All values were normalized to the control (OD_{450} 0.284) using GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, California, United States of America). Data expressed as the mean ± two (2) standard deviations, compiled from three experiments run in triplicate $^{**}p <$ 0.01 and $^{****}p < 0.0001$ compared to control.

Figures 18A and 18B show growth of wild-type *B. pertussis* strains BP338 and BPSM over 96 hours in shakling culture and ACT and FHA protein expression in these strains at 24 hours. Figure 18A is a time course of biofilm growth of the wild-type *B. pertussis* strains BP338 (squares) and BPSM (circles). Figure 18B is an image of ACT and FHA protein expression of BP338 and BPSM at 24 hours in shaking culture. 20 µL of OD_{600} normalized bacteria were run on SDS-PAGE gel and protein expression was determined by western blot analysis; polyclonal anti-ACT antibody was used to detect ACT and monoclonal anti-CRD antibody (Noel *et al.*, 2012) was used to detect FHA.

Figures 19A and 19B are bar graphs showing the inhibitory effects of ACT and anti-MCD antibodies on biofilm production. Figure 19A is a bar graph showing that the MCD of FHA must be present and properly folded for ACT inhibition of biofilm. FHA mutant proteins were generated in the wild-type *B. pertussis* BPSM parent strain. BPSM JS20 (ΔMCD) had the entire MCD sequence deleted. BPSM T-N had a transposon inserted into the prodomain sequence, precluding prodomain cleavage and processing of the MCD, leaving the MCD unfolded in the final FHA molecule. *B. pertussis* strains were allowed to form biofilm for 96 hours in the presence or absence of 100 ng/ml ACT. Biofilm was measured by crystal violet assay. Data expressed as the mean ± two (2) standard deviations, compiled from 3 experiments run in triplicate. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to wild-type (BPSM). Figure 19B is a bar graph showing that anti-MCD antibodies blocked biofilm production. Anti-MCD antibodies were added at 1:1000 and 1:100 dilutions to anti-MCD antibodies *B. pertussis* (BP338) cultures in 96-well microtiter plates to observe their effects on biofilm formation. Biofilm formation was measured at 96 hours using the crystal violet assay. All values were normalized to the control (OD_{450} 0.284) using Graphpad Prism6 software (GraphPad Software, Inc., La Jolla, California, United States of America). Data are expressed as the mean ± two (2) standard deviations, compiled from three (3) experiments run in

triplicate $^{**}p < 0.01$ compared to anti-MCD antibodies alone. n.s.: not significant.

Figure 20 is a bar graph showing that the MCD of FHA must be present and properly folded for ACT inhibition of *B. bronchiseptica* biofilm. FHA mutants were generated in the *B. bronchiseptica* RBX11 parent strain. RBX11 was derived from RB50; it is a Δ*fhaS* mutant. RBX11 JS20 (ΔMCD) had the entire MCD sequence deleted. RBX11 T-N had a transposon inserted into the prodomain sequence, precluding prodomain cleavage and processing of the MCD, leaving the MCD unfolded in the final FHA molecule. *B. bronchiseptica* strains were allowed to form biofilm for 96 hours in the presence or absence of ACT. Biofilm was measured by crystal violet assay. Data expressed as the mean ± two (2) standard deviations, compiled from three (3) experiments run in triplicate. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to wild-type (RB50). n.s.: not significant.

Figure 21 is a model of biofilm inhibition by ACT. In the model, the AC domain of ACT binds FHA via the MCD at the distal tip of the FHA molecule. This binding blocks FHA function in biofilm, either through FHA-FHA interactions within biofilm or FHA-surface interactions as previously suggested, or possibly through some signaling event due to conformation change in the FHA protein.

Figures 22A-22C are a series of graphs showing that ACT and AC Domain inhibited *P. aeruginosa* biofilm in a concentration-dependent manner. Figures 22A and 22B are bar graphs of wild-type *P. aeruginosa* PA01 (Figure 22A) and wild-type *P. aeruginosa* PA14 (Figure 22B) biofilm formation in the presence of increasing concentrations of recombinant purified ACT (0.1, 1, and 10 μg/ml; bars 2, 3, and 4, respectively, in each of Figures 22A and 22B) and AC domain ACT (0.1, 1, and 10 μg/ml; bars 5, 6, and 7, respectively, in each of Figures 22A and 22B) was assessed at 12 hours. Biofilm formation was measured by crystal violet assay. Data expressed as the mean ± two (2) standard deviations, compiled from four (4) experiments run in triplicate. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$ compared to wild-type *P. aeruginosa* PA01 (bar 1 in Figure 22A) and PA14 (bar 1 in Figure 22B) without ACT or AC domain added. Figure 22C is a graph of the growth of wild-type *P. aeruginosa* PA01 and wild-type *P. aeruginosa* PA14 over 12 hours in the presence or absence of 10 μg/ml recombinant purified ACT or 10 μg/ml AC Domain. Inverted triangle: PA01 alone; diamond: PA01 + 10 μg/ml AC Domain; solid black circle: PA01 + 10 μg/ml recombinant purified ACT;

solid gray circle: PA14 alone; square: PA14 + 10 µg/ml AC Domain; triangle: PA14 + 10 µg/ml recombinant purified ACT.

Figure 23 is a graph showing that exogenous AC domain inhibited *P. aeruginosa* PA14 biofilm, but not biofilm of the CdrA transposon mutant, PA14 32575 (tn::*cdrA*). The graph is a time course of biofilm formation of the wild-type PA14 (solid black line with solid circles) and CdrA transposon mutant, PA14 32575 (solid gray line with inverted triangles) measured over 12 hours via the crystal violet assay. Concurrently, 10 µg/m AC domain was added at time zero to PA14 (dotted black line with solid circles) and the PA14 32575 mutant (dotted gray line with inverted triangles) and biofilm was measured at the same time points. Mean values represented by circles and error bars represent standard deviations. Data compiled from three (3) experiments run in triplicate. Statistics are not included on graph for clarity were as follows: at 12 hours, PA14 vs. PA14 + AC domain: $p < 0.0001$; PA14 vs. PA14 32575: $p < 0.001$; and PA14 32575 vs. PA14 32575 + AC domain: not significant.

Figure 24 is a bar graph showing that anti-MCD antibodies inhibited *P. aeruginosa* PA14 biofilm. Anti-MCD antibodies were added at 1:1000 and 1:100 dilutions to wild-type *P. aeruginosa* PA14 cultures in 96 well microtiter plates to observe their effects on biofilm formation. Biofilm formation was measured at 12 hours using the crystal violet assay. Data expressed as the mean ± two (2) standard deviations, compiled from three (3) experiments run in triplicate. **** $p < 0.0001$ compared to wild-type PA14 alone.

Figures 25A-25C are a series of bar graphs showing that the AC Domain inhibited *E. coli and S. typhimurium* biofilm. Figure 25A shows a lack of inhibition with respect to wild-type *E. coli* MC4100, Figure 25B shows inhibition of wild-type *E. coli* 87-23 and Figure 25C shows inhibition of wild-type *S. typhimurium* S1344 biofilm formation in the presence (gray bars in Figures 25A-25C) or the absence (black bars in Figures 25A-25C) of 10 µg/ml AC domain assessed at 12 hours. Biofilm formation was measured by crystal violet assay. Data expressed as the mean ± two (2) standard deviations, compiled from four (4) experiments run in triplicate. *p* values are indicated on graphs. n.s.: not significant.

Figures 26A and 26B relate to experiments showing that the T18 and the T25 peptides inhibited *B. pertussis* biofilm in a concentration-dependent manner.

Figure 26A is a schematic depiction of the ACT holoenzyme, showing the locations of the AC domain, the T18 peptide, and the T25 peptide. Figure 26B is a bar graph of wild-type *B. pertussis* BP338 biofilm formation in the presence of increasing concentrations of recombinant purified T18 (0.1, 1, and 10 µg/ml; bars 2, 3, and 4, respeectively) and T25 peptides (0.1, 1, and 10 µg/ml; bars 5, 6, and 7, repectively) was assessed at 12 hours. Biofilm formation was measured by crystal violet assay. Data expressed as the mean ± two (2) standard deviations, compiled from two (2) experiments run in triplicate. **** $p < 0.0001$ compared to wild-type *P. aeruginosa* grown without peptides added.

BRIEF DESCRIPTION OF THE SEQUENCE LISTING

SEQ ID NO: 1 is an amino acid sequence of a *Bordetella pertussis* ACT holoenzyme polypeptide. It corresponds to Accession No. NP_879578.1 in the GENBANK® biosequence database.

SEQ ID NO: 2 is an amino acid sequence of a *Bordetella pertussis* AC Domain polypeptide. It corresponds to amino acids 1-400 of SEQ ID NO: 1.

SEQ ID NOs: 3 and 4 are amino acid sequences of the T25 and T18 peptides, respectively, derived from the *Bordetella pertussis* AC Domain polypeptide of SEQ ID NO: 1. They correspond to amino acids 1-225 and 226-400 of SEQ ID NO: 1, respectively.

SEQ ID NO: 5 is an amino acid sequence of an inactivated *Bordetella pertussis* AC Domain polypeptide. The polypeptide has been inactivated by substituting the aspartic acid at amino acid 188 of SEQ ID NO: 1 with a cysteine and the isoleucine at amino acid 189 of SEQ ID NO: 1 with a threonine.

SEQ ID NOs: 6-25 are the nucleotide sequences of the primers listed in Table 1.

SEQ ID NOs: 26-30 are the amino acid sequences of subunits 1-5, respectively, of a *Bordetella pertussis* PT polypeptide. SEQ ID NOs: 26-30 correspond to GENBANK® biosequence database Accession Nos. NP_882282.1, NP_882283.1, NP_882286.1, NP_882284.1, and NP_882285.1, respectively.

SEQ ID NOs: 31-35 are the amino acid sequences of subunits 1-5, respectively, of a *Bordetella bronchiseptica* PT polypeptide. SEQ ID NOs: 31-35 correspond to GENBANK® biosequence database Accession Nos. WP_033452809.1, WP_033452812.1, WP_015064783.1, WP_033468323.1, and

WP_033446920.1, respectively.

SEQ ID NOs: 36 and 37 are the amino acid sequences of FHA polypeptides from *Bordetella pertussis* and *Bordetella bronchiseptica*, respectively. SEQ ID NOs: 36 and 37 correspond to GENBANK® biosequence database Accession Nos. NP_880571.1 and YP_006966876.1, respectively.

SEQ ID NOs: 38 and 39 are the amino acid sequences of Fim2 and Fim3 polypeptides, respectively, from *Bordetella pertussis*. SEQ ID NOs: 38 and 39 correspond to GENBANK® biosequence database Accession Nos. NP_879898.1 and NP_880302.1, respectively.

SEQ ID NOs: 40 and 41 are the amino acid sequences of Fim2 and Fim3 polypeptides, respectively, from *Bordetella bronchiseptica*. SEQ ID NOs: 40 and 41 correspond to GENBANK® biosequence database Accession Nos. YP_006967303.1 and YP_006967865.1, respectively.

SEQ ID NOs: 42 and 43 are the amino acid sequences of PRN polypeptides from *Bordetella pertussis* and *Bordetella bronchiseptica*, respectively. SEQ ID NOs: 42 and 43 correspond to GENBANK® biosequence database Accession Nos. NP_879839.1 and WP_033839724.1, respectively.

SEQ ID NO: 44 is an amino acid sequence of a *Bordetella bronchiseptica* ACT holoenzyme polypeptide. SEQ ID NO: 44 corresponds to Accession No. WP_080702041.1 in the GENBANK® biosequence database.

SEQ ID NO: 45 is an amino acid sequence of a *Bordetella bronchiseptica* AC Domain polypeptide. SEQ ID NO: 45 corresponds to amino acids 1-400 of SEQ ID NO: 44.

SEQ ID NOs: 46 and 47 are amino acid sequences of the T25 and T18 peptides, respectively, derived from the *Bordetella bronchiseptica* AC Domain polypeptide of SEQ ID NO: 1. SEQ ID NOs: 46 and 47 correspond to amino acids 1-225 and 226-400 of SEQ ID NO: 1, respectively.

SEQ ID NO: 48 is an amino acid sequence of an inactivated *Bordetella bronchiseptica* AC Domain polypeptide. The polypeptide has been inactivated by substituting the aspartic acid at amino acid 188 of SEQ ID NO: 44 with a cysteine and the isoleucine at amino acid 189 of SEQ ID NO: 44 with a threonine.

SEQ ID NO: 49 is an amino acid sequence of a *Bordetella parapertussis* ACT holoenzyme polypeptide (hemolysin). SEQ ID NO: 49 corresponds to

Accession No. WP_010927405.1 in the GENBANK® biosequence database.

SEQ ID NO: 50 is an amino acid sequence of a *Bordetella parapertussis* AC Domain polypeptide. SEQ ID NO: 50 corresponds to amino acids 35-434 of SEQ ID NO: 49.

SEQ ID NOs: 51 and 52 are amino acid sequences of the T25 and T18 peptides, respectively, derived from the *Bordetella parapertussis* AC Domain polypeptide of SEQ ID NO: 49. SEQ ID NOs: 51 and 52 correspond to amino acids 35-249 and 250-434 of SEQ ID NO: 49, respectively.

SEQ ID NO: 53 is an amino acid sequence of an inactivated *Bordetella parapertussis* AC Domain polypeptide. The polypeptide has been inactivated by substituting the aspartic acid at amino acid 222 of SEQ ID NO: 49 with a cysteine and the isoleucine at amino acid 223 of SEQ ID NO: 49 with a threonine.

SEQ ID NOs: 54-58 are the amino acid sequences of subunits 1-5, respectively, of a *Bordetella parapertussis* PT polypeptide. SEQ ID NOs: 54-58 correspond to GENBANK® biosequence database Accession Nos. WP_010929490.1, YP_006898153.1, YP_006898156.1, YP_006898154.1, and YP_006898155.1, respectively.

SEQ ID NO: 59 is the amino acid sequence of an FHA polypeptide from *Bordetella parapertussis*. SEQ ID NO: 59 corresponds to GENBANK® biosequence database Accession No. YP_006896577.1.

SEQ ID NOs: 60 and 61 are the amino acid sequences of Fim2 and Fim3 polypeptides, respectively, from *Bordetella parapertussis*. SEQ ID NOs: 60 and 61 correspond to GENBANK® biosequence database Accession Nos. YP_006895663.1 and YP_006895400.1, respectively.

SEQ ID NO: 62 is the amino acid sequence of a PRN polypeptide from *Bordetella parapertussis*. SEQ ID NO: 62 corresponds to GENBANK® biosequence database Accession No. YP_006897297.1.

DETAILED DESCRIPTION

Bordetella pertussis is the causative agent of whooping cough (pertussis) and a reemerging health threat in the United States and globally, as illustrated by the increasing number of cases reported each year. Despite high vaccination rates of children and adolescents, there were approximately 33,000 cases in the United States reported to the United States Centers for Disease Control and

Prevention (CDC) in 2014. The most striking shift in the age-specific incidence of pertussis has been in subjects aged 15 and older (Strebel *et al.*, 2001), who are now more frequently infected with *B. pertussis*. In contrast to the potential fatality of pertussis in infants and young children, adolescents and adults develop a persistent cough with fewer systemic manifestations of the disease (Birkebaek *et al.*, 1999; Wendelboe *et al.*, 2007) and often serve as sources of pertussis transmission (Nelson, 1978; Cherry & Olin, 1999; Bisgard *et al.*, 2004; Quinn & McIntyre, 2011).

Bordetella pertussis has been shown to form biofilm *in vitro* on abiotic surfaces and *in vivo*, primarily on nasal septum and the trachea (Sloan *et al.*, 2007; Serra *et al.*, 2007; Conover *et al.*, 2010; Serra *et al.*, 2011). Ongoing studies support the concept that *B. pertussis* forms biofilm during infection; recent clinical isolates form more biofilm compared to a lab-passaged isolate, BP338. The closely related animal pathogen, *B. bronchiseptica*, forms biofilm *in vitro* on abiotic surfaces and biofilm formation contributes to its chronic infection of dogs and other mammals (Fenwick, 2013). Although the specific role of biofilm in human infections with *B. pertussis* has not yet been established, the fact that these organisms produce biofilm both *in vitro* and *in vivo* provides a modality to investigate the production of biofilm as well as possible ways to interrupt biofilm formation.

Biofilms are complex structures controlled by a variety of bacterial signaling systems. They are comprised of aggregative bacteria surrounded by a matrix of polysaccharides, proteins, and extracellular DNA (eDNA). *Bordetella* biofilm has been shown to require eDNA (Conover *et al.*, 2011), Bps (*Bordetella* polysaccharide) (Conover *et al.*, 2010), which resembles *S. aureus* poly-N-acetyl-beta-(1-6)-glucosamine, and multiple proteins. Of significance to the presently disclosed subject matter is the observation that filamentous haemagglutinin (FHA) is an important component of *B. pertussis* and *B. bronchiseptica* biofilm formation. This surface displayed adhesin promotes the formation and maintenance of biofilm by mediating bacteria-substrate as well as bacteria–bacteria interactions. Serra *et al.* showed that anti-FHA antibodies blocked biofilm formation by *B. pertussis*, and a strain lacking FHA (*ΔfhaB* BPGR4) made less biofilm *in vitro* and *in vivo* on mouse trachea and nasal septum compared to wild-type BPSM (Serra

et al., 2011).

Although less is known about the regulation of biofilm in *Bordetellae* compared to other medically relevant biofilm-forming bacterial species, several modes of regulation have been implicated. Nutrient limitation and oxidative stress activate (p)ppGpp signaling to enhance biofilm formation in *B. pertussis* (Sugisaki *et al.*, 2013), while c-di-GMP signaling regulates motility and biofilm formation in *B. bronchiseptica* (Sisti *et al.*, 2013). The machinery for synthesis of the Bps matrix component is encoded by the *bpsABCD* operon and is under control of the BpsR repressor, but the factor, process, or signal that relieves BpsR repression is unknown (Conover *et al.*, 2012). Multiple signals, including those from the "master regulator" of virulence, *Bordetella* virulence gene two-component regulatory system, BvgAS, are integrated to control *Bordetella* biofilm. Biofilm formation occurs in the Bvg(+) phase and Bvg(i) phase, but biofilm is not observed in the Bvg(-) phase. Irie *et al.* showed biofilm formation was maximal in the Bvg(i) phase in *B. bronchiseptica* (Irie *et al.*, 2004). In contrast, Mishra *et al.* found biofilm formation was equal in the Bvg(+) and Bvg(i) phases for *B. pertussis* (Mishra *et al.*, 2005). Irie *et al.* also showed that a *B. bronchiseptica* strain lacking adenylate cyclase toxin (ACT; Δ*cyaA* RB58) made more biofilm than the parental wild-type RB50 strain and in light of the earlier observation demonstrating a direct interaction between ACT and FHA (Zaretzky *et al.*, 2002), suggested that this protein-protein interaction could function to regulate biofilm production in *Bordetella*.

ACT is an important virulence factor of both *B. pertussis* and *B. bronchiseptica*. The 177 kDa protein toxin is secreted by a type I secretion system and remains surface-associated or is released as a function of free calcium concentration in the medium (Bumba *et al.*, 2016). ACT that has been released from the bacterial surface is the active form of the toxin, which affects target cells (Gray *et al.*, 2004). ACT uses complement receptor 3, the heterodimeric aMb2 integrin (CD11b/CD18 or Mac-1), as its receptor (Guermonprez *et al.*, 2001; Osička *et al.*, 2015), but can also intoxicate cells that lack this integrin heterodimer (Eby *et al.*, 2010). Following binding to the host cell, the catalytic domain of the toxin is translocated across the plasma membrane and into the host cytoplasm, where calmodulin (CaM) binds the enzymatic (catalytic) domain, activating it to

convert ATP to cAMP (Guermonprez *et al.*, 2001; El-Azami-El-Idrissi *et al.*, 2003; Perkins *et al.*, 2007; Martin *et al.*, 2010; Osickova *et al.*, 2010; Eby *et al.*, 2012; Uribe *et al.*, 2013). This in turn leads to supraphysiological levels of cAMP and can cause a massive reduction in intracellular ATP (Basler *et al.*, 2006; Hewlett *et al.*, 2006; Bumba *et al.*, 2010; Eby *et al.*, 2012). Through these mechanisms, ACT inhibits phagocytosis, chemotaxis, and superoxide generation by neutrophils, is required for the establishment of infection in the mouse model and human infections with the attenuated strain, BPZE1 (Thorstensson *et al.*, 2014; Lim *et al.*, 2014) and serves as a protective antigen (Confer & Eaton, 1982; Weiss *et al.*, 1983; Pearson *et al.*, 1987; Cherry & Heinninger, 2004; Vojtova *et al.*, 2006; Basler *et al.*, 2006; Hewlett *et al.*, 2006; Fiser *et al.*, 2012; Costache *et al.*, 2013; Fedele *et al.*, 2013; Thorstensson *et al.*, 2014; Bumba *et al.*, 2016). In previous studies, the secretion, release, binding to host cells, interaction with host cells, functional effects of ACT on host cells, and its role in establishing an infection have been characterized. This host-directed protein bacterial toxin has not, however, been studied for effects on the bacterium itself.

The 177 kD Adenylate Cyclase Toxin (ACT) of *Bordetella* and fragments thereof, including but not limited to the 40 kD derived catalytic domain (AC domain) and peptides T18 and T25, are disclosed herein to be potent biofilm inhibitors in *B. pertussis* and *B. bronchiseptica*. While not wishing to be bound by any particular theory of operation, the inhibitors appear to function by directly binding the distal tip of the surface-displayed biofilm adhesin, Filamentous Hemagglutinin (FHA). Many bacterial species express FHA-like proteins that display high structural or sequence homology to *Bordetella* FHA, and some of these proteins are involved in biofilm formation. The ACT holoenzyme, the AC domain, and peptides derived from the AC domain were tested for their abilities to inhibit biofilm of *Bordetellae, Pseudomonas aeruginosa, Escherichia coli,* and *Salmonella typhimurium in vitro*. The AC domain demonstrated an inhibitory effect on biofilm formation for several bacteria tested. Using a *Pseudomonas aeruginosa* transposon mutant that lacks CdrA, the FHA-like protein of *Pseudomonas*, it has been demonstrated that the AC domain might inhibit biofilm of other bacteria via a similar mechanism, which involves the AC domain binding FHA-like proteins. These data, taken together, showed that the AC domain and derived peptides

could serve as general biofilm inhibitors, specifically for bacteria that express FHA-like proteins.

The presently disclosed subject matter now will be described more fully hereinafter, in which some, but not all embodiments of the presently disclosed subject matter are described. Indeed, the presently disclosed subject matter can be embodied in many different forms and should not be construed as limited to the embodiments set forth herein; rather, these embodiments are provided so that this disclosure will satisfy applicable legal requirements.

I. Definitions

While the following terms are believed to be well understood by one of ordinary skill in the art, the following definitions are set forth to facilitate explanation of the presently disclosed subject matter.

All technical and scientific terms used herein, unless otherwise defined below, are intended to have the same meaning as commonly understood by one of ordinary skill in the art. Mention of techniques employed herein are intended to refer to the techniques as commonly understood in the art, including variations on those techniques or substitutions of equivalent techniques that would be apparent to one of skill in the art. While the following terms are believed to be well understood by one of ordinary skill in the art, the following definitions are set forth to facilitate explanation of the presently disclosed subject matter. Thus, unless defined otherwise, all technical and scientific terms and any acronyms used herein have the same meanings as commonly understood by one of ordinary skill in the art in the field of the presently disclosed subject matter. Although any compositions, methods, kits, and means for communicating information similar or equivalent to those described herein can be used to practice the presently disclosed subject matter, particular compositions, methods, kits, and means for communicating information are described herein. It is understood that the particular compositions, methods, kits, and means for communicating information described herein are exemplary only and the presently disclosed subject matter is not intended to be limited to just those embodiments.

The articles “a”, “an”, and “the” are used herein to refer to one or to more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, “an element” means one element or more than one element.

As used herein, the term “about” means approximately, in the region of, roughly, or around. When the term “about” is used in conjunction with a numerical range, it modifies that range by extending the boundaries above and below the numerical values set forth. For example, in one aspect, the term “about” is used herein to modify a numerical value above and below the stated value by a variance of in some embodiments ± 20%, in some embodiments ± 15%, in some embodiments ± 10%, in some embodiments ± 5%, in some embodiments ± 1%, in some embodiments ± 0.5%, in some embodiments ± 0.1%, and in some embodiments less than ± 0.1%.

As use herein, the terms “administration of” and or “administering” with respect to a compound, peptide, composition, etc. should be understood to mean providing a compound, peptide, composition, etc. of the presently disclosed subject matter or a prodrug of a compound, peptide, composition, etc. of the presently disclosed subject matter to a subject in need thereof, in some embodiments to ameliorate at least one symptom of a disease, disorder, or condition in the subject, to prevent the occurrence of at least one symptom of a disease, disorder, or condition in the subject, and/or to prevent the further development of at least one symptom of a disease, disorder, or condition in the subject.

As used herein, the term “aerosol” refers to suspension in the air. In particular, aerosol refers to the particlization or atomization of a formulation of the presently disclosed subject matter and its suspension in the air.

As used herein, an “agonist” is a composition of matter which, when administered to a mammal such as a human, enhances or extends a biological activity attributable to the level or presence of a target compound, peptide, composition, molecule of interest, etc. in the mammal.

An “antagonist” is a composition of matter which when administered to a mammal such as a human, inhibits a biological activity attributable to the level or presence of a target compound, peptide, composition, molecule of interest, etc. in the mammal.

As used herein, the phrase “alleviating a disease or disorder symptom” refers to reducing the severity of the symptom or the frequency with which such a symptom is experienced by a subject, or both. In some embodiments, “alleviating

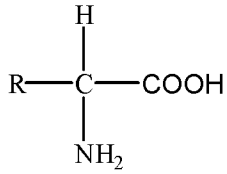
a disease or disorder symptom" refers to eliminating the symptom experienced by the subject.

As used herein, amino acids are represented by the full name thereof, by the three-letter code corresponding thereto, and/or by the one-letter code corresponding thereto, as indicated in the following:

Full Name	Three-Letter Code	One-Letter Code
Aspartic Acid	Asp	D
Glutamic Acid	Glu	E
Lysine	Lys	K
Arginine	Arg	R
Histidine	His	H
Tyrosine	Tyr	Y
Cysteine	Cys	C
Asparagine	Asn	N
Glutamine	Gln	Q
Serine	Ser	S
Threonine	Thr	T
Glycine	Gly	G
Alanine	Ala	A
Valine	Val	V
Leucine	Leu	L
Isoleucine	Ile	I
Methionine	Met	M
Proline	Pro	P
Phenylalanine	Phe	F
Tryptophan	Trp	W

The phrase "amino acid" is used interchangeably with "amino acid residue", and may refer to a free amino acid and/or to an amino acid residue of a peptide. It will be apparent from the context in which the term is used whether it refers to a free amino acid or a residue of a peptide.

Amino acids have the following general structure:



They may be classified into seven groups based on the side chain R: (1) aliphatic side chains; (2) side chains containing a hydroxylic (OH) group; (3) side chains containing sulfur atoms; (4) side chains containing an acidic or amide group; (5) side chains containing a basic group; (6) side chains containing an aromatic ring; and (7) proline, an imino acid in which the side chain is fused to the amino group.

The nomenclature used to describe the peptide compounds of the presently disclosed subject matter follows the conventional practice wherein the amino group is presented to the left and the carboxy group to the right of each amino acid residue. In the formulae representing selected specific embodiments of the presently disclosed subject matter, the amino- and carboxy-terminal groups, although not specifically shown, will be understood to be in the form they would assume at physiologic pH values, unless otherwise specified.

The term "basic" and the phrase "positively charged" as they relate to amino acids refer herein to amino acids in which the R groups have a net positive charge at pH 7.0, and include, but are not limited to, the standard amino acids lysine, arginine, and histidine.

As used herein, an "analog" of a chemical compound is a compound that, by way of example, resembles another in structure but is not necessarily an isomer (*e.g.*, 5-fluorouracil is an analog of thymine).

The term "antigen" as used herein refers to a molecule that provokes an immune response *in vitro* and/or *in vivo*. This immune response can involve antibody production, the activation of specific immunologically-competent cells, or both. An antigen can be derived from an organism, a subunit of a protein, a killed or inactivated whole cell or lysate, or any other source to which an organism's immune system or a component thereof (*e.g.*, an immune cell) can react.

The term "binding" refers to the adherence of molecules to one another, such as, but not limited to, enzymes to substrates, ligands to receptors, antibodies to antigens, DNA binding domains of proteins to DNA, and DNA or RNA strands to complementary strands.

As used herein, the phrase "binding partner" refers to a molecule capable

of binding to another molecule. In some embodiments, binding partner bind to each other *in vitro*, *ex vivo*, *in vivo*, and/or under physiological conditions.

The term "biocompatible", as used herein, refers to a material that does not elicit a substantial detrimental response in the host.

As used herein, the phrases "biologically active fragment" and "bioactive fragment" of polypeptides encompass natural and synthetic portions of full-length polypeptides that have one or more desirable characteristics of the full-length polypeptides, including but not limited to specific binding to their natural ligand(s) and/or performing desirable functions of the polypeptides.

The phrase "biological sample", as used herein, refers to samples obtained and/or otherwise isolated from a subject, including, but not limited to, skin, hair, tissue, blood, plasma, cells, sweat, and urine.

A "coding region" of a gene includes the nucleotide residues of the coding strand of the gene and/or genetic locus and the nucleotides of the non-coding strand of the gene which are homologous with or complementary to, respectively, the coding region of an mRNA molecule which is produced by transcription of the gene. A "coding region" thus comprises the "open reading frame" of the genetic locus.

A "compound", as used herein, refers to any type of substance or agent that is commonly considered a drug, or a candidate for use as a drug, as well as combinations and mixtures of the above.

As used herein, the phrase "conservative amino acid substitution" is defined herein as an amino acid exchange within one of the following five groups:

A. Small aliphatic, nonpolar, or slightly polar residues: Ala, Ser, Thr, Pro, Gly;

B. Polar, negatively charged residues and their amides: Asp, Asn, Glu, Gln;

C. Polar, positively charged residues: His, Arg, Lys;

D. Large, aliphatic, nonpolar residues: Met, Leu, Ile, Val, Cys; and

E. Large, aromatic residues: Phe, Tyr, Trp.

Thus, a conservative amino acid substitution includes a substitution of in some embodiments any small aliphatic, nonpolar, or slightly polar residue for any other small aliphatic, nonpolar, or slightly polar residues; in some embodiments any

polar, negatively charged residue and its amide for any other polar, negatively charged residue and its amide; in some embodiments any polar, positively charged residue for any other polar, positively charged residue; in some embodiments any large, aliphatic, nonpolar residue for any other large, aliphatic, nonpolar residue; and/or in some embodiments any large, aromatic residue for any other large, aromatic residue.

As used herein, a "derivative" of a compound refers to a chemical compound that may be produced from another compound of similar structure in one or more steps, as in replacement of H by an alkyl, acyl, or amino group.

A "disease" is a state of health of an animal wherein the animal cannot maintain homeostasis, and wherein if the disease is not ameliorated then the animal's health would be expected to deteriorate.

In contrast, a "disorder" in an animal is a state of health in which the animal is able to maintain homeostasis, but in which the animal's state of health is less favorable than it would be in the absence of the disorder. Left untreated, a disorder does not necessarily cause a further decrease in the animal's state of health.

As used herein, the term "domain" refers to a part of a molecule or structure that shares common physicochemical features, such as, but not limited to, hydrophobic, polar, globular, and helical domains, and/or properties such as ligand binding, signal transduction, cell penetration, and the like. Specific examples of binding domains include, but are not limited to, DNA binding domains and ATP binding domains.

As used herein, an "effective amount" or "therapeutically effective amount" means an amount sufficient to produce a desired effect, such as ameliorating or alleviating symptoms of a disease or disorder. In the context of administering compounds in the form of a combination, such as multiple compounds, the amount of each compound, when administered in combination with another compound(s), may be different from when that compound is administered alone. Thus, an effective amount of a combination of compounds refers collectively to the combination as a whole, although the actual amounts of each compound may vary. The term "more effective" means that the selected effect is ameliorated and/or alleviated to a greater extent by one treatment relative to a second

treatment to which it is being compared.

The term "elixir", as used herein, refers in general to a clear, sweetened, alcohol-containing, usually hydroalcoholic liquid containing flavoring substances and sometimes active medicinal agents.

"Encoding" refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (*i.e.*, rRNA, tRNA, and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a genetic locus encodes a protein if transcription and translation of mRNA corresponding to that genetic locus produces the protein in a cell or other biological system. Both the coding strand (*i.e.*, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings), and the non-coding strand, used as the template for transcription of a gene or cDNA, can be referred to as encoding the protein or other product of that genetic locus or cDNA.

The term "epitope" as used herein is defined as a small chemical group on an antigen molecule that can elicit and react with an antibody. An antigen can have one or more epitopes. Most antigens have many epitopes; *i.e.*, they are multivalent. In general, epitopes are roughly five to eight amino acids or sugars in size. One skilled in the art understands that generally the overall three-dimensional structure, rather than the specific linear sequence of the molecule, is the main criterion of antigenic specificity.

As used herein, an "essentially pure" preparation of a particular protein or peptide is a preparation wherein in some embodiments at least about 95%, in some embodiments at least about 97%, and in some embodiments at least about 99%, by weight, of the total protein or total peptide in the preparation is the particular protein or peptide of interest.

A "fragment" or "segment" is a portion of an amino acid sequence (*i.e.*, a subsequence) comprising at least one amino acid or a portion of a nucleic acid sequence comprising at least one nucleotide. The terms "fragment" and "segment" are used interchangeably herein.

As used herein, a "functional" biological molecule is a biological molecule in

a form in which it exhibits a desirable property by which it can be characterized. A functional enzyme, for example, is one which exhibits the characteristic catalytic activity by which the enzyme is characterized.

As used herein, the term "homologous" refers to the subunit sequence similarity between two polymeric molecules, *e.g.*, between two nucleic acid molecules, *e.g.*, two DNA molecules or two RNA molecules, or between two polypeptide molecules. When a subunit position in both of the two molecules is occupied by the same monomeric subunit, *e.g.*, if a position in each of two DNA molecules is occupied by adenine, then they are homologous at that position. The homology between two sequences is a direct function of the number of matching or homologous positions, *e.g.*, if half (*e.g.*, five positions in a polymer ten subunits in length) of the positions in two compound sequences are homologous then the two sequences are 50% homologous, if 90% of the positions, *e.g.*, 9 of 10, are matched or homologous, the two sequences share 90% homology. By way of example, the DNA sequences 3'ATTGCC5' and 3'TATGGC share 50% homology.

As used herein, the term "homology" is used synonymously with the term "identity". Similarly, the term "homologous" is used synonymously with the term "identical".

The determination of percent identity between two nucleotide or amino acid sequences can be accomplished using a mathematical algorithm. For example, a mathematical algorithm useful for comparing two sequences is the algorithm of Karlin & Altschul, 1990, modified as in Karlin & Altschul, 1993. This algorithm is incorporated into the NBLAST and XBLAST programs of Altschul *et al.*, 1990a, and can be accessed, for example at the National Center for Biotechnology Information (NCBI) world wide web site having the universal resource locator using the BLAST tool at the NCBI website. BLAST nucleotide searches can be performed with the NBLAST program (designated "blastn" at the NCBI web site), using the following parameters: gap penalty = 5; gap extension penalty = 2; mismatch penalty = 3; match reward = 1; expectation value 10.0; and word size = 11 to obtain nucleotide sequences homologous to a nucleic acid described herein. BLAST protein searches can be performed with the XBLAST program (designated "blastn" at the NCBI web site) or the NCBI "blastp" program, using the following parameters: expectation value 10.0, BLOSUM62 scoring matrix to obtain amino

acid sequences homologous to a protein molecule described herein. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.*, 1997. Alternatively, PSI-Blast or PHI-Blast can be used to perform an iterated search which detects distant relationships between molecules (Altschul *et al.*, 1997) and relationships between molecules which share a common pattern. When utilizing BLAST, Gapped BLAST, PSI-Blast, and PHI-Blast programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used.

The percent identity between two sequences can be determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, typically exact matches are counted. In some embodiments, a percent identity is computed over a subsequence of the nucleic acid or amino acid, and in some embodiments the percent identity relates to comparing the full length sequence of a first nucleic acid or amino acid to either a subsequence of a second nucleic acid or amino acid or the full length sequence of the second nucleic acid or amino acid.

As used herein, the term “inhaler” refers both to devices for nasal and pulmonary administration of a drug, *e.g.*, in solution, powder, and the like. For example, the term “inhaler” is intended to encompass a propellant driven inhaler, such as is used to administer antihistamine for acute asthma attacks, and plastic spray bottles, such as are used to administer decongestants.

As used herein “injecting” or “applying” includes administration of a compound (*e.g.*, a peptide) of the presently disclosed subject matter by any number of routes including, but not limited to, topical, oral, buccal, intravenous, intramuscular, intraarterial, intramedullary, intrathecal, intraventricular, transdermal, subcutaneous, intraperitoneal, intranasal, enteral, topical, sublingual, vaginal, ophthalmic, pulmonary, and rectal routes of administration.

A “ligand” is a molecule that specifically binds to a target molecule such as but not limited to a receptor. A “receptor” is a molecule that specifically binds to a ligand. In some embodiments, the attribution of a given molecule as being a “ligand” or a “receptor” is merely one of convenience in the event that the “receptor” can be a molecule that is not recognized as a “receptor” as that term might be understood with respect to cell biology and/or signal transduction.

As such, in some embodiments a ligand or a receptor (*e.g.*, an antibody) "specifically binds to" or "is specifically immunoreactive with" a compound when the ligand or receptor functions in a binding reaction which is determinative of the presence of the compound in a sample of heterogeneous compounds. Thus, under designated assay (*e.g.*, immunoassay) conditions, the ligand or receptor binds preferentially to a particular compound and does not bind in a significant amount to other compounds present in the sample. For example, a polynucleotide specifically binds under hybridization conditions to a compound polynucleotide comprising a complementary sequence; an antibody specifically binds under immunoassay conditions to an antigen bearing an epitope against which the antibody was raised. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically immunoreactive with a protein. *See* Harlow & Lane, 1988 for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity.

The phrase "nasal administration" in all its grammatical forms refers to administration of at least one compound of the presently disclosed subject matter through the nasal mucous membrane to the bloodstream for systemic delivery of at least one compound of the presently disclosed subject matter. The advantages of nasal administration for delivery are that it does not require injection using a syringe and needle, it avoids necrosis that can accompany intramuscular administration of drugs, and trans-mucosal administration of a drug is highly amenable to self-administration.

As used herein, "parenteral administration" of a pharmaceutical composition includes any route of administration characterized by physical breaching of a tissue of a subject and administration of the pharmaceutical composition through the breach in the tissue. Parenteral administration thus includes, but is not limited to, administration of a pharmaceutical composition by injection of the composition, by application of the composition through a surgical incision, by application of the composition through a tissue-penetrating non-surgical wound, and the like. In particular, parenteral administration is contemplated to include, but is not limited to, subcutaneous, intraperitoneal,

intramuscular, intrasternal injection, and kidney dialytic infusion techniques.

The term "peptide" typically refers to short polypeptides. In some embodiments, a peptide of the presently disclosed subject matter is thus at least or about 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 amino acids long, including but not limited to at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 amino acids long. The peptides of the presently disclosed subject matter can in some embodiments also have a length that falls in the ranges of 6-8, 8-10, 9-12, 10-13, 11-14, 12-15, 15-20, 20-25, 25-30, 30-35, 35-40, and 45-50 amino acids. In some embodiments, exactly, about, or at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17 or more of the amino acid residues within a recited sequence of a target peptide contains an O-GlcNAc moiety, a hexose-GlcNAc moiety, or any combination thereof.

The phrase "per application" as used herein refers to administration of a drug or compound to a subject.

The phrase "pharmaceutical composition" refers to a composition comprising at least one active ingredient, whereby the composition is amenable to administration for a specified, efficacious outcome to a mammal (for example, without limitation, a human). Those of ordinary skill in the art will understand and appreciate the techniques appropriate for determining whether an active ingredient has a desired efficacious outcome based upon the needs of the artisan.

As used herein, the phrase "pharmaceutically-acceptable carrier" means a chemical composition with which an appropriate compound and/or derivative can be combined and which, following the combination, can be used to administer the appropriate compound to a subject. In some embodiments, a pharmaceutically-acceptable carrier is pharmaceutically acceptable for use in a human, which means that the carrier is in some embodiments generally recognized as being safe (GRAS) for human consumption and/or administration. "Pharmaceutically acceptable" thus means physiologically tolerable, for either human or veterinary application.

As used herein, the phrase "physiologically acceptable" ester or salt means an ester or salt form of the active ingredient which is compatible with any other ingredients of the pharmaceutical composition, which is not deleterious to the

subject to which the composition is to be administered.

As used herein, "pharmaceutical compositions" include formulations for human and veterinary use.

As used herein, the term "plurality" means at least two and, unless specifically limited herein, has no upper boundary.

As used herein, the term "polypeptide" refers to a polymer composed of amino acid residues, related naturally occurring structural variants, and/or synthetic non-naturally occurring analogs thereof linked via peptide bonds, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof.

As used herein, the phrase "synthetic peptides or polypeptides" refers to non-naturally occurring peptides and polypeptides. Synthetic peptides and polypeptides can be synthesized, for example, using an automated polypeptide synthesizer. Various solid phase peptide synthesis methods are known to those of skill in the art.

The term "prevent", as used herein, means to stop something from happening, or taking advance measures against something possible or probable from happening. In the context of medicine, "prevention" generally refers to action taken to decrease the chance of getting a disease or condition.

A "preventive" or "prophylactic" treatment is a treatment administered to a subject who does not exhibit signs, or exhibits only early signs, of a disease or disorder. A prophylactic or preventative treatment is administered for the purpose of decreasing the risk of developing pathology associated with developing the disease or disorder.

As used herein, the terms "primer" and "oligonucleotide" refer to a polynucleotide that is capable of specifically hybridizing to a designated polynucleotide template and providing a point of initiation for synthesis of a complementary polynucleotide. Such synthesis occurs when the polynucleotide primer is placed under conditions in which synthesis is induced, *i.e.*, in the presence of nucleotides, a complementary polynucleotide template, and an agent for polymerization such as DNA polymerase. A primer is typically single-stranded, but may be double-stranded. Primers are typically deoxyribonucleic acids, but a wide variety of synthetic and naturally occurring primers are useful for many

applications. A primer is complementary to the template to which it is designed to hybridize to serve as a site for the initiation of synthesis, but need not reflect the exact sequence of the template. In such a case, specific hybridization of the primer to the template depends on the stringency of the hybridization conditions. Primers can be labeled with, *e.g.*, chromogenic, radioactive, or fluorescent moieties and used as detectable moieties.

As used herein, the term "purified" and like terms relate to an enrichment of a molecule or compound relative to other components normally associated with the molecule or compound in a native environment. The term "purified" does not necessarily indicate that complete purity of the particular molecule has been achieved during the process. A "highly purified" compound as used herein refers to a compound that is greater than 90% pure.

A "recombinant polypeptide" is one which is produced upon expression of a recombinant polynucleotide.

A "sample", as used herein, refers preferably to a biological sample from a subject, including, but not limited to, normal tissue samples, diseased tissue samples, biopsies, blood, saliva, feces, semen, tears, and urine. A sample can also be any other source of material obtained from a subject which contains cells, tissues, or fluid of interest. A sample can also be obtained from cell or tissue culture.

By the term "specifically binds to", as used herein, is meant when a compound or ligand functions in a binding reaction or assay conditions which is determinative of the presence of the compound in a sample of heterogeneous compounds.

The term "standard", as used herein, refers to something used for comparison. For example, it can be a known standard agent or compound which is administered and used for comparing results when administering a test compound, or it can be a standard parameter or function which is measured to obtain a control value when measuring an effect of an agent or compound on a parameter or function. Standard can also refer to an "internal standard", such as an agent or compound which is added at known amounts to a sample and is useful in determining such things as purification or recovery rates when a sample is processed or subjected to purification or extraction procedures before a marker

of interest is measured. Internal standards are often a purified marker of interest which has been labeled, such as with a radioactive isotope, allowing it to be distinguished from an endogenous marker.

A "subject" of analysis, diagnosis, or treatment is an animal. Such animals include in some embodiments mammals, which in some embodiments can be a human.

As used herein, a "subject in need thereof" is a subject, animal, mammal, or human, who will benefit from the method of the presently disclosed subject matter.

The term "substantially pure" describes a compound, *e.g.*, a protein or polypeptide which has been separated from components which naturally accompany it. Typically, a compound is substantially pure when at least 10%, more preferably at least 20%, more preferably at least 50%, more preferably at least 60%, more preferably at least 75%, more preferably at least 90%, and most preferably at least 99% of the total material (by volume, by wet or dry weight, or by mole percent or mole fraction) in a sample is the compound of interest. Purity can be measured by any appropriate method, *e.g.*, in the case of polypeptides by column chromatography, gel electrophoresis, or HPLC analysis. A compound, *e.g.*, a protein, is also substantially purified when it is essentially free of naturally associated components or when it is separated from the native contaminants which accompany it in its natural state.

The term "symptom", as used herein, refers to any morbid phenomenon or departure from the normal in structure, function, or sensation, experienced by the subject and indicative of disease. In contrast, a "sign" is objective evidence of disease. For example, in some embodiments a bloody nose is a sign. It is evident to the subject, doctor, nurse and other observers.

A "therapeutic" treatment is a treatment administered to a subject who exhibits signs of pathology for the purpose of diminishing or eliminating those signs.

A "therapeutically effective amount" of a compound is that amount of compound which is sufficient to provide a beneficial effect to the subject to which the compound is administered.

The term to "treat", as used herein, means reducing the frequency and/or

severity of at least one symptom that is experienced by a subject or subject or administering an agent or compound to reduce the frequency and/or severity of at least one symptom that is experienced.

II. Compositions

II.A. Generally

In some embodiments, the presently disclosed subject matter provides compositions for inhibiting bacterial biofilm development and/or for reducing or eliminating a bacterial biofilm present on a surface. In some embodiments, the compositions comprise an effective amount of a peptide or polypeptide comprising a peptide or polypeptide derived from and/or that is a fragment of an Adenylate Cyclase Toxin (ACT) polypeptide of *Bordetella*, such as but not limited to a catalytic domain (AC domain) thereof, a T25 peptide thereof, a T18 peptide thereof, or any combination thereof. In some embodiments, the peptide or polypeptide comprises, consists essentially of, or consists of an amino acid sequence selected from the group consisting of SEQ ID NOs: 1-5 and 44-53.

As used herein, the phrases "Adenylate Cyclase Toxin" and "ACT" refer to a bifunctional hemolysin-adenylate cyclase gene and/or gene product encoding or having an amino acid sequence emplified by, but not limited to, that set forth in GENBANK® biosequence database Accession Nos. NP_879578.1 (SEQ ID NO: 1), WP_080702041.1 (SEQ ID NO: 44), and WP_010927405.1 (SEQ ID NO: 49). This amino acid sequence is set forth in SEQ ID NO: 1. While this particular amino acid sequence represents the amino acid sequence of ACT from *Bordetella pertussis* Tohama I, it is recognized that the genomes of other isolates of *B. pertussis*, *B. bronchiseptica*, and/or *B. parapertussis* might encode ACT polypeptides with one or more modifications of the sequence of SEQ ID NO: 1. For example, the ACT polypeptide of certain isolates of *B. pertussis* include a serine at position 304 in place of the asparagine of SEQ ID NO: 1. The presently disclosed subject matter is understood to encompass all such ACT polypeptides, both naturally occurring and artificially produced.

As used herein, the phrases "Adenylate Cyclase domain" and "AC domain" refer to the catalytic domain of an ACT polypeptide. The AC domain of *B. pertussis* ACT includes the N-terminal approximately 400 amino acids of an ACT polypeptide. Exemplary AC domains include in some embodiments amino acids

1-398, in some embodiments amino acids 1-399, in some embodiments amino acids 1-400 of SEQ ID NO: 1 (*i.e.*, SEQ ID NO: 2), amino acids 1-400 of SEQ ID NO; 45 (*i.e.*, SEQ ID NO: 45), and amino acids 35-434 of SEQ ID NO: 49 (*i.e.*, SEQ ID NO: 50). Here as well, it is recognized that the genomes of other isolates of *B. pertussis*, *B. bronchiseptica*, and/or *B. parapertussis* might encode AC domain polypeptides with one or more modifications of the sequence of SEQ ID NOs: 2, 45, and 50, and the presently disclosed subject matter is understood to encompass all such AC domain polypeptides, both naturally occurring and artificially produced.

As used herein, the term "T25 peptide" refers to a subsequence of the AC domain of *B. pertussis* that includes in some embodiments amino acids 1-225 of SEQ ID NO: 1 or SEQ ID NO: 2. SEQ ID NO: 3 presents an amino acid sequence of an exemplary T25 peptide. Similarly, "T25 peptide" also refers to a subsequence of the AC domain of *B. bronchiseptica* that includes in some embodiments amino acids 1-225 of SEQ ID NO: 44 or SEQ ID NO: 45. SEQ ID NO: 46 presents an amino acid sequence of an exemplary T25 peptide. Also similarly, "T25 peptide" refers to a subsequence of the AC domain of *B. parapertussis* that includes in some embodiments amino acids 35-249 of SEQ ID NO: 49 or SEQ ID NO: 50. SEQ ID NO: 51 presents an amino acid sequence of an exemplary T25 peptide.

The term "T18 peptide" refers to a subsequence of the AC domain of *B. pertussis* that includes in some embodiments amino acids 226-400 of SEQ ID NO: 1 or SEQ ID NO: 2. Similarly, "T18 peptide" also refers to a subsequence of the AC domain of *B. bronchiseptica* that includes in some embodiments amino acids 226-400 of SEQ ID NO: 44 or SEQ ID NO: 45. SEQ ID NO: 47 presents an amino acid sequence of an exemplary T18 peptide. Also similarly, "T18 peptide" refers to a subsequence of the AC domain of *B. parapertussis* that includes in some embodiments amino acids 250-434 of SEQ ID NO: 49 or SEQ ID NO: 50. SEQ ID NO: 52 presents an amino acid sequence of an exemplary T18 peptide.

Thus, in some embodiments, a peptide or polypeptide of the presently disclosed subject matter comprises, consists essentially of, or consists of an amino acid sequence selected from the group consisting of SEQ ID NOs: 1-5 and 44-53.

Variants, both naturally occurring and artificially produced, are also encompassed by the presently disclosed subject matter. In some embodiments, a variant of a peptide or polypeptide of the presently disclosed subject matter is characterized by having a substantially identical amino acid sequence to one or more of SEQ ID NOs: 1-5 and 44-53. As used herein, a "substantially identical amino acid sequences" includes those amino acid sequences which have in some embodiments at least about 80% amino acid sequence identity, in some embodiments at least about 85% amino acid sequence identity, in some embodiments at least about 90% amino acid sequence identity, in some embodiments at least about 91% amino acid sequence identity, in some embodiments at least about 92% amino acid sequence identity, in some embodiments at least about 93% amino acid sequence identity, in some embodiments at least about 94% amino acid sequence identity, in some embodiments at least about 95% amino acid sequence identity, in some embodiments at least about 96% amino acid sequence identity, in some embodiments at least about 97% amino acid sequence identity, in some embodiments at least about 98% amino acid sequence identity, and in some embodiments at least about 99% or more amino acid sequence identity to an amino acid sequence as set forth herein. Amino acid sequence amino acid sequence identity, similarity, or identity can be computed by using the BLASTP and TBLASTN programs which employ the BLAST (basic local alignment search tool) 2.0.14 algorithm (Altschul *et al.*, 1990a; Altschul *et al.*, 1990b). The default settings used for these programs are suitable for identifying substantially similar amino acid sequences for purposes of the presently disclosed subject matter.

II.A. Pharmaceutical Compositions

In some embodiments, a composition of the presently disclosed subject matter is intended for use as a therapeutic or preventative. As such, in some embodiments the composition is a pharmaceutical composition comprising or consisting essentially of one or more peptides and/or polypeptides in combination with one or more pharmaceutically acceptable excipients and/or carriers. In some embodiments, a peptide or polypeptide of the presently disclosed subject matter is present in the pharmaceutical composition in an inactivated form.

As used herein, the terms "inactivated" and grammatical variants thereof

refer to a biomolecule that has been modified in order to reduce or eliminate a biological activity that, under certain circumstances, is undesirable. By way of example and not limitation, an ACT polypeptide or a fragment thereof (such as but not limited to an AC domain) is characterized by adenylate cyclase activity, which is known to be involved in the virulence of *B. pertussis*. This adenylate cyclase activity is in some embodiments undesirable, and thus inactivated versions of the presently disclosed peptides and polypeptides are employed in the compositions and methods of the presently disclosed subject matter. It has been determined that the adenylate cyclase activity of the peptides and polypeptides of the presently disclosed subject matter can be reduced or eliminated by making substitutions in the amino acid sequences of the peptides and polypeptides of the presently disclosed subject matter. An exemplary such modification is to substitute the aspartic acid at amino acid position 188 of SEQ ID NO: 1 or SEQ ID NO: 2 with a cysteine and to substitute the isoleucine at amino acid position 189 of SEQ ID NO: 1 or SEQ ID NO: 2 with a threonine. An inactivated ACT polypeptide or AC domain of the presently disclosed subject matter thus in some embodiments comprises, consists essentially of, or consists of SEQ ID NO: 5. Other methods of inactivation are also encompassed by the presently disclosed subject matter, including but not limited to treatment with chemical crosslinkers such as formaldehyde as is currently performed on many components of vaccines (*see e.g.*, U.S. Patent Nos. 4,057,626; 8,444,992).

In some embodiments, a pharmaceutical composition of the presently disclosed subject matter comprises a delivery vehicle, and in some embodiments the peptide or polypeptide is optionally associated with, conjugated to, and/or encapsulated by a delivery vehicle. In some embodiments, a he delivery vehicle comprises a liposome, a microparticle, or a nanoparticle, optionally wherein the liposome, microparticle, or nanoparticle is designed to be biodegradable in a subject. Biodegradable delivery vehicles are known in the art and can be designed to release the peptide(s) and/or polypeptide(s) of the presently disclosed subject matter under various release parameters. Exemplary delivery vehicles are described, for example, in U.S. Patent Nos. 7,393,924; 8,404,662; 9,028,864; 9,089,677; and 9,566,247; and in U.S. Patent Application Publication No. 2010/0021391, each of which is incorporated by reference in its entirety.

In some embodiments, a composition or pharmaceutical composition of the presently disclosed subject matter further comprises one or more pharmaceutically acceptable excipients and/or carriers (discussed in more detail herein below). In some embodiments, the one or more pharmaceutically acceptable excipients and/or carriers are pharmaceutically acceptable for use in a human (*i.e.*, are biocompatible with human administration).

The pharmaceutical compositions of the presently disclosed subject matter can be formulated for whatever route of administration that might be desirable. By way of example and not limitation, the pharmaceutical compositions of the presently disclosed subject matter can be formulated for oral administration, intravenous administration, intramuscular administration, intrathecal administration, cutaneous administration, topical administration, transdermal administration, systemic administration, subcutaneous administration, sublingual administration, buccal administration, ocular administration, otic administration, nasal administration, inhalation, nebulization, or any combination thereof.

The compositions of the presently disclosed subject matter have activity in inhibiting bacterial biofilm development and/or in reducing or eliminating a bacterial biofilm present on a surface. The compositions of the presently disclosed subject matter thus have activity in various different bacterial species that form biofilms. By way of example and not limitation, compositions of the presently disclosed subject matter have activity against bacterial biofilms comprising a strain of bacteria selected from the group consisting of *Bordetella spp.*, optionally *Bordetella pertussis* or *Bordetella bronchiseptica*; *Salmonella spp.*, optionally *Salmonella typhimurium*; *Pseudomonas sp.*, optionally *Pseudomonas aeruginosa*; coliform bacterial including *E. coli spp.*; *Listeria spp.*; *Neisseria spp.*; *Streptococcus spp.*; *Staphylococcus spp.*; *Yersinia spp.*; *Campylobacter spp.*; *Helicobacter spp.*; *Aeromonas spp.*; atypical Mycobacteria; and *Legionella spp.* It is noted, however, that compositions of the presently disclosed subject matter also have activity against other biofilm-producing microorganisms such as, but not limited to *Candida*, *Giardia*, and *Cryptosporidium*.

III. Methods of Use in Prevention and/or Treatment of Disorders, Diseases, and Conditions Associated with the Formation and/or Presence of Bacterial Biofilms

III.A. Generally

Bacterial biofilms are surface-associated communities of bacteria embedded in a self-produced matrix of polysaccharides, extracellular DNA (eDNA), and proteins. These communities are the most widely distributed and successful modes of life on earth, found in humans, plants, animals, and surfaces in the environment. While biofilms can drive important bio-geochemical processes, such as organic matter decomposition, nitrogen fixation, nitrification, denitrification, iron reduction, and sulfate reduction, harmful types of biofilms are associated with persistent infections in humans, plants, and animals, including infections associated with the use of medical devices and implants. In humans and animals, antibiotics are a common treatment for bacterial infections caused by biofilm, but bacteria in biofilms are frequently resistant to antibiotics due to changes in metabolism and antibiotic resistance, rendering antibiotic treatment ineffective.

The presently disclosed subject matter thus provides in some embodiments methods for using the presently disclosed compositions to prevent and/or treat a disease, disorder, or condition associated with the presence and/or development of bacterial biofilm in a subject. In some embodiments, the presently disclosed methods comprise administering to a subject a composition as disclosed herein in an effective amount and via a route sufficient for preventing and/or reducing the severity of at least one symptom of the disease, disorder, or condition. Various diseases, disorders, and conditions are known to be associated with the presence of bacterial biofilms. In some embodiments, a disease, disorder, or condition associated with a bacterial biofilm is selected from the group consisting of whooping cough, cystic fibrosis, bacterial vaginosis, urinary tract infections, infections associated with catheter use, middle ear infections, formation of dental plaque, gingivitis, eye infections associated with contact lens use, endocarditis, and infections resulting from use of medical and/or dental implants such as but not limited to joint prostheses and heart valves.

As such, in some embodiments the biofilm is present on a surface or could possibly grow on the surface, and the presently disclosed methods comprise contacting the surface with a composition of the presently disclosed subject matter. In some embodiments, the contacting comprises administering a pharmaceutical composition comprising the peptide or polypeptide to the subject in an amount and via a route of administration whereby the peptide or polypeptide contacts the surface or the biofilm present thereon and inhibits bacterial biofilm development on the surface and/or reduces or eliminates the existing bacterial biofilm present thereon.

Thus, in the instant context, in some embodiments a surface is a cellular surface, a tissue surface, and/or an organ surface present within a subject, including but not limited to a surface of the respiratory system such as but not limited to a nasal surface, a tracheal surface, and a lung surface. With respect to respiratory system surfaces, in some embodiments the respiratory surface is a nasal surface, a tracheal surface, and/or a lung surface, and the pharmaceutical composition is configured for inhalation and/or insufflation by the subject. In some embodiments, the composition comprises a delivery vehicle, optionally wherein the peptide or polypeptide is associated with, conjugated to, and/or encapsulated by a delivery vehicle in the pharmaceutical composition. In some embodiments, the delivery vehicle comprises a liposome, a microparticle, or a nanoparticle, optionally wherein the liposome, microparticle, or nanoparticle is designed to be biodegradable in the subject.

In some embodiments, a peptide and/or polypeptide composition of the presently disclosed subject matter is administered as part of a combination therapy with another therapeutic composition or method, which in some embodiments can be an anti-biofilm therapeutic composition or method. Exemplary anti-biofilm therapeutic compositions and methods include, but are not limited to those described in U.S. Patent Nos. 6,726,898; 6,830,745; 9,339,525; and 9,566,247; U.S. Patent Application Publication Nos. 2002/0037260; 2006/0105025; 2010/0322872; and references cited therein, each of which is incorporated by reference in its entirety.

III.B. Administration of Compositions

III.B.1. Routes of Administration

The compositions of the presently disclosed subject matter can be administered parenterally, systemically, topically, or any combination thereof. By way of example and not limitation, administration of a composition of the presently disclosed subject matter can be performed by intravenous (i.v.) injection, subcutaneous (s.c.) injection, intradermal (i.d.) injection, intraperitoneal (i.p.) injection, and/or intramuscular (i.m.) injection. One or more such routes can be employed. Parenteral administration can be, for example, by bolus injection or by gradual perfusion over time. Alternatively or in addition, administration can be by the oral route.

For delivery to nasal or lung surfaces, the compositions of the presently disclosed subject matter are in some embodiments suitable for administration via inhalation or insufflation.

Other acceptable routes of administration include but are not limited to oral (enteral), nasal, ophthal, and transdermal. In some embodiments, the administration is subcutaneous, and in some embodiments the subcutaneous administration is by an infusion pump.

III.B.2. Formulations

Pharmaceutical carriers, diluents, and excipients are generally added to the compositions of the presently disclosed subject matter (or kits comprising the same) that are compatible with the active ingredients and acceptable for pharmaceutical use (including but not limited to pharmaceutical use in a human). Examples of such carriers include but are not limited to water, saline solutions, dextrose, and/or glycerol. Combinations of carriers can also be used.

The compositions of the presently disclosed subject matter can further incorporate additional substances to stabilize pH and/or to function as adjuvants, wetting agents, and/or emulsifying agents, which can serve to improve the effectiveness of the pharmaceutical compositions.

In some embodiments, a composition can include one or more sugars, sugar alcohols, amino acids such but not limited to glycine, arginine, glutamic acid, and/or others as framework formers. The sugars can be mono-, di-, or trisaccharides. These sugars can be used alone and/or in combination with sugar

alcohols. Exemplary sugars include glucose, mannose, galactose, fructose, or sorbose as monosaccharides; sucrose, lactose, maltose, and trehalose as disaccharides; and raffinose as a trisaccharide. A sugar alcohol can be, for example, mannitose. In some embodiments, the composition comprises sucrose, lactose, maltose, trehalose, mannitol, and/or sorbitol. In some embodiments, the composition comprises mannitol.

Furthermore, in some embodiments compositions can include physiological well-tolerated excipients (*see* Strickley, 2004; Rowe *et al.*, 2006) such as antioxidants like ascorbic acid or glutathione; preserving agents such as phenol, m-cresol, methyl- or propylparabene, chlorobutanol, thiomersal/thimerosal, and/or benzalkoniumchloride; stabilizers, framework formers such as sucrose, lactose, maltose, trehalose, mannitose, mannitol, and/or sorbitol; mannitol and/or lactose and solubilizers such as polyethylene glycols (PEG; *e.g.*, PEG 3000, 3350, 4000, or 6000), cyclodextrins (*e.g.*, hydroxypropyl-β-cyclodextrin, sulfobutylethyl-β-cyclodextrin, or γ-cyclodextrin), dextranes, or poloxamers (*e.g.*, poloxamer 407 or poloxamer 188); or TWEEN® 20 or TWEEN® 80. In some embodiments, one or more well-tolerated excipients can be included, optionally selected from the group consisting of antioxidants, framework formers, and stabilizers.

III.B.3. Dosages

It is understood that a suitable dosage of a composition of the presently disclosed subject matter can depend upon the age, sex, health, and/or weight of the recipient, the kind of concurrent treatment, if any, the frequency of treatment, and the nature of the effect desired. However, it is understood that dosages can be tailored to the individual subject, as determined by the researcher or clinician. The total dose required for any given treatment will in some embodiments be determined with respect to a standard reference dose based on the experience of the researcher or clinician, such dose being administered either in a single treatment or in a series of doses, the success of which will depend on the production of a desired therapeutic response.

Thus, in some embodiments the overall administration schedule is considered in determining the success of a course of treatment and not whether a single dose, given in isolation, would or would not produce the desired immunologically therapeutic result or effect. As such, a therapeutically effective

amount can depend on the composition used, the nature of the disease condition, the severity of the disease condition, the extent of any need to prevent such a condition where it has not already been detected, the manner of administration dictated by the situation requiring such administration, the weight and state of health of the individual receiving such administration, and/or the sound judgment of the clinician or researcher. In some embodiments, the efficacy of administering additional doses and/or of increasing or decreasing the interval can be continually re-evaluated in view of the recipient's response.

The compositions of the present presently disclosed subject matter can in some embodiments also be contained in artificially created structures such as liposomes, which structures in some embodiments can contain additional molecules such as but not limited to proteins or polysaccharides, inserted in the outer membranes of said structures and having the effect of targeting the liposomes to particular areas of the body and/or to particular cells within a given organ or tissue. Such targeting molecules can in some embodiments comprise an immunoglobulin. Antibodies can work particularly well for targeting of liposomes and/or other scaffolds to tumor cells.

Single doses of in some embodiments about 1 to 50 µg, in some embodiments about 1 to 100 µg, in some embodiments about 1 to 500 µg, in some embodiments about 1 to 1000 µg, in some embodiments about 1 to 50 mg, in some embodiments about 1 to 100 mg, in some embodiments about 1 to 500 mg, or in some embodiments about 1 to 1000 mg of a peptide and/or polypeptide composition of the presently disclosed subject matter can be given and can depend from the respective compositions of compositions with respect to total amount for all peptides and/or polypeptides in the composition or alternatively for each individual peptide and/or polypeptide in the composition. A single dose of a peptide and/or polypeptide composition of the presently disclosed subject matter can in some embodiments have a target peptide amount (*e.g.*, total amount for all peptides and/or polypeptides in the composition or alternatively for each individual peptide and/or polypeptide in the composition) of about or at least 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 525, 550, 575, 600, 625, 650, 675, 700, 725, 750, 775, 800, 825, 850, 875, 900, or 950 µg. In some

embodiments, a single dose of a peptide and/or polypeptide composition of the presently disclosed subject matter can have a total peptide and/or polypeptide amount (*e.g.*, total amount for all peptides and/or polypeptides in the composition or alternatively for each individual peptide and/or polypeptide in the composition) of about or at least 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 525, 550, 575, 600, 625, 650, 675, 700, 725, 750, 775, 800, 825, 850, 875, 900, or 950 mg. In some embodiments, the peptides and/or polypeptides of a composition of the presently disclosed subject matter are present in equal amounts of about 100 micrograms per dose.

In a single dose of a peptide and/or polypeptide composition of the presently disclosed subject matter, the amount of each peptide and/or polypeptide in the composition is in some embodiments equal or substantially equal. Alternatively, a ratio of the peptides and/or polypeptides present in the least amount relative to the peptide and/or polypeptide present in the greatest amount is about or at least 1:1.25, 1:1.5, 1:1.75, 1:2.0, 1:2.25, 1:2.5, 1:2.75, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, 1:9, 1:10, 1:20, 1:30; 1:40, 1:50, 1:100, 1:200, 1:500, 1:1000, 1:5000; 1:10,000; or 1:100,000. Alternatively, a ratio of the peptides and/or polypeptides present in the least amount relative to the peptide and/or polypeptide present in the greatest amount is about or at least 1 or 2 to 25; 1 or 2 to 20; 1 or 2 to 15; 1 or 2 to 10; 1 to 3; 1 to 4; 1 to 5; 1 to 6; 1 to 7; 1 to 10; 2 to 3; 2 to 4; 2 to 5; 2 to 6; 2 to 7; 2 to 10; 3 to 4; 3 to 5; 3 to 6; 3 to 7; 3 to 10; 5 to 10; 10 to 15; 15 to 20; 20 to 25; 1 to 40; 1 to 30; 1 to 20; 1 to 15; 10 to 40; 10 to 30; 10 to 20; 10 to 15; 20 to 40; 20 to 30; or 20 to 25; 1 to 100; 25 to 100; 50 to 100; 75 to 100; 25 to 75, 25 to 50, or 50 to 75; 25 to 40; 25 to 50; 30 to 50; 30 to 40; or 30 to 75.

Single dosages can be given to a subject about or at least 1, 2, 3, 4, or 5 times per day. Single dosages can be given to a subject about or at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 18, 19, 20, 21, 22, 23, 24, 36, 48, 60, or 72 hours subsequent to a previous dose.

Single dosages can be given to a subject about or at least 1, 2, 3, 4, 5, 6, or 7 times per week, or every other, third, fourth, or fifth day. Single doses can also be given every week, every other week, or only during 1, 2, or 3 weeks per month. A course of treatment can in some embodiments last about or at least 1, 2,

3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 months.

In some embodiments, the single dosages of the compositions of the presently disclosed subject matter can be provided to a subject in at least two phases: *e.g.*, during an initial phase and then during a subsequent phase. An initial phase can be about or at least 1, 2, 3, 4, 5, or 6 weeks in length. The subsequent phase can last at least or about 1, 2, 3, 4, 5, 6, 7, or 8 times as long as the initial phase. The initial phase can be separated from the subsequent phase by about or at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 weeks or months.

The composition dosage during the subsequent phase can be at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1000 times greater than during the initial phase.

The composition dosage during the subsequent phase can be at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1000 times less than during the initial phase.

In some embodiments, the initial phase is about three weeks and the second phase is about 9 weeks. The compositions can be administered to the subject on or about days 1, 8, 15, 36, 57, and 78.

III.C. Kits and Storage

In some embodiments, a kit is disclosed comprising (a) a container that contains at least one composition as described herein, in solution or in lyophilized form; (b) optionally, a second container containing a diluent or reconstituting solution for the lyophilized formulation; and (c) optionally, instructions for (i) use of the solution or (ii) reconstitution and/or use of the lyophilized formulation. The kit can further comprise one or more of (iii) a buffer, (iv) a diluent, (v) a filter, (vi) a needle, or (v) a syringe. In some embodiments, the container is selected from the group consisting of a bottle, a vial, a syringe, a test tube, or a multi-use container. In some embodiments, the composition is lyophilized.

The kits can contain exactly, about, or at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 45, 46, 47, 48, 49, 50, 51, or more compositions. Each composition in the kit can be administered at the same time or at different times.

In some embodiments, the kits can comprise a lyophilized formulation of

the presently disclosed compositions in a suitable container and instructions for its reconstitution and/or use. Suitable containers include, for example, bottles, vials (*e.g.,* dual chamber vials), syringes (such as dual chamber syringes), and test tubes. The container can be formed from a variety of materials such as glass or plastic. In some embodiments, the kit and/or the container contain(s) instructions on or associated therewith that indicate(s) directions for reconstitution and/or use of a lyophilized formulation. For example, the label can indicate that the lyophilized formulation is to be reconstituted to concentrations as described herein. The label can further indicate that the formulation is useful or intended for subcutaneous administration. Lyophilized and liquid formulations are typically stored at -20ºC to -80ºC.

The container holding the composition(s) can be a multi-use vial, which in some embodiments allows for repeat administrations (*e.g.*, from 2-6 or more administrations) of the reconstituted formulation. The kit can further comprise a second container comprising a suitable diluent (*e.g.*, sodium bicarbonate solution).

In some embodiments, upon mixing of the diluent and the lyophilized formulation, the final concentration of an active agent in the reconstituted formulation is at least or about 0.15, 0.20, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 2.25, 2.5, 2.75, 3.0, 3.25, 3.50, 3.75, 4.0, 4.25, 4.5, 4.75, 5.0, 6.0, 7.0, 8.0, 9.0, or 10 mg/mL/target peptide. In some embodiments, upon mixing of the diluent and the lyophilized formulation, the concentration of an active agent in the reconstituted formulation is at least or about 0.15, 0.20, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 2.25, 2.5, 2.75, 3.0, 3.25, 3.50, 3.75, 4.0, 4.25, 4.5, 4.75, 5.0, 6.0, 7.0, 8.0, 9.0, or 10 µg/mL/active agent.

The kit can further include other materials desirable from a commercial and/or user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with or without instructions for use.

The kits can have a single container that contains the formulation of the target peptide compositions with or without other components (*e.g.*, other compounds or compositions of these other compounds) or can have a distinct container for each component.

Additionally, the kits can include a formulation of the presently disclosed compositions packaged for use in combination with the co-administration of a

second compound. One or more of the components of the kit can be pre-complexed or one or more components can be in a separate distinct container prior to administration to a subject. One or more of the components of the kit can be provided in one or more liquid solutions. In some embodiments, the liquid solution is an aqueous solution. In a further embodiment, the liquid solution is a sterile aqueous solution. One or more of the components of the kit can also be provided as solids, which in some embodiments can be converted into liquids by addition of suitable solvents, which in some embodiments can be provided in another distinct container.

The container of a therapeutic kit can be a vial, a test tube, a flask, a bottle, a syringe, or any other structure suitable for enclosing a solid or liquid. Typically, when there is more than one component, the kit contains a second vial or other container that allows for separate dosing. The kit can also contain another container for a pharmaceutically acceptable liquid. In some embodiments, a therapeutic kit contains an apparatus (*e.g.*, one or more needles, syringes, eye droppers, pipette, etc.), which enables administration of the peptide and/or polypeptide compositions of the disclosure that are components of the kit.

IV. Methods for Inhibiting the Development and/or Reducing the Presence of Biofilm on Abiotic Surfaces

The presence of biofilm on abiotic surfaces is also detrimental in the medical, dental, and industrial contexts. Thus, in some embodiments the presently disclosed subject matter provides compositions and methods for inhibiting the development and/or reducing the presence of biofilm on abiotic surfaces.

In some embodiments, the presently disclosed subject matter provides methods for inhibiting bacterial biofilm development and/or for reducing or eliminating a bacterial biofilm present on an abiotic surface that comprises contacting the abiotic surface or the biofilm present thereon with an effective amount of a peptide or polypeptide derived from Adenylate Cyclase Toxin (ACT) of *Bordetella* or a catalytic domain (AC domain) thereof, optionally wherein the peptide or polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 1-5 and 44-53, whereby bacterial biofilm development on the abiotic surface is inhibited and/or existing bacterial biofilm on the on the abiotic surface is reduced or eliminated. In some embodiments, the

bacterial biofilm comprises a strain of bacteria selected from the group consisting of *Bordetella spp.*, optionally *Bordetella pertussis* or *Bordetella bronchiseptica*; *Salmonella spp.*, optionally *Salmonella typhimurium*; and *Pseudomonas spp.*, optionally *Pseudomonas aeruginosa*. Other exemplary microorganisms associated with biofilm formation for which the presently disclosed methods is applicable include coliform bacterias, *Yersinia, Campylobacter, Helicobacter, Aeromonas*, atypical *Mycobacteria*, *Giardia* cysts, *Cryptosporidium* oocysts, *Klebsiella pneumoniae*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Porphyromonas gingivalis*, and *Legionella*.

IV.A. Methods for Reducing the Incidence of Nosocomial Infection

Biofilms are also associated with nosocomial (*i.e.*, hospital-acquired) infections, and thus use the presently disclosed subject matter provides methods for reducing the incidence of nosocomial infection associated with the presence of or the development of biofilm. Exemplary biofilm-forming organisms associated with nosocomial infections include *Klebsiella pneumoniae*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Porphyromonas gingivalis*, *Pseudomonas aeruginosa*, and others. Whereas antibiotics are typically employed to combat these bacteria, antibiotic resistance in these species is increasing such that the frontline strategies are becoming less effective and in fact could be contributing to the problems associated with the presenece of biofilms inn medical and/or dental facilities.

In some embodiments, the presently disclosed subject matter relates to methods for reducing the incidence of nosocomial infection, wherein the method comprises contacting a surface present in a medical and/or dental facility with a composition as described herein in an amount sufficient to inhibit bacterial biofilm development and/or reduce or eliminate bacterial biofilm present on the surface, thereby reducing the incidence of a nosocomial infection associated with the incidence of nosocomial infection is a subject. In some embodiments, the surface is a door surface, a door handle surface, a sink surface, a toilet surface, a faucet surface, a furniture surface, optionally a bed surface, and a window surface.

IV.B. Methods for Reducing Biofilms on Medical and Dental Devices

In some embodiments, an abiotic surface is a part of a device selected from the group consisting of a medical device, a dental device, and an industrial

device.

In some embodiments, the medical/dental device is selected from the group consisting of a surgical tool, an implant, a catheter, a stent, a replacement for a bone or joint (*e.g.*, a hip, knee, ankle, elbow, or shoulder replacement device), and ventilator tubing. In some embodiments, the medical device is a cardiac implant. Reducing or eliminating biofilm on any such device can reduce infections associated with the use of the devices.

IV.C. Methods for Reducing Biofilms on Industrial Devices

Biofilms present on industrial devices are also associated with various deleterious effects. This is particularly common with respect to devices employed in water treatment, sewage treatment, petroleum manufacturing and/or storage, and recycling. As such, in some embodiments the industrial device is selected from the group consisting of a pipe, a tube, a valve, an air-cooled tower, a warm water system, a coolant circuit, a silo, a fermenter, a colander, a piece of furniture, and a sink.

Irrespective of the location and/or type of surface, in some embodiments the presently disclosed methods further comprise contacting the surface with one or more additional compositions that inhibit bacterial biofilm development and/or reduces or eliminates bacterial biofilm present on the surface.

EXAMPLES

The following Examples provide further illustrative embodiments. In light of the present disclosure and the general level of skill in the art, those of skill will appreciate that the following EXAMPLES are intended to be exemplary only and that numerous changes, modifications, and alterations can be employed without departing from the scope of the presently disclosed subject matter.

Materials and Methods for the EXAMPLES

Bacterial strains and growth conditions. *Bordetella pertussis* wild-type BP338 (Tohama I); Bvg(2) BP347 (TN5::*bvgS* mutant derived from BP338); wild-type BPSM (Tohama I); BPSM JS20 (*fhaB* ΔMCD and C-terminal prodomain derivative of BPSM); and BPSM T-N (*fhaB* with transposon insertion in C-terminal prodomain derivative of BPSM) were grown on Bordet-Gengou (BG) agar (GIBCO™ brand, available from Thermo Fisher Scientific Inc., Waltham, Massachusetts, United States of America) supplemented with 15% defibrinated

sheep blood (Cocalico Biologicals Inc., Reamstown, Pennsylvania, United States of America) for 48 hours at 37ºC. The same growth conditions were used for *B. bronchiseptica* strains, wild-type RB50; Bvg(–) RB54; RBX11-JS20 (*fhaB* ΔMCD and C-terminal prodomain); and RBX11 T-N (*fhaB* with transposon insertion in C-terminal domain). These *B. pertussis* strains have been previously reported (Weiss *et al.*, 1983; Mazar & Cotter, 2006), as have *B. bronchiseptica* strains (Mazar & Cotter, 2006). Bacteria were then transferred to liquid culture in modified synthetic Stainer-Scholte liquid medium (SSM) and grown for 24 hours at 37ºC, shaking at 150 RPM. Bacteria were pelleted and washed in SSM, and then resuspended to an OD_{600} of 0.1 for biofilm experiments.

Strain construction (ΔAC domain). Two DNA fragments (517 and 513 bps), corresponding to the 5' and the 3' flanking region of the in-frame deletion, were amplified from *B. pertussis* genomic DNA as a template by PCR using the following two pairs of primers:

F1: 5'-TTTACTAGTGGGATTGAGGAGGGAGGGC-3' (SEQ ID NO: 6);
R1: 5'-TTTATGCATGTGGATCTGTCGATAAGTAGTC-3' (SEQ ID NO: 7);
F2: 5'-TTTATGCATAAGTTCTCGCCGGATGTACTG-3' (SEQ ID NO: 8);
R2: 5'-TTTGAATTCGCCGCCTCCCAGCGCCAT-3' (SEQ ID NO: 9).

The primers were cut with the *Spe*I/*Nsi*I and *Nsi*I/*Eco*RI respectively, and ligated with the *Spe*I/*Eco*RI-cleaved pSS4245 vector (*see* Inatusuka *et al.*, 2010; U.S. Patent No. 9,187,754). Bacteria were mated following the previously described methods for *B. pertussis*. Briefly, *B. pertussis* BP338 was passaged 2-3 days on BG and grown over night in SSM. The OD_{600} was between 0.7 and 0.8. RHO3 *E. coli* were grown in antibiotic selection media, LB + 150 µg/ml ampicillin (AMP) supplemented with 400 µg/ml DAP to mid-log phase. A 2:1 donor to recipient ratio was used. After washing, the bacteria were resuspended in 100 ml SSM and plated on BG + $MgSO_4$ + DAP plates. After an overnight incubation (approximately 15 hours), the plates were swabbed, the material was washed to remove residual DAP and resuspended in 100 ml SSM. Bacteria were plated on BG + $MgSO_4$ + AMP. Colonies were isolated and confirmed to lack the AC domain via western blot and PCR.

ACT and ACT mutant protein purification. As previously described, calmodulin columns were used to isolate holotoxin ACT from whole-cell urea

extracts of XL1-Blue *E. coli* (Agilent Technologies, Santa Clara, California, United States of America) transformed with plasmid *pT7cACT1* (Osička *et al.*, 2000) Delivery of CD8+ T-Cell Epitopes into Major Histocompatibility Complex Class I Antigen Presentation Pathway by Bordetella pertussis Adenylate Cyclase: Delineation of Cell Invasive Structures and Permissive Insertion Sites. Infect Immun. 68:247-256). Similar methods were used to purify the ACT deletion mutants expressed from the previously described plasmid constructs with similar backbone, whole-cell urea extracts of XL1-Blue *E. coli* transformed with the following plasmids and urea-extracted material was used in assays described below; $ACT_{\Delta AC}$ (pCACT Δ-373), $ACT_{\Delta HR}$ (pCACT Δ385-1489), $ACT_{\Delta H}$ (pCACT Δ385-1006), $ACT_{\Delta R}$ (pCACT ΔC217), and iACT (pGW44/188; Lee *et al.*, 1999). The AC domain was purified from the *E. coli* BL21 (kDE3) strain expressing the AC-int-CBD fusion protein (Sadilkova *et al.*, 2008). Briefly, bacteria were grown at 30ºC in MDO medium supplemented with 150 mg/ ml of ampicillin. Cultures were induced with IPTG (1 mM), the cells were washed and resuspended in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl (TN buffer) containing 1 mM EDTA (EDTA buffer), and disrupted by sonication. For protein purification by chitin affinity chromatography, the cell extract was cleared at 20,000 x g and loaded on a chitin bead column. After washing, EDTA buffer containing 50 mM dithiothreitol was loaded on the column to promote self-excision of the intein-CBD from the AC-intein-CBD fusion protein during overnight incubation at 4ºC. The AC domain was then eluted with EDTA buffer and dithiothreitol was removed by dialysis against TN buffer. Purified proteins were stored at -70ºC until use.

FHA and FHA44 purification. FHA was produced using *B. pertussis* Tohama I, while *B. pertussis* Fha44-stop was used to produce the ~80 kDa N-terminal fragment of FHA that comprises residues 72-862 of FhaB (the fhaB allele having a stop codon inserted at position 863 was constructed according to Renaud-Mongenie). Bacteria were grown in 1 liter (l) shaking liquid cultures in SSM supplemented with 1 g/1 of (2,6-O-dimethyl)-b-cyclodextrin (Sigma-Aldrich Corp., St. Louis, Missouri, United States of America). At OD_{600} ≥4.0, culture supernatants were collected by centrifugation at 10,000 x g for 20 minutes at 4ºC and were sterilized by passage through filters with 0.22 µm pore diameter before loading onto 5 ml CELLUFINE™ brand sulfate columns (JNC Corporation, Tokyo,

Japan) equilibrated with 10 mM sodium phosphate buffer at pH 7.6 (buffer A). After sample application, both matrices containing adsorbed FHA or FHA44 were washed with 80 column volumes (cv) of buffer A, after which a second wash of 20 cv was performed with buffer A containing 300 mM NaCl. The purified FHA and FHA44 proteins were eluted with 700 mM NaCl in buffer A and were stored frozen at -70ºC until use. The entire purification procedure on chromatography column was performed at 4ºC.

Microtiter crystal violet assay. *Bordetella* biofilm was measured using the microtiter plate assay, coupled with crystal violet staining, as previously described (O'Toole, 2011). Briefly, *Bordetella* bacteria were grown in a total volume of 100 µl of Stainer-Scholte medium at 37ºC in 96-well polyvinylchloride (PVC), round bottom, non-tissue-culture treated microtiter plates (NUNC™ brand, available from Thermo Fisher Scientific Inc., Waltham, Massachusetts, United States of America). *B. pertussis* biofilm was measured at 96 hours and *B. bronchiseptica* biofilm was measured at 72 hours. Wells were washed at the final time point to remove planktonic bacteria. Bacterial cells that remained attached to the wells were stained with a 0.1% solution of crystal violet (CV) and were incubated at room temperature for 30 minutes. The washing process was repeated, and the CV stain was solubilized from bacterial cells with 200 µl of 95% ethanol. Biofilm formation was quantitated by measuring OD_{595}.

Scanning electron microscopy. Biofilms were grown statically in 24-well non-tissue culture treated polypropylene plates (0.5 ml cultures per well), and a circular microscope cover glass (12CIR.-1.5) was inserted into each well. Bacteria successfully adhered to the coverslip, as initially confirmed by Gram stain, and matrix was present as determined by Calcofluor White staining. Coverslips were placed in 4% paraformaldehyde to fix samples. *B. pertussis* biofilm was observed on the surfaces of the coverslips at 96 hours under various conditions.

Aggregation assay. Bacterial aggregation was measured using previously described methods for *B. pertussis* (Arnal *et al.*, 2015). Briefly, BP338 was grown in the presence and absence of AC domain for 24 hours at 37ºC shaking. At 24 hours, six 1 ml samples were collected. Three were homogenized by vigorous vortexing and three were not homogenized before centrifugation at 650 x g for 2

minutes. The OD_{600} was then measured from the supernatant, and the aggregation index (AI) was calculated based on the following equation:

$$(OD_{homogenized} - OD_{nonhomogenized})/OD_{homogenized}$$

Three independent experiments were performed in triplicate.

Surface plasmon resonance (SPR). SPR measurements were performed at 25ºC using a ProteOn XPR36 Protein Interaction Array System (Bio-Rad, Hercules, California, United States of America). FHA and FHA44 were diluted to a final concentration of 1 mg/ml in PBS containing 0.005% TWEEN® 20 brand non-ionic detergent and immobilized as ligands to a GLC sensor chip (Bio-Rad Laboratories, Hercules, California, United States of America) at flow rate of 30 µl/min. SPR measurements were carried out in the running buffer containing PBS supplemented with 0.005% TWEEN® 20 brand non-ionic detergent at the flow rate of 30 µl/min for association and dissociation phase of sensograms. The purified AC domain was diluted in the running buffer to the indicated concentrations and injected in parallel ("one-shot kinetics") over the chip surface. The sensograms were corrected for sensor background by interspot referencing (the sites within the 6 x 6 array which are not exposed to ligand immobilization but are exposed to analyte flow), and double referenced by subtraction of analyte (channels 1-5) using a 'blank' injection (channel 6). The data were analyzed globally by fitting both the association and the dissociation phases simultaneously for five different AC domain concentrations using a 1:1 Langmuir-type binding model. An apparent equilibrium dissociation constant (K_D) was determined as KD = k_d/k_a.

ELISA-based binding assay. ELISA-specific MaxiSorp 96-well immunoplates (Thermo Fisher Scientific Inc., Waltham, Massachusetts, United States of America) were coated with 0.5 µg/ml FHA overnight in 100 µl bicarbonate solution. Before beginning the assay, the wells were washed and then blocked in 5% milk 1X PBS 0.05% Tween for 1 hour. Wells were washed and control (no protein), ACT, AC domain, or $ACT_{\Delta AC}$ (50 µl) was added for 30 minutes. Anti-MCD antibodies described previously (Noel *et al.*, 2012; 50 µl of 1:100,000 dilution) were added to wells for 30 minutes after the addition of the first 50 µl solution. Wells were washed and a secondary anti-rabbit-HRP linked antibody was added to wells for 1 hour. Wells were washed again and the

detection solution (SUREBLUE™ TMB Microwell Peroxidase Substrate; VWR, Radnor, Pennsylvania, United States of America) was added for 15 minutes. HCl 1N was added to stop the detection solution reaction and the absorbance at OD_{450} was read using an μQUANT™ brand ELISA reader (BIOTEK® Instruments, Inc., Winooski, Vermont, United States of America).

Statistics. Statistical analysis was performed using Student's unpaired t test with Welch's correction, assuming Gaussian distribution (parametric test), these tests were performed on data sets to compare conditions within experiments.

EXAMPLE 1

Exogenous ACT Inhibits Biofilm in a Concentration-dependent Manner

Whether ACT had an inhibitory effect on *B. pertussis* biofilm was tested. The *B. pertussis* mutant, BP348, lacking ACT by virtue of a transposon insertion in *cyaA,* and the parental wild-type strain, BP338, were compared for their abilities to form biofilm. BP338, BP348, and BP347 were grown in 96-well plates and biofilm formation was measured by bacterial accumulation on wells using the crystal violet assay. As shown in Figure 1, BP348 made more biofilm than BP338. Bvg(-) BP347, a negative control, formed no biofilm; the low, but measurable, OD_{595} reflected basal levels of bacterial adherence, and was previously observed for Bvg(-) *B. pertussis* (Mishra *et al.*, 2005). These differences in biofilm formation were not due to differences in bacteria growth, in 10 ml SSM shaking cultures all strains grew at the same rate, while under 100 μl static culture conditions in 96-well plates, BP338 and BP348 grew at similar rates. BP347, which does not make biofilm, reached a higher OD_{600} more quickly (Figures 2A and 2B), excluding impaired bacterial growth as the cause of differences in biofilm formation.

Exogenous, purified, recombinant ACT was then tested for its ability to inhibit biofilm formation by *B. pertussis* and *B. bronchiseptica*. Exogenous ACT was added at concentrations of 10, 100, or 1000 ng/ml (56 pM, 565 pM, and 5650 pM respectively) to BP338 cultures. The concentration-dependent inhibition of biofilm is shown in Figure 3 (IC_{50} 17.32 ng/ml to reduce biofilm to negative control OD_{595}). *B. pertussis* grown in the presence of ACT or urea, a major component of the solution in which ACT was stored, grew at the same rate as in media alone, further showing that differences in growth rate did not account for reduced biofilm formation (Figure 4). The same concentration-dependent inhibition of biofilm by

ACT occurred with *B. bronchiseptica* (Figure 5). Importantly, the concentrations of ACT (approximately 100 ng/ml) employed were comparable to calculated concentrations found in nasopharyngeal washes from baboons and infants infected with *B. pertussis* (Eby *et al.*, 2013).

EXAMPLE 2

The AC Domain is Necessary and Sufficient for Inhibition of Biofilm

ACT is a 177 kDa, multi-domain protein, and to determine which part(s) of the ACT molecule were involved in the inhibitory effect, truncated ACT variants and an ACT protein mutant lacking AC enzymatic activity (iACT) were tested for their abilities to inhibit biofilm formation (Figure 6 is a schematic representation of modifications). These variants, which were partial deletions from full-length Δ*cyaA* (Sebo & Ladant, 1993; Sadilkova *et al.*, 2008), were previously used to characterize monoclonal antibodies (Lee *et al.*, 1999) and have been used previously in functional assays (Sakamoto *et al.*, 1992; Iwaki *et al.*, 1995; Macdonald-Fyall *et al.*, 2004; Eby *et al.*, 2014). All ACT variants, with one exception, inhibited biofilm formation to the same extent as full-length ACT (Figure 6). $ACT_{\Delta AC}$, ACT lacking the catalytic domain, was without an inhibitory effect at 100 ng/ml, while the 43 kDa AC domain was comparable to native ACT in inhibiting biofilm formation at just 10 ng/ml (comparable molar concentrations), and did so in a concentration-dependent manner (Figure 7). iACT, the enzymatically inactive form of the toxin (1000-fold reduction in enzymatic activity in comparison to ACT holotoxin), inhibited biofilm comparably to ACT at 100 ng/ml (Figure 8), establishing that the enzymatic activity of the toxin was not required for the inhibitory effect. Thus the AC domain was both necessary and sufficient for biofilm inhibition, yet its catalytic activity contained in the AC domain was not required.

The AC domain is the most conserved portion of ACT among *Bordetella* species that encode the toxin (Park *et al.*, 2012), indicating the possibility of a comparable inhibitory role for the AC domain in biofilm regulation among the *Bordetellae*. The AC domain is also necessary and sufficient to inhibit *B. bronchiseptica* RB50 biofilm (Figure 9). To confirm the role of endogenous ACT and its catalytic domain, BP338 ΔAC was constructed. The portion of *cyaA* encoding the AC domain (Δ1-373) was deleted and the resultant strain, BP338

ΔAC, was tested for biofilm formation as in *in vitro* assays described above. A Western blot was completed to show that the BP338 ΔAC mutant expressed truncated ACT peptide (Figure 10). As expected, BP338 ΔAC made more biofilm than the parental wild-type BP338 (Figure 11), confirming the necessity for and specificity of the AC domain to inhibit biofilm in *B. pertussis*.

In that the crystal violet assay is an indirect quantification of biofilm, scanning electron microscopy was employed to determine the effects of AC domain on BP338 biofilm, using BP347 as a biofilm-negative control. *B. pertussis* was allowed to form biofilm on glass coverslips in the absence and presence of exogenous AC domain to complement data from the crystal violet assay. Samples obtained under these conditions were imaged using a Zeiss Sigma VP HD field emission scanning electron microscope (SEM). Figures 12A-12C illustrate the dramatic effects of AC domain under these conditions. Wild-type BP338 (Figure 12A) and Bvg(-) BP347 (Figure 12B) biofilm were compared to BP338 grown in the presence of 10 ng/ml AC domain (Figure 12C). The exogenous AC domain precluded biofilm accumulation on glass coverslips, such that BP338 plus AC domain was equivalent to the negative control, BP347. In the images of BP347 and BP338 plus AC domain, there were few bacteria adherent to the coverslip. The lack of bacterial accumulation under these conditions suggested a defect in the initial binding of bacteria, which then impairs subsequent biofilm accumulation. Thus, the initial step of binding to the abiotic surface would be one determinant of the ability of *B. pertussis* to produce robust biofilm.

EXAMPLE 3

AC Domain Inhibits Bacterial Aggregation and Disrupts Preformed *B. pertussis* Biofilm

To address the underlying mechanisms of ACT inhibition of biofilm, other steps in the biofilm life cycle were tested for susceptibility to ACT inhibition. Bacterial aggregates form in shaking culture and positively correlate with biofilm formation in many bacterial species (Sorroche *et al.*, 2012; Kragh *et al.*, 2016). Exogenous AC domain was added to growing cultures of *B. pertussis* and the aggregation index was determined at 24 hours, as previously described for *B. pertussis* (Arnal *et al.*, 2015). Exogenous AC domain decreased bacteria aggregation by 75% (Figure 13A).

The final stages of the biofilm lifecycle involve dispersal of the bacteria from the biofilm structure. In order to investigate further the regulatory effect of ACT on biofilm, the effect of AC domain when added to existing biofilm was tested. Figure 13B shows the time course of biofilm formation in the presence (gray line) and absence of AC domain (black line). When AC domain was added to BP338 biofilm at 72 hours post-inoculation and measured 24 hours later (96 hours), biofilm formation was reduced 76% (dotted line) compared to BP338 alone at 96 hours, resulting in quantities comparable to biofilm formed in the continuous presence of AC domain (gray line). This disruption of existing biofilm did not occur when full-length ACT was added at 72 hours. Although the mechanism of disruption is unknown, it appeared that the lack of effect of ACT was due to limitations in the ability of the large hydrophobic protein to access the necessary site(s) within the biofilm. Collectively, these data suggested that ACT played several roles in regulation of *Bordetella* biofilm, not only during the initial steps, but also during later stages.

EXAMPLE 4

The Inhibition of Biofilm Formation by ACT Was Specific

To characterize the inhibitory effects of exogenous ACT and AC domain on *B. pertussis* biofilm formation, molecules that interact with ACT were tested for their abilities to affect ACT-mediated inhibition. Calmodulin (CaM) binds the AC domain of ACT with high affinity (K_d ~2 nM) and activates its enzymatic activity (Guo *et al.*, 2005). It has been previously demonstrated that addition of CaM to ACT, prior to incubation with cells, blocks translocation of the AC domain into the cell cytosol, thereby precluding cAMP production (Mouallem *et al.*, 1990; Gray *et al.*, 2001). In the present studies, purified ACT or AC domain and CaM were combined before addition to bacteria. Under these conditions, a molar excess of CaM prevented the inhibitory effect of ACT or AC domain on biofilm formation (Figure 14). Similarly, an antibody directed against the catalytic domain of ACT blocked the inhibitory effects of ACT and AC domain on biofilm (Figure 14). The fact that CaM or an antibody blocked the inhibitory effect of ACT suggested the possibility that CaM caused a disruption of a physical interaction between ACT and another bacterial factor, such as filamentous haemagglutinin (FHA), which is involved in biofilm formation.

EXAMPLE 5

The AC Domain Interacted with FHA

In a *B. pertussis* mutant lacking FHA, ACT is present in the media as opposed to remaining surface-associated (Weiss *et al.*, 1983) and ACT interacts with FHA on the surface of bacteria (Zaretzky *et al.*, 2002). These data suggested the possibility that ACT directly interacts with FHA to inhibit biofilm formation. In light of this collection of observations and the fact that AC domain was necessary and sufficient for inhibition of biofilm, the interaction of the AC domain with FHA was examined by surface plasmon resonance (SPR).

The FHA (~220 kDa) protein, purified from *B. pertussis* culture supernatant (Figure 15), was immobilized on GLC sensor chip and real-time kinetics of the interaction of the recombinant AC domain with FHA was analyzed by parallel injection of diluted AC protein over the sensor chip surface at a constant flow rate of 30 µl/min (Figure 16A). Interaction of the AC domain with FHA was specific, since negligible binding of the AC domain was observed to the chip coated with FHA44, a truncated FHA protein corresponding to residues 72-862 of FHA, which did not contain the c-terminal domain (Figure 16B). Kinetic parameters of the AC-FHA interaction were calculated from global fitting of concentration-dependent binding curves. As shown in Figure 16A, the binding curves fit well to a Langmuir-type binding model indicating a simple 1:1 interaction between the AC domain and FHA with equilibrium dissociation constant (K_D) of approximately 650 nM. These data suggested that the AC domain only interacted with FHA when the C-terminal segment was present.

To complement the functional data which showed CaM blocked the inhibitory effects of AC domain on biofilm formation, AC domain was mixed with CaM in molar ratios of 10:1, 1:1 or 1:10 and the capacity of AC domain-CaM complex to interact with FHA was probed by SPR. As shown in Figure 16C, binding of the AC domain to FHA in the presence of CaM (10:10) was reduced approximately 75% as compared to AC domain alone, suggesting that CaM and FHA competed for the same site on AC domain, or that CaM binding altered conformation of the AC domain thereby interfering with the ACT-FHA interaction. The data presented in Figure 14 showed that an excess of CaM blocked AC domain inhibition of biofilm, and the data in Figure 16C showed that equal molar

ratios of AC domain:CaM reduced binding to FHA by 75%. Based on these data, it was hypothesized that the molar excess of CaM used in the biofilm assay blocked the inhibitory effects of ACT on biofilm formation by blocking the physical interaction between ACT and FHA.

In that the C-terminal portion of FHA was required for AC domain binding to FHA (Figure 16B, FHA44), it was hypothesized that ACT and the AC domain would block specific antibody interactions with FHA. To test this hypothesis, an ELISA-based assay to characterize the interaction between ACT and the C-terminal segment of FHA was developed. Plates were coated with full length FHA and incubated with buffer, ACT, or AC domain over a range of concentrations (0.1–10 μg/ml), or $ACT_{\Delta AC}$ at 10 μg/ml. Monoclonal antibodies directed against the mature C-terminal domain (MCD) of FHA (residues 1870–2362) were used to determine the accessibility of the C-terminal segment of FHA. This anti-MCD antibody has been used previously to detect FHA and study FHA processing (Mazar & Cotter, 2006; Noel *et al.*, 2012), and was used in the experiments disclosed herein because it recognizes a large portion of FHA that was deleted from the truncated FHA44 mutant protein.

The presence of ACT or AC domain blocked anti-MCD antibodies from binding to FHA (Figure 17). Both ACT and the AC domain produced a concentration-dependent inhibition of anti-MCD antibody binding to FHA, but, in accordance with the earlier functional data on inhibition of biofilm, $ACT_{\Delta AC}$ had no effect (Figure 17). Furthermore, incubation of ACT or the AC domain with CaM prior to addition to FHA-coated plates precluded them from blocking the binding of MCD antibody to FHA (striped bars; Figure 17). Because the data regarding ACT inhibition of biofilm correlated with the physical binding of the AC domain and FHA, the consequences of their physical interaction were investigated to better understand the molecular mechanisms involved in biofilm inhibition.

In summary, the data presented in Figures 16A-16C demonstrated that with higher concentrations of the the AC domain, there was more of an SPR response recorded because more of the AC domain was binding the FHA-coated sensor chip.

EXAMPLE 6

The MCD of FHA Was Required for ACT Inhibition of Biofilm

FHA is delivered to the bacterial surface via a two-partner secretion pathway. This process involves translocation of FhaB, the FHA precursor, through FhaC, an FhaB-specific outer-membrane transporter (Fan *et al.*, 2012). FhaB enters the FhaC channel as a hairpin and then begins folding in an N-to-C-terminal manner on the cell surface, creating a β-helical shaft (Mazar & Cotter, 2006; Mazar & Cotter, 2007). After the region distal to the β-helical shaft reaches the cell surface, the C-terminal prodomain is proteolyzed in the periplasm, creating the "mature C-terminal domain" (MCD), which is located on the distal portion of FHA (Noel *et al.*, 2012).

To better understand the functional domains involved in ACT binding and inhibition of biofilm, *B. pertussis* and *B. bronchiseptica* mutants with altered secretion and processing of FHA were examined.

Since the MCD of FHA is required for AC domain binding (Figure 16B), it was hypothesized that the MCD must be present in order for ACT inhibition to occur. A *B. pertussis* mutant lacking the MCD was assessed for biofilm formation in the presence and absence of ACT to determine the role of the MCD in the inhibitory process. BPSM JS20, a derivative of parental strain BPSM, produces a truncated FHA by virtue of deletion of the MCD and C-terminal prodomain and, is therefore, composed only of the β-helical shaft. BPSM and BPSM JS20 were grown in the presence and absence of ACT and biofilm formation was measured at 96 hours. wild-type BPSM formed biofilm that was susceptible to inhibition by ACT. Importantly, the BPSM isogenic strain and the BP338 isogenic strain, both of which are Tohama I derivatives, were compared for biofilm formation, ACT expression, and FHA expression. No significant differences were observed in biofilm formation between the parental wild-type strains (Figure 18A). FHA protein expression was similar between the two strains, although there was slightly more ACT protein expression in BPSM (Figure 18B). BPSM JS20 formed equivalent amounts of biofilm in the presence and absence of exogenous ACT (Figure 19A) and made more biofilm than the parental BPSM strain in the absence of exogenous ACT. Without wishing to be bound by any theory, this might have been due to the inability of endogenous ACT to have an effect on BPSM JS20 biofilm.

The equivalent BPSM JS20 mutant strain in *B. bronchiseptica*, which was derived from RBX11 and lacked the MCD and C-terminal prodomain, produced biofilm that was not inhibited by ACT (Figure 20). Although the MCD was not required for biofilm formation, it appeared to be necessary for ACT-mediated inhibition of biofilm to occur. These data were consistent with the SPR results showing ACT did not bind FHA44, which lacks the MCD (Figure 16B).

To validate the role of the MCD in ACT inhibition of biofilm, a mutant in which the MCD is improperly folded was tested. BPSM T-N, also derived from BPSM parental wild-type strain, contained a mutation in *fhaB* such that a stop codon was introduced in the region encoding the N-terminus of the prodomain. As a result, the MCD was present and located distally from the cell surface, but was not folded in its native conformation (Mazar & Cotter, 2006; Noel *et al.*, 2012). BPSM T-N formed similar amounts of biofilm compared to BPSM, yet like BPSM JS20, ACT did not inhibit biofilm formation of this strain (Figure 19A). The same was true for RBX11 T-N, the equivalent *B. bronchiseptica* strain with a misfolded MCD (Figure 20). Although the MCD itself was not required for biofilm formation, the MCD of FHA had to be present and in the proper conformation for the inhibition of biofilm by ACT to occur. These data directly linked the ACT-FHA interaction to inhibition of biofilm by ACT in *B. pertussis* and *B. bronchiseptica*.

In light of the inhibitory effects of ACT and the fact that ACT and anti-MCD antibody both bound to the MCD, the ability of the anti-MCD antibody to block biofilm was tested. Indeed, when BP338 was grown in the presence of anti-MCD antibodies, there was a reduction in biofilm (Figure 19B). These data suggested that the anti-MCD antibody might block biofilm formation in a similar manner to ACT, support the competition between the ACT and the anti-MCD antibodies for FHA binding, and corroborated previous studies showing that polyclonal antibodies directed against FHA blocked biofilm formation (Serra *et al.*, 2011). It is thus clear that the MCD of FHA played a part in inhibition of biofilm. When binding partners, either anti-MCD antibodies or the AC domain, were present, biofilm formation was inhibited.

EXAMPLE 7

ACT and the AC domain Inhibit *Pseudomonas aeruginosa* Biofilm Formation

Although there are multiple bacterial species that express FHA-like proteins, *P. aeruginosa* is one of the best-studied biofilm forming bacteria due to its prevalence in Cystic Fibrosis and wound infections. *P. aeruginosa* biofilm was selected for testing because the FHA-like protein, CdrA, is involved in biofilm formation, and because of the striking structural homology between *P. aeruginosa* CdrA and *Bordetella* FHA (*compare* Figure 1 of Borlee *et al.*, 2010 with Figure 4 of Kajava *et al.*, 2001).

First, the ability of full-length holotoxin ACT to inhibit biofilm formation of *P. aeruginosa* was tested. Two lab-adapted parental strains were used: PA14 and PA01. Bacteria were grown over night as shaking cultures, and following procedures described for measuring biofilm in the microtiter assay for *P. aeruginosa,* bacteria were diluted to an OD_{600} of 0.05 before inoculating 100 µL Luria Broth (LB) cultures in 96 well plates. Recombinant purified *B. pertussis* ACT was added exogenously to *P. aeruginosa* cultures at concentrations of 0.1, 1, and 10 µg/ml. Biofilm formation, or bacterial accumulation in wells, was determined at 12 hours by crystal violet assay as described herein above. A concentration-dependent inhibition of biofilm by ACT was observed in both PA01 and PA14 (Figures 22A and 22B; bars 5-7).

The ability of AC domain to inhibit biofilm was also tested, as the AC domain is necessary and sufficient for biofilm inhibition in *Bordetellae*. AC domain was added at 0.1, 1, and 10 µg/ml to PA01 and PA14 cultures in 96 well plates and biofilm formation was assessed at 12 hours using the crystal violet assay (Figures 22A and 22B; bars 2-4). The AC domain inhibited biofilm formation of both *P. aeruginosa* strains in a concentration-dependent manner. There were no observed differences in growth between PA14 or PA01 grown in the presence or absence of the highest concentration of ACT or AC domain (10 µg/ml), as measured by OD_{600}; ACT and the AC domain did not inhibit biofilm by altering growth rates under these conditions (Figure 22C).

In order to test the hypothesis that ACT and AC domain inhibited biofilm formation by bacteria that expressed FHA-like proteins via a conserved mechanism, a transposon mutant from the PA14 transposon library, PA14 32575

(tn::*cdrA*, transposon insertion at 249 codons from start) which lacks CdrA, the *Pseudomonas* FHA-like protein, was obtained. Biofilm formation of PA14 and the CdrA transposon mutant was determined over a 12-hour time course in the presence and absence of 10 μg/ml AC domain (Figure 23). PA14 32575 (gray solid line) made less biofilm than the parental wild type strain PA14 (black solid line), which is consistent with previous observation that a Δ*cdrA* mutant made less biofilm than the parental wild type strain. The AC domain inhibited PA14 biofilm throughout the experiment (black dotted line), but did not significantly decrease biofilm formation of PA14 32575 (gray dotted line) at anytime point during the experiment (Figure 23). These data suggested that the AC domain inhibited biofilm by targeting (and possibly binding) the FHA-like protein, CdrA, in *P. aeruginosa.*

Because the AC domain was able to disrupt preformed *Bordetella* biofilm, the AC domain was also tested to determine its ability to disrupt preformed *P. aeruginosa* biofilm. PA14 was allowed to form biofilm for 6, 8, and 10 hours before the AC domain was added to cultures. Biofilm formation was measured at 12 hours by the crystal violet assay. No differences in biofilm formation were observed; the AC domain does not disrupt biofilm of *Pseudomonas.* While not wishing to be bound by any theory of operation, it is possible that the AC domain is too large to access the correct sites once biofilm formation has already occurred in *P. aeruginosa,* as the matrix material is denser than that of *B. pertussis,* or because once mature biofilm has formed, *P. aeruginosa* has other matrix components and proteins that reinforce the biofilm independent of CdrA. It is possible that a smaller peptide might be able to disrupt biofilm.

EXAMPLE 8

Anti-MCD Antibodies Inhibit *P. aeruginosa* Biofilm

As demonstrated in *B. pertussis*, anti-FHA antibodies prevent biofilm formation and more specifically, anti-MCD monoclonal antibodies prevent biofilm formation. Because the AC domain and anti-MCD antibodies both bind the MCD, the two binding events might prevent biofilm in a similar manner. The anti-MCD antibody was tested for its ability to inhibit *P. aeruginosa* PA14 biofilm, and was added at dilutions of 1:100 and 1:1000 to PA14 cultures in 96 well plates, similar to concentrations used in *B. pertussis* biofilm experiments. Biofilm formation was

assessed at 12 hours using the crystal violet assay (Figure 24). Anti-MCD antibodies inhibited PA14 biofilm formation in a concentration-dependent manner, suggesting that a direct binding event to the distal globular tip of CdrA, or some other MCD-like epitope, prevented biofilm formation.

EXAMPLE 9

The AC Domain Inhibits Biofilm Formation of Other Bacterial Species

In addition to testing the inhibitory ability of the AC domain on *P. aeruginosa*, the ability of AC domain to decrease or inhibit biofilm was tested on other bacterial species, including *Escherichia coli* and *Salmonella typhimurium*. Species were selected based upon availability and presence of FHA-like proteins found in their genomes. *Escherichia coli* expresses Filamentous Hemagglutinin (hypothetical protein, member of the ShlA/HecA/Fha exoprotein family) that shares sequence and structural homology to *Bordetella* FHA (including the portion of the protein which maps to the MCD of *Bordetella* FHA). *E. coli* also expressed amyloid proteins that are involved in biofilm formation. These amyloid proteins react with congo red dye, as does *Bordetella* FHA. *Salmonella enterica* serovars express BapA, which shares structural homology to *Bordetella* FHA and is involved in biofilm development (biofilm associated protein, BapA).

Biofilm formation of *E. coli* and *S. typhimurium* was assessed in the presence or absence of 10 µg/ml AC domain using the crystal violet assay. Bacteria were grown overnight in liquid LB culture and diluted to an OD_{600} of 0.05 in the morning. 100 µL cultures of bacteria were added to wells in 96 well plates and biofilm was measured at 12 hours for both *E. coli* and *S. typhimurium.* Two strains of *E. coli* were tested: *E. coli* MC4100, which has been studied in the context of curli proteins and biofilm, and *E. coli* 87-23, which is a shigatoxin negative 0157:H7 *E. coli* strain. The results are shown in Figures 25A-25C.

The AC domain was able to inhibit biofilm of one strain of *E. coli,* 87-23 (Figure 25A), but was ineffective against biofilm formation of the other *E. coli* strain tested, MC4100 (Figure 25B).

It is unclear why AC domain only inhibited one of the *E. coli* strains tested, but in some bacteria, such as *Salmonella*, curli proteins can compensate for biofilm defects. To analyze this, the presence of FHA-like proteins in both of the *E. coli* strains are tested using polyclonal anti-*B. pertussis* FHA antibodies (and the

anti-MCD antibody). The requirement of these predicted FHA-like proteins in biofilm formation and inhibition by AC domain is determined as well.

The AC domain did inhibit biofilm formation of *S. typhimurium* at 10 µg/ml at 12 hours, as measured by the crystal violet assay (Figure 25C).

EXAMPLE 10

A Fragment of the AC Domain Inhibited Biofilm

Because the AC domain is a smaller, more potent biofilm inhibitory peptide than full length holotoxin ACT, whether an even smaller peptide that retained the inhibitory properties would be more potent than the AC domain was tested. The AC domain has a natural trypsin cleavage site, which cleaves the AC domain into two pieces referred to herein as T25 and T18 (*see* Figure 26A).

The ability of trypsin-treated AC domain to inhibit biofilm was first examined. The smaller peptides, T18 and T25, although still mixed together, were able to inhibit biofilm. To resolve which of these peptides retained the inhibitory activity, plasmids encoding the genes for the T18 and T25 peptides were utilized. The commercially available BACTH plasmids (bacterial adenylate cyclase two-hybrid; Euromedex, Strasbourg, France), containing the T18 and T25 peptides, were obtained from collaborators at the University of Virginia. The plasmids were transformed into and expressed in *E. coli* BL21 (DE3) and methods similar to those that were used to purify full-length holotoxin and the AC domain were used to purify the T18 and T25 plasmids. Briefly, plasmids contained an IPTG inducible promoter and peptide expression was induced in early log phase. During mid log phase, bacteria were pelleted and sonicated to release cellular contents. Bacterial membranes and cell debris were spun down and the supernatent fraction was removed. Preliminary data demonstratd that the plasmids were functional and the T18 and T25 peptides remained membrane-associated. Urea extraction of bacterial membranes resulted in enrichment for the T18 and T25 peptides, but did not remove all other *E. coli* membrane proteins (similar to urea extracts from BL21 (DE3) *E. coli* expressing the plasmid encoding holotoxin ACT (*pT7cACT1*)).

The T18 and T25 peptides were added separately to *B. pertussis* BP338 (wild-type) cultures during biofilm growth in 96 well plates. Both of these peptides retained inhibitory activity on *B. pertussis* biofilm formation, which was concentration dependent (Figure 26B).

Discussion of the EXAMPLES

In addition to biofilms which form on surfaces associated with living organisms, biofilm can also occur on man-made structures in the environment, such as but not limited to water and sewage pipelines, bathroom drains and faucets, water holding tanks, etc. These biofilms can lead to complications with the efficiency of processes that involve these structures and materials. Thus, eradication of these unwanted communities of bacteria, either by disrupting preformed biofilm or preventing biofilm from occurring in the first place, would be desirable.

Virtually all surfaces, both man-made and naturally occurring, are susceptible to bacterial deposition and subsequent biofilm formation and thus prevention has been difficult. Disruption of biofilm typically consists of abrasive mechanical treatment and the application of harsh chemicals, which kills all living cells. While this is an option for abiotic sites such as sewage pipelines and sinks, these types of treatments are not suited for use in humans, animals, or plants.

Disclosed herein is the discovery that the 177 kD *Bordetella* adenylate cyclase toxin (ACT) and several fragments thereof including, but not limited to the 40 kDa AC domain (which comprises the catalytic domain of the toxin) as well as the T18 and T25 peptides were able to inhibit biofilm formation. ACT is a bacterial adenylate cyclase expressed by some *Bordetella* species (*B. pertussis*, *B. bronchiseptica, B. parapertussis, B. hinzii,* and *B. ansorpii).* The holotoxin is a major virulence factor of *Bordetellae,* which kills macrophages, blocks neutrophil function, and helps *B. pertussis* and *B. bronchiseptica* establish infection in hosts (mice and humans). The inhibitory effect on biofilm was mediated by a direct binding event between ACT and the 220 kD surface displayed adhesin, Filamentous Hemagglutinin (FHA). *Bordetella* FHA is involved in a variety of processes, such as binding to epithelial cells in the nares, trachea and lungs, and altering the host immune response, but is also described as one of the major protein components of *Bordetella* biofilm. *B. pertussis* and *B. bronchiseptica* lacking FHA do not form biofilm compared to their parental wild type strains *in vitro* or *in vivo*. The ACT-FHA interaction occurs between the 40 kDa catalytic domain of ACT (amino acids 1-400) (AC domain) and the distal tip of FHA, the mature C-terminal domain (MCD amino acids 1870-2362). The AC domain is

sufficient for FHA-binding, and necessary and sufficient for biofilm inhibition. The c-terminal portion of FHA is required for AC domain-FHA binding, and the MCD must be present and properly folded for biofilm inhibition. We hypothesize that the ACT-FHA interaction results in some hindrance of FHA for biofilm formation, either through a conformational change of FHA or through spatial hindrance of FHA, as opposed to sending a signal via the binding event. In addition to inhibitory properties of the AC domain, the peptide is able to disrupt preformed *B. pertussis* biofilm. The full-length holotoxin lacks this biofilm disruptive activity, which may be due to the size difference between ACT and AC domain and inability of full-length ACT to access FHA within mature biofilm structures.

The MCD is just one of many domains within the FHA protein. Several of the proteins domains, such as the carbohydrate recognition domain, confer binding to substrates, such as epithelial cells, macrophages, leukocytes, and monocytes. These domains and the overall structure of FHA are highly conserved amongst Bordetellae (B. ansorpii do not express FHA) and bacterial species outside of the Bordetella genus. Many of these FHA-like proteins act as adhesins, similar to FHA of B. pertussis, and some have been implicated in biofilm or aggregative growth, a precursor to biofilm.

In other bacterial species, specifically Pseudomonas fluorescens and Pseudomonas aeruginosa, the FHA-like protein, CdrA, is major components of the biofilm matrices. CdrA directly binds the polysaccharide component of Pseudomonas matrix, Psl, to reinforce biofilm structure. Strains that lack CdrA form less complex biofilm structures than the parental wild type strains. Biofilm of the ΔcdrA strain grows as a thin field, as opposed to growing into a complex three-dimensional structure. Biofilm of the CdrA deficient mutant accumulates at low levels, likely because Pseudomonads have other mechanisms to partially compensate for this defect. P. aeruginosa CdrA shows high structural similarity to Bordetella FHA, although the homology between the two genes encoding the proteins is low. Both the predicted structures for FHA and CdrA contain β-helical shafts, a globular c-terminal domain located at the distal tip of the protein, and CRD binding domains. The peptides are both inserted into a specific transporter in a hairpin structure, begin folding into β-helical sheets, and via proteolytic processing, the protein reaches its final structure and is displayed on the surface

of the bacterial outer membrane. FHA requires a two-partner secretion system, while CdrA is an RTX toxin secreted via the Type 1 Secretion System. Both FHA and CdrA can be released into the extracellular milieu, albeit via different mechanisms. CdrA is cleaved by the LapG protease, located in the periplasm, and is dependent on c-di-GMP levels within the cell. The mechanism by which FHA is released into the media is unknown, but does not require a proteolytic cleavage event. Because of the similarities between FHA and CdrA, we hypothesized that Pseudomonas, and possibly other bacteria that express FHA-like proteins, may be susceptible to biofilm inhibition by AC domain.

Thus, disclosed herein are experiments linking the ACT-FHA interaction to inhibition of biofilm formation by *Bordetella pertussis* and *Bordetella bronchiseptica*. The AC domain was necessary and sufficient, yet the catalytic activity of the toxin was not required for this inhibitory phenomenon. These effects of ACT could be blocked by CaM or by a catalytic domain-specific antibody. Thus, the AC domain was been identified as a sufficient binding partner for FHA, and the MCD as necessary for this binding and the inhibitory effect on biofilm to occur. The inhibitory effect could result from the AC domain – MCD interaction simply blocking the FHA molecule in its yet-to-be-identified role in *Bordetella* biofilm production, or by inducing a conformational change in FHA that has this and other effects.

An exemplary working model for the inhibition of *B. pertussis* biofilm by ACT is diagrammed in Figure 21, in which the AC domain of ACT binds the MCD of FHA to interfere with inter-bacterial FHA-FHA interactions, which have been previously described as important for biofilm formation. The inhibition by ACT might start in the early steps of biofilm formation, by ACT blocking initial bacteria-substrate, as well as bacteria-bacteria interactions and thus limiting subsequent biofilm accumulation. These observations are, however, in contrast to the observations by Perez Vidakovics *et al.* showing that the absence of ACT reduces *B. pertussis* binding to alveolar epithelial cells (Perez Vidakovics *et al.*, 2006).

The data illustrating inhibition of *Bordetella* biofilm by ACT through its interaction with FHA presented herein raise the important question of how this phenomenon fits with the current concepts of *Bordetella* pathogenesis and biofilm production. Others have shown that multiple factors, ranging from (p)ppGpp and

c-di-GMP to transcriptional regulators of Bps polysaccharide production, control biofilm production by *Bordetellae* (*see e.g.*, Conover *et al.*, 2012; Sugisaki *et al.*, 2013; Sisti *et al.*, 2013). Specifically, Irie *et al.* and Mishra *et al.* demonstrated that BvgAS modulates the formation of biofilm and that there is an increase in *B. bronchiseptica* biofilm under Bvg(i) conditions (Irie *et al.*, 2004). This scenario can now be explained, at least in part, by a reduction in the amount of inhibitory ACT in the presence of a constant level of FHA in the Bvg(i) phase (Cotter & Miller, 1997; Mattoo & Cherry, 2005; Vergara-Irigaray *et al.*, 2005). Thus, during active phase of infection in which conditions are optimal for the bacteria, ACT is actively produced for its inhibitory effects on the host immune response and biofilm production is suppressed (Figure 21). Under less favorable conditions, during which a defensive posture might be beneficial, a reduction in ACT production could be one of several mechanisms by which production of biofilm is initiated.

Given the active production of ACT during the Bvg(+) phase, it is appropriate to ask why there was any biofilm produced during these *in vitro* assays. It is now apparent that the quantity and distribution of ACT was different than what occurs *in vivo*. Previously, it has been demonstrated that in *ex vivo* samples obtained during active infection, concentrations of ACT can reach approximately 100 ng/ml and all of the ACT is in the supernatant fraction, as opposed to being surface associated (Eby *et al.*, 2013). This is in contrast to *B. pertussis* cultured *in vitro* in SSM, in which >90% of the ACT remained surface-associated and concentrations rarely got as high as seen in the *ex vivo* samples (Eby *et al.*, 2013). It has also been demonstrated that the functional form of the toxin is that which is released into the media (Gray *et al.*, 2004), while the surface-associated toxin is likely an improperly folded, inert pool.

Finally, Dr. Constance Jeffery has described and catalogued (*see* the Moonlight Proteins (MoonProt) Database, accessible through the World Wide Web at <<moonlightingproteins>>.<<org>>; *see also* Mani *et al.*, 2015) a number of dual function protein molecules, in which a single protein performs multiple physiologically relevant biochemical or biophysical roles (Jeffery, 1999; Jeffery, 2003; Jeffery, 2009; Jeffery, 2014; and Jeffery, 2015). On the basis of recognizing additional functions for known proteins, these fascinating molecules, which are from both prokaryotic and eukaryotic sources, have been designated

"moonlighting proteins". Their study has facilitated identification of novel biochemical pathways and protein functions, and allowed systems biologists to better understand cellular processes.

Prior to the present disclosure, ACT had been studied and characterized solely as a host-directed protein bacterial toxin that modulates function and is cytotoxic for some target cells by increasing cAMP levels and, depending on concentration, depleting ATP levels. ACT is also a hemolysin and member of the RTX family of pore-forming toxins, which includes *E. coli* hemolysin, HlyA (Menestrina *et al.*, 1994). The poreforming function, which for ACT is involved in delivery of its catalytic domain to the target cell interior, has an additional effect of compromising membrane integrity and polarization and contributes to cytotoxicity. The additional role for this protein bacterial toxin, contained within its catalytic domain, namely interaction with a surface adhesion to impair formation of biofilm makes it unlike any other moonlighting protein that has been described in the MoonProt Database (Mani *et al.*, 2015). This information can now be used to study *Bordetella* biofilm and to hypothesize when formation may occur *in vivo*.

Summarily, disclosed herein are experiments that tested the hypothesis that the ACT-FHA interaction inhibits biofilm by adding purified ACT to cultures of *B. pertussis* and *B. bronchiseptica in vitro*. Indeed, exogenous ACT inhibited biofilm formation, adding to the effect of endogenously produced and secreted ACT. This effect of added ACT occurred through binding of the catalytic AC domain, independently of its enzyme activity, to the mature C-terminal domain (MCD) of FHA, which must be properly folded for the inhibitory effect of ACT to occur. An exemplary relationship of this novel regulatory role for a bacterial toxin to the hypothetical "life cycle" of *B. pertussis*, controlled by BvgAS, is also provided.

As such, the present disclosure demonstrates that *Bordetella pertussis*, the causative agent of whooping cough, secretes and releases adenylate cyclase toxin (ACT), a protein bacterial toxin that targets host cells and disarms immune defenses. ACT binds filamentous haemagglutinin (FHA), a surface-displayed adhesin, and until now, the consequences of this interaction were unknown. Disclsoed herein are characterizations of the physical interaction of ACT with FHA as well as evidence linking that interaction to inhibition of biofilm *in vitro*.

Exogenous ACT inhibited biofilm formation in a concentration-dependent manner and the N-terminal catalytic domain of ACT (AC domain) was necessary and sufficient for this inhibitory effect. AC Domain interacted with the C-terminal segment of FHA with ~650 nM affinity. ACT did not inhibit biofilm formation by *Bordetella* lacking the mature C-terminal domain (MCD), suggesting the direct interaction between AC domain and the MCD was required for the inhibitory effect. Additionally, AC domain disrupted preformed biofilm on abiotic surfaces. The demonstrated inhibition of biofilm formation by a host-directed protein bacterial toxin represents a novel regulatory mechanism and provides an unprecedented role for ACT.

REFERENCES

All references listed in the instant disclosure, including but not limited to all patents, patent applications and publications thereof, scientific journal articles, and database entries (including but not limited to GENBANK® biosequence database entries and including all annotations available therein) are incorporated herein by reference in their entireties to the extent that they supplement, explain, provide a background for, and/or teach methodology, techniques, and/or compositions employed herein. The discussion of the references is intended merely to summarize the assertions made by their authors. No admission is made that any reference (or a portion of any reference) is relevant prior art. Applicants reserve the right to challenge the accuracy and pertinence of any cited reference.

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Headings are included herein for reference and to aid in locating certain sections. These headings are not intended to limit the scope of the concepts described therein under, and these concepts can have applicability in other sections throughout the entire specification.

It will be understood that various details of the presently disclosed subject matter may be changed without departing from the scope of the presently disclosed subject matter. Furthermore, the foregoing description is for the purpose of illustration only, and not for the purpose of limitation.

CLAIMS

What is claimed is:

1. A composition for inhibiting bacterial biofilm development and/or for reducing or eliminating a bacterial biofilm present on a surface, the composition comprising an effective amount of a peptide or polypeptide derived from Adenylate Cyclase Toxin (ACT) of *Bordetella* or a catalytic domain (AC domain) thereof, optionally wherein the peptide or polypeptide comprising an amino acid sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to an amino acid sequence selected from the group consisting of SEQ ID NOs: 1-5 and 44-53, optionally wherein the percent identity exists over the full length of one of SEQ ID NOs: 1-5 and 44-53.
2. The composition of claim 1, wherein the peptide or polypeptide comprises, consists essentially of, or consists of an amino acid sequence selected from the group consisting of SEQ ID NOs: 1-5 and 44-53.
3. The composition of claim 1, wherein the composition is a pharmaceutical composition comprising or consisting essentially of the peptide or polypeptide and one or more pharmaceutically acceptable excipients and/or carriers.
4. The composition of any of claims 1-3, comprising a delivery vehicle, optionally wherein the peptide or polypeptide is associated with, conjugated to, and/or encapsulated by a delivery vehicle.
5. The composition of claim 4, wherein the delivery vehicle comprises a liposome, a microparticle, or a nanoparticle, optionally wherein the liposome, microparticle, or nanoparticle is designed to be biodegradable in a subject.
6. The composition of any of claims 3-5, wherein the one or more pharmaceutically acceptable excipients and/or carriers are pharmaceutically acceptable for use in a human.
7. The composition of any of claims 3-6, wherein the pharmaceutical composition is formulated for oral administration, intravenous administration, intramuscular administration, intrathecal administration, cutaneous administration, topical administration, transdermal

administration, systemic administration, subcutaneous administration, sublingual administration, buccal administration, ocular administration, otic administration, nasal administration, inhalation, nebulization, or any combination thereof.

8. The composition of any of claims 1-7, wherein the bacterial biofilm comprises a strain of bacteria selected from the group consisting of *Bordetella spp.*, optionally *Bordetella pertussis* or *Bordetella bronchiseptica*; *Salmonella spp.*, optionally *Salmonella typhimurium*; *Pseudomonas sp.*, optionally *Pseudomonas aeruginosa*; coliform bacterial including *E. coli spp.*; *Listeria spp.*; *Neisseria spp.*; *Streptococcus spp.*; *Staphylococcus spp.*; *Yersinia spp.*; *Campylobacter spp.*; *Helicobacter spp.*; *Aeromonas spp.*; atypical Mycobacteria; and *Legionella spp.*

9. A method for preventing and/or treating a disease, disorder, or condition associated with the presence and/or development of bacterial biofilm in a subject, the method comprising administering to the subject a composition of any of claims 1-8 in an effective amount and via a route sufficient for preventing and/or reducing the severity of at least one symptom of the disease, disorder, or condition.

10. The method of claim 9, wherein the disease, disorder, or condition is selected from the group consisting of whooping cough, cystic fibrosis, bacterial vaginosis, urinary tract infections, infections associated with catheter use, middle ear infections, formation of dental plaque, gingivitis, eye infections associated with contact lens use, endocarditis, and infections resulting from use of medical and/or dental implants such as but not limited to joint prostheses and heart valves.

11. A method for reducing the incidence of nosocomial infection, the method comprising contacting a surface present in a medical and/or dental facility with a composition of any of claims 1-7 in an amount sufficient to inhibit bacterial biofilm development and/or reduce or eliminate bacterial biofilm present on the surface, wherein the bacterial biofilm is associated with the incidence of nosocomial infection.

12. The method of claim 11, wherein the surface is a door surface, a door handle surface, a sink surface, a toilet surface, a faucet surface, a furniture surface, optionally a bed surface, and a window surface.

13. A method of inhibiting bacterial biofilm development and/or for reducing or eliminating a bacterial biofilm present on a surface, the method comprising contacting the surface or the biofilm present thereon with an effective amount of a composition of claim 1, whereby bacterial biofilm development on the surface is inhibited and/or existing bacterial biofilm on the on the surface is reduced or eliminated.

14. The method of claim 13, wherein the bacterial biofilm comprises a strain of bacteria selected from the group consisting of *Bordetella spp.*, optionally *Bordetella pertussis* or *Bordetella bronchiseptica*; *Salmonella spp.*, optionally *Salmonella typhimurium*; *Pseudomonas sp.*, optionally *Pseudomonas aeruginosa*; coliform bacterial including *E. coli spp.*; *Listeria spp.*; *Neisseria spp.*; *Streptococcus spp.*; *Staphylococcus spp.*; *Yersinia spp.*; *Campylobacter spp.*; *Helicobacter spp.*; *Aeromonas spp.*; atypical Mycobacteria; and *Legionella spp.*

15. The method of claim 13, wherein the surface is a part of a device selected from the group consisting of a medical device, a dental device, and an industrial device.

16. The method of claim 15, wherein the medical device is selected from the group consisting of a surgical tool, an implant, a catheter, a stent, a ventilator tubing, and a bone or joint implant, optionally a hip, knee, ankle, wrist, elbow, or shoulder prosthesis.

17. The method of claim 16, wherein the implant is a cardiac implant.

18. The method of claim 15, wherein the industrial device is selected from the group consisting of a pipe, a tube, a valve, an air-cooled tower, a warm water system, a coolant circuit, a silo, a fermenter, a colander, a piece of furniture, and a sink.

19. The method of claim 15, wherein the industrial device is part of device used for water treatment, sewage treatment, petroleum manufacturing and/or storage, or recycling.

20. The method of claim 13, wherein the surface is a cellular surface, a tissue surface, and/or an organ surface present within a subject.

21. The method of claim 20, wherein the contacting comprises administering a pharmaceutical composition comprising the peptide or polypeptide to the subject in an amount and via a route of administration whereby the peptide or polypeptide contacts the surface or the biofilm present thereon and inhibits bacterial biofilm development on the surface and/or reduces or eliminates the existing bacterial biofilm present thereon.

22. The method of claim 21, wherein the surface is a nasal surface and/or a lung surface and the pharmaceutical composition is configured for inhalation and/or insufflation by the subject.

23. The method of claim 21, wherein the composition comprises a delivery vehicle, optionally wherein the peptide or polypeptide is associated with, conjugated to, and/or encapsulated by a delivery vehicle in the pharmaceutical composition.

24. The method of claim 23, wherein the delivery vehicle comprises a liposome, a microparticle, or a nanoparticle, optionally wherein the liposome, microparticle, or nanoparticle is designed to be biodegradable in the subject.

25. The method of claim 13, further comprising contacting the surface with one or more additional compositions that inhibit bacterial biofilm development and/or reduces or eliminates bacterial biofilm present on the surface.

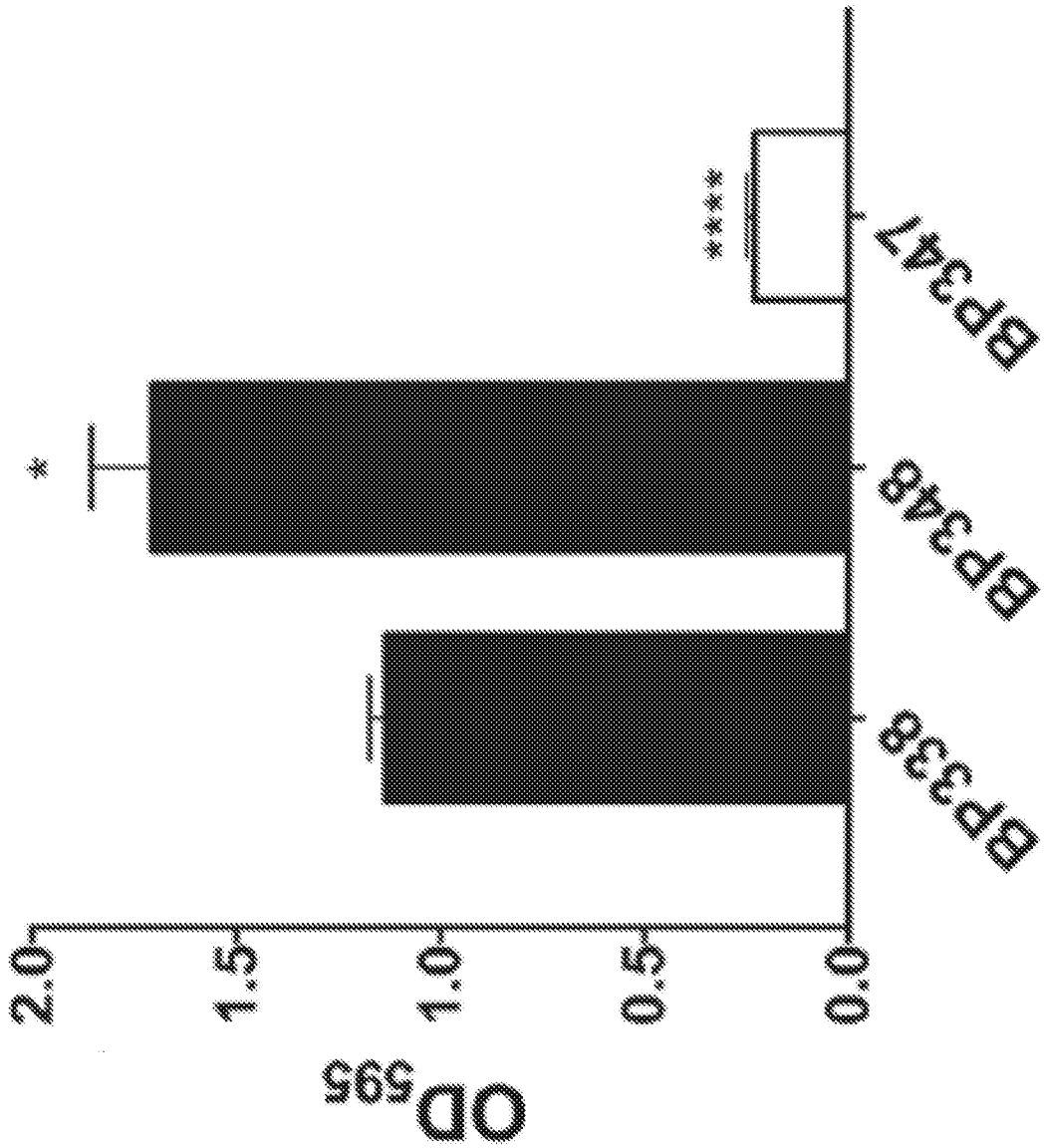
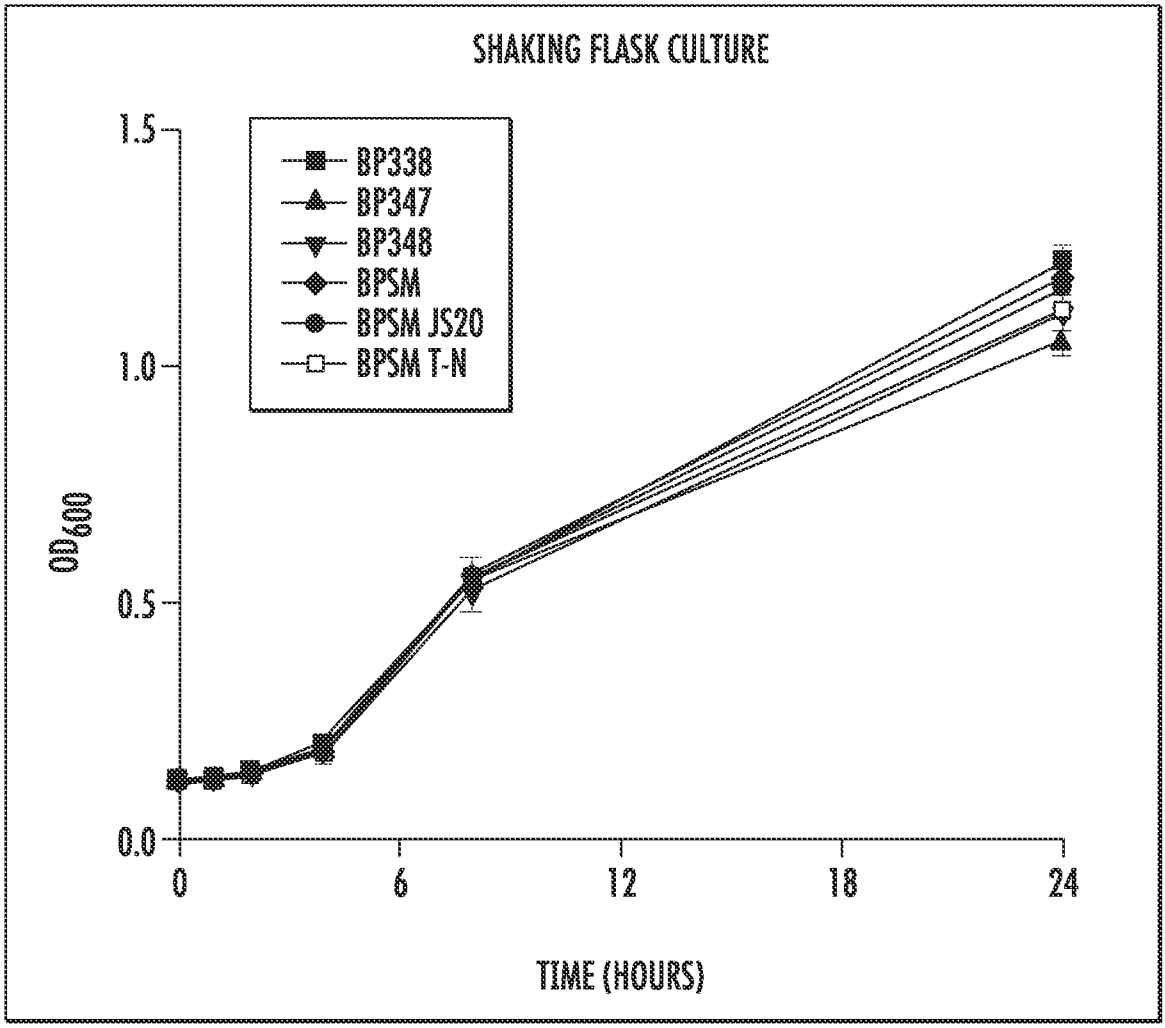


Fig. 1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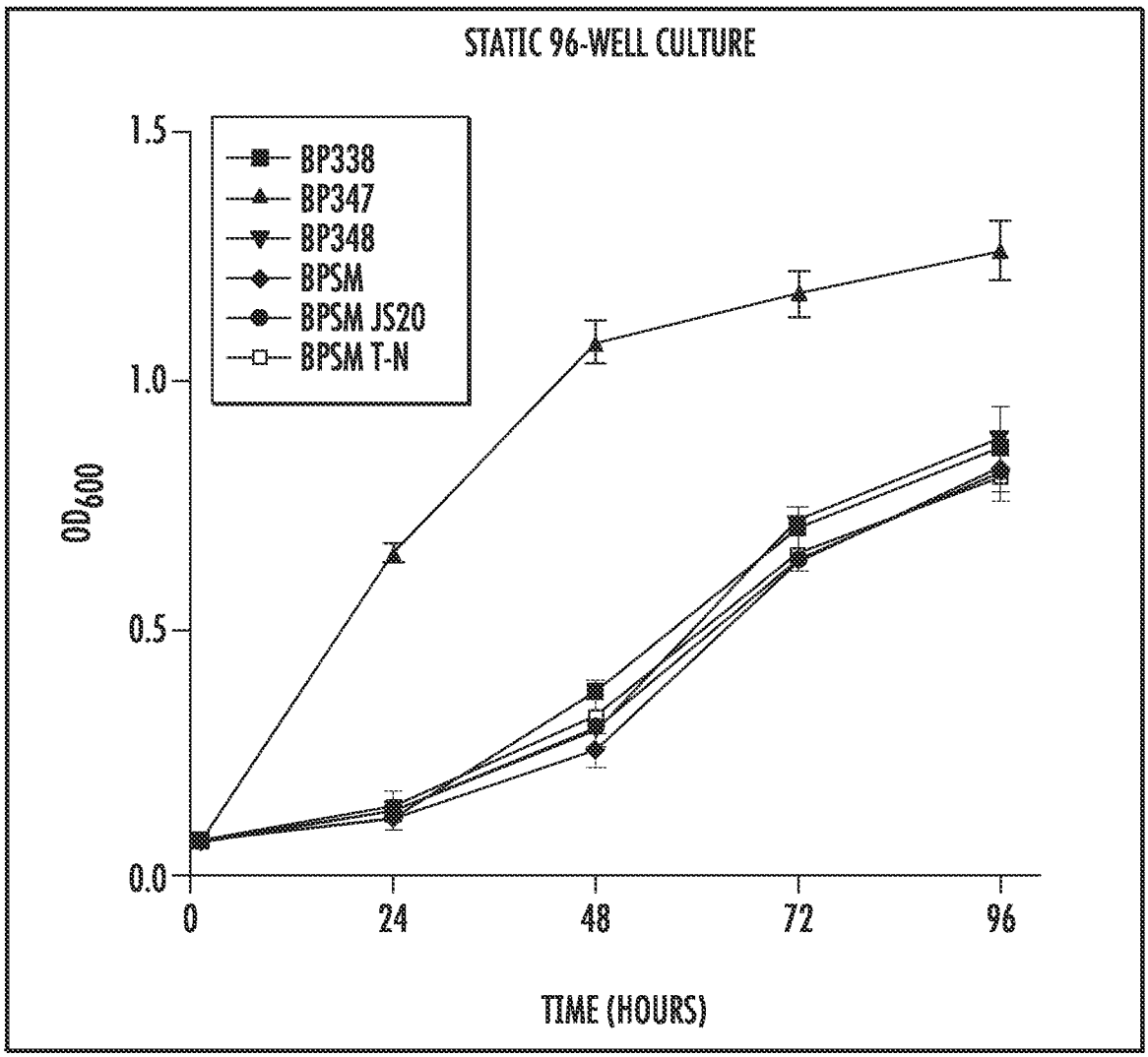
1.5
1.0
0.5
0.0
OD600
BP338
BP347
BP348
BPSM
BPSM JS20
BPSM T-N
0
6
12
18
24
TIME (HOURS)

FIG. 2A


STATIC 96-WELL CULTURE
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BP347
BP348
BPSM
BPSM JS20
BPSM T-N
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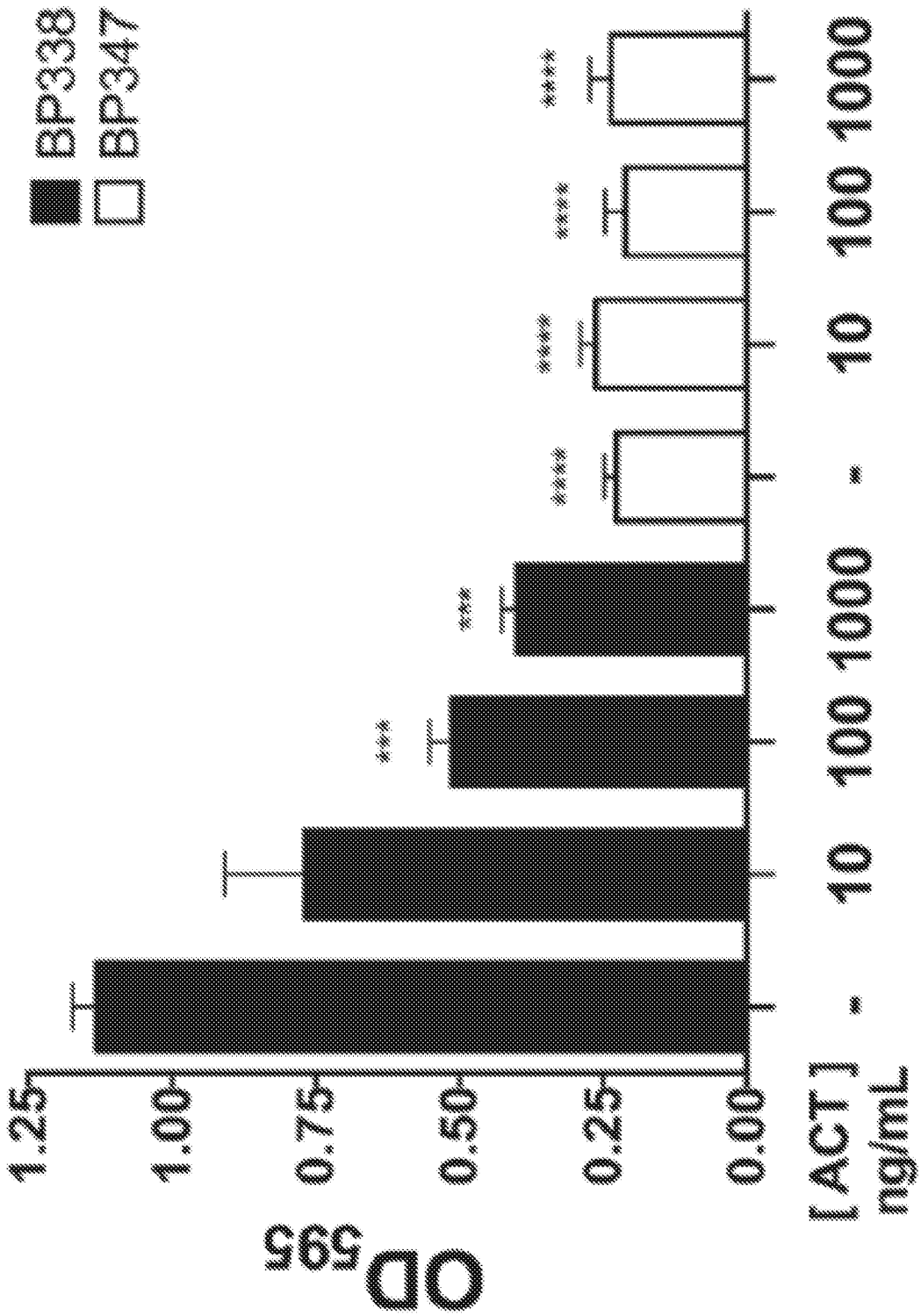
24
48
72
96
TIME (HOURS)

FIG. 2B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BP347
OD595
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1.00
0.75
0.50
0.25
0.00
[ACT] ng/mL
-
10
100
1000
-
10
100
1000
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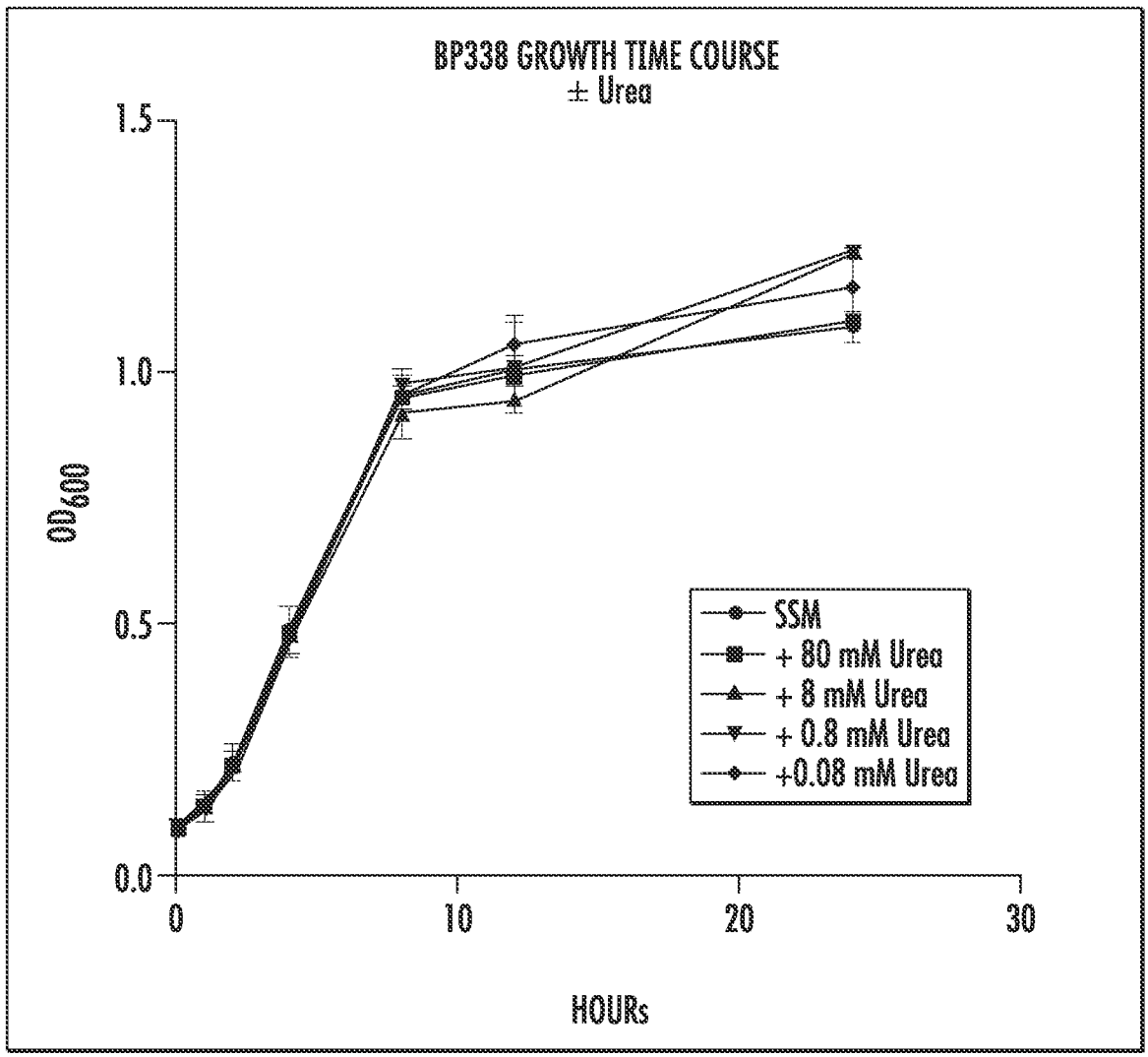
Fig. 3


BP338 GROWTH TIME COURSE
± Urea
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1.0
0.5
0.0
OD600
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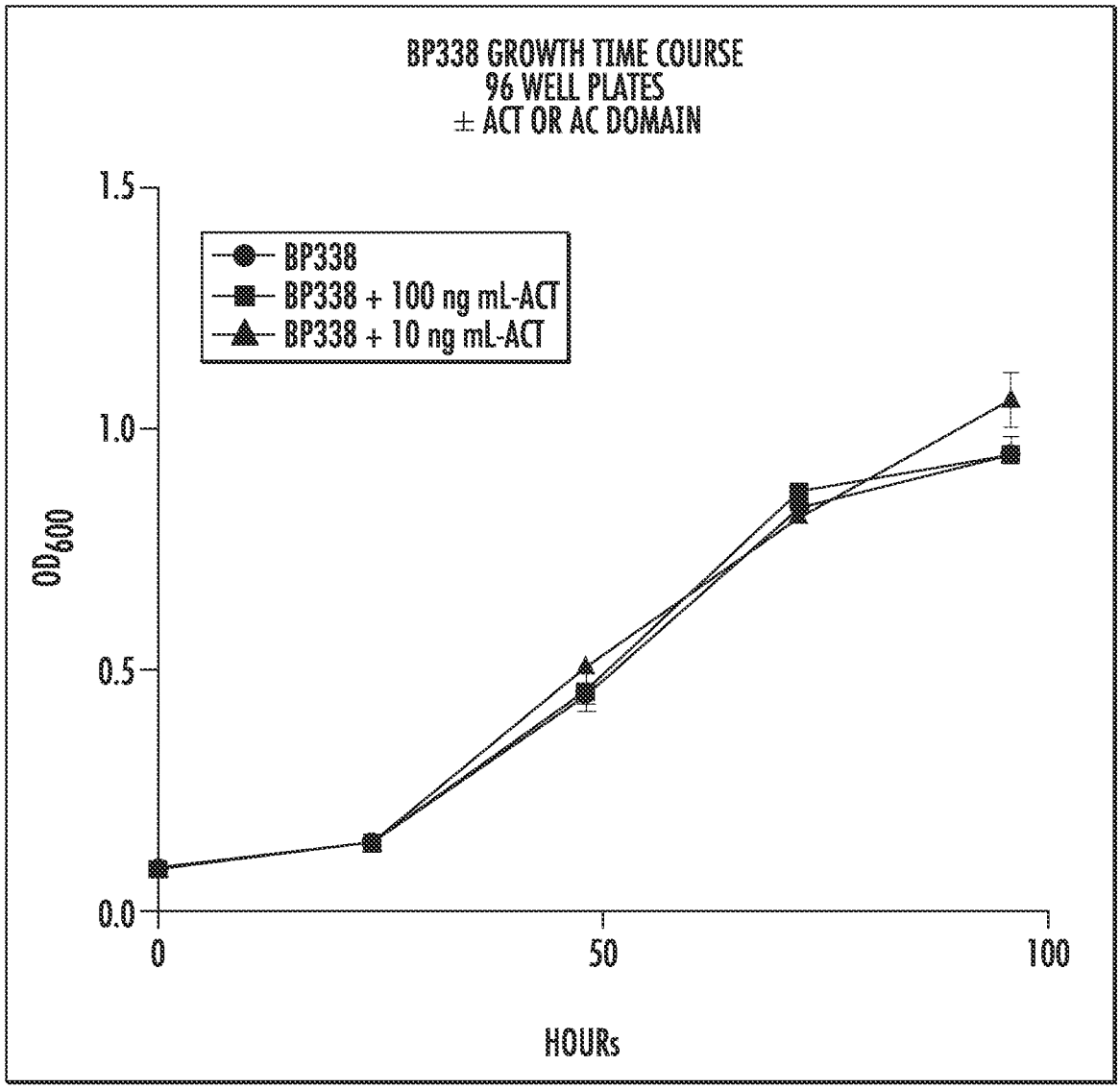
30
HOURs
SSM
+ 80 mM Urea
+ 8 mM Urea
+ 0.8 mM Urea
+0.08 mM Urea

FIG. 4A


BP338 GROWTH TIME COURSE
96 WELL PLATES
± ACT OR AC DOMAIN
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BP338 + 100 ng mL-ACT
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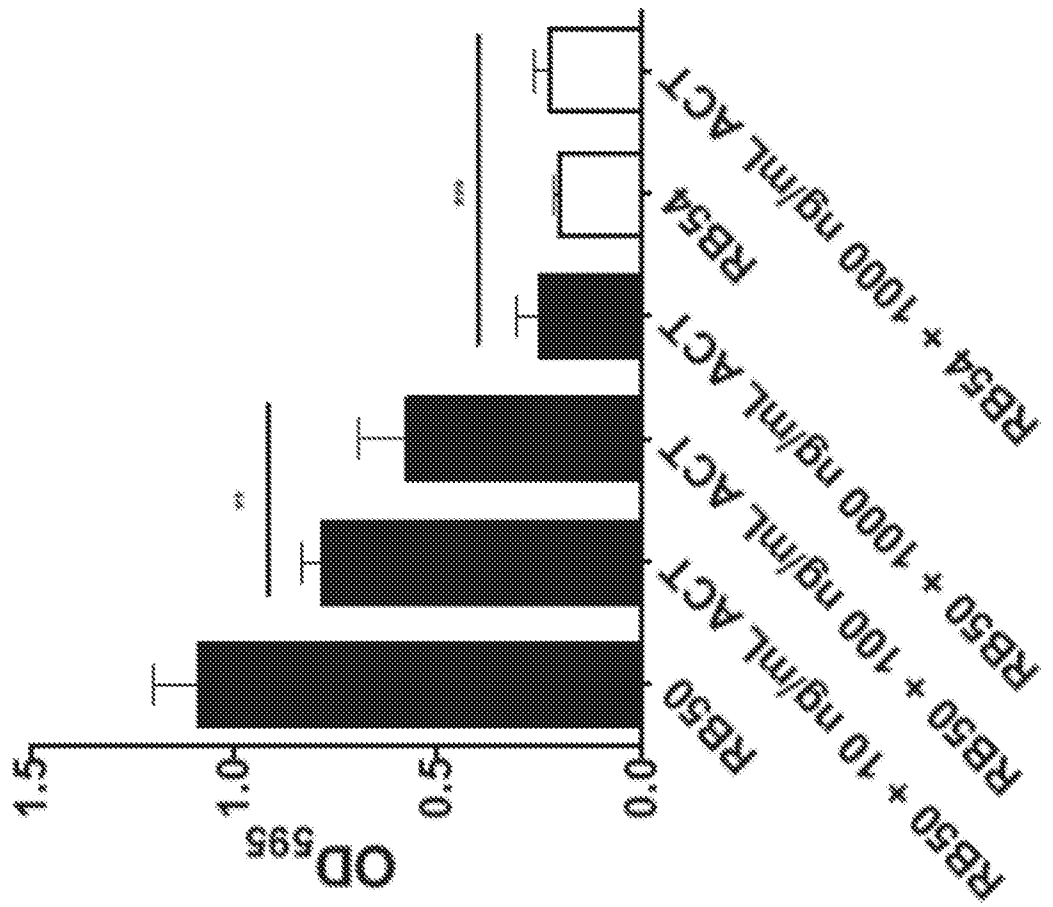
0.0
0
50
100
HOURs

FIG. 4B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RB50 + 100 ng/mL ACT
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RB54
RB54 + 1000 ng/mL ACT
*
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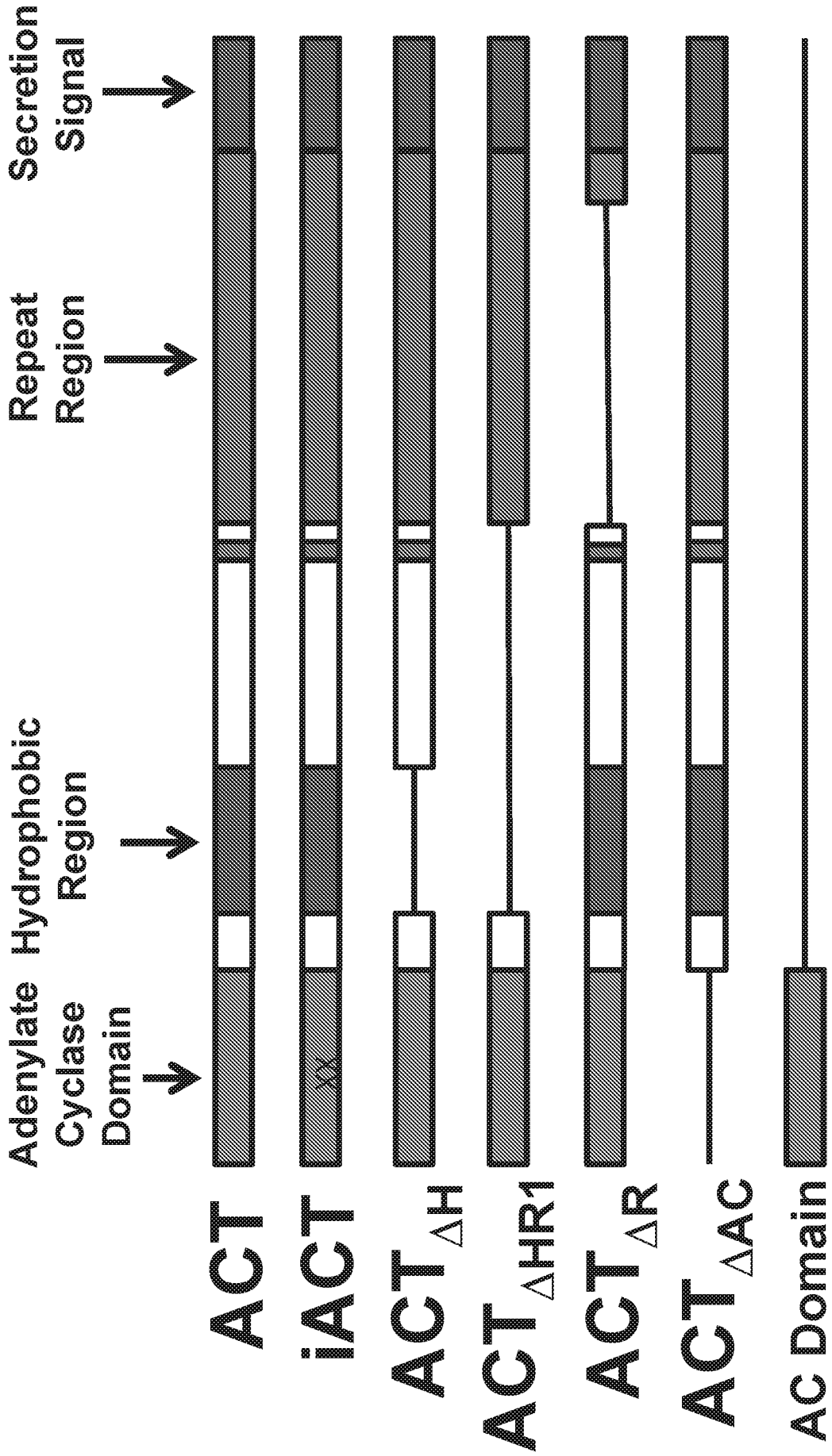
Fig. 5


Adenylate Cyclase Domain
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Repeat Region
Secretion Signal
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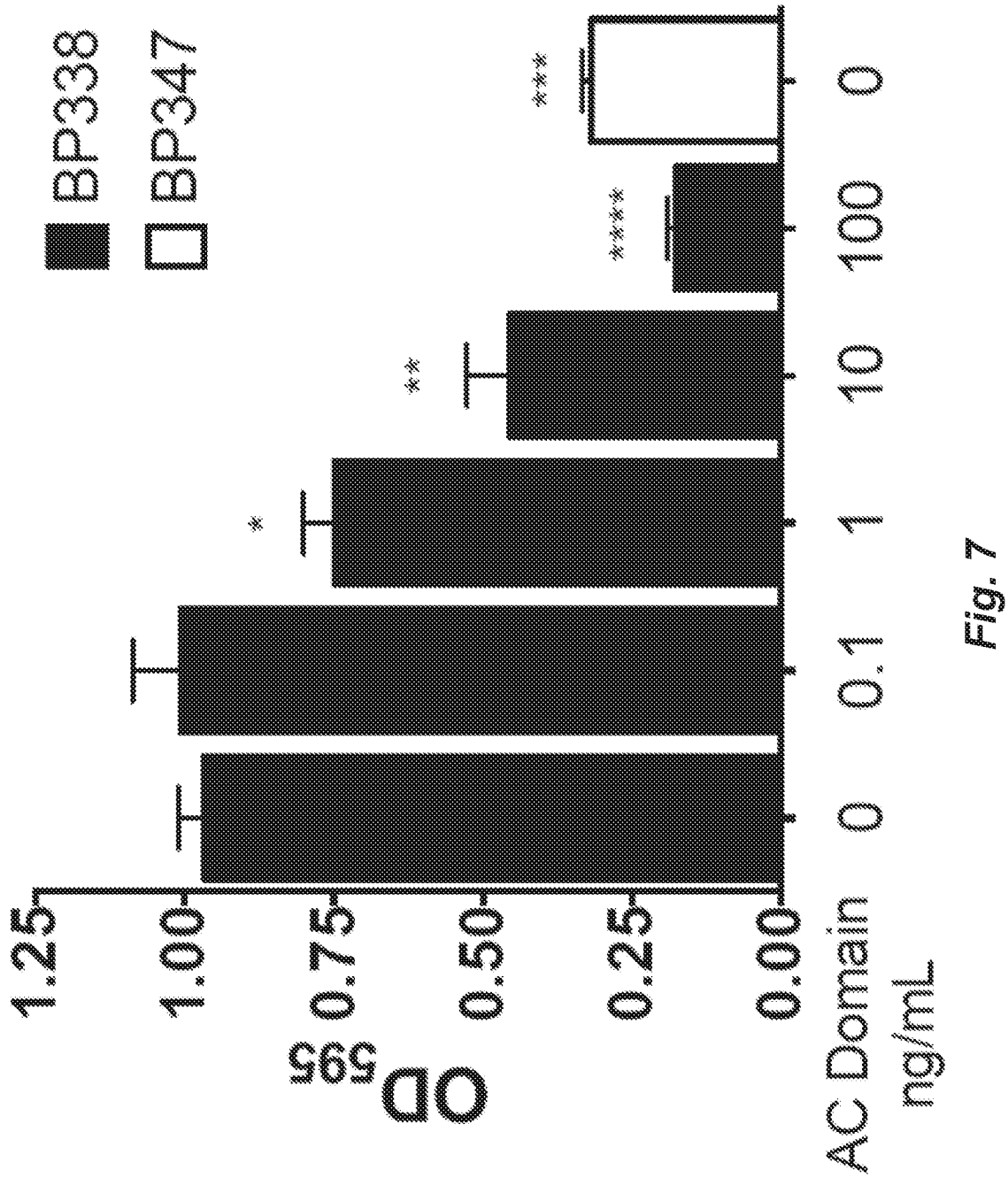
XX
ACT ΔH
ACT ΔHR1
ACT ΔR
ACT ΔAC
AC Domain

Fig. 6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BP347
OD595
1.25
1.00
0.75
0.50
0.25
0.00
*
**
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***
0
0.1
1
10
100
0
AC Domain
ng/mL

Fig. 7

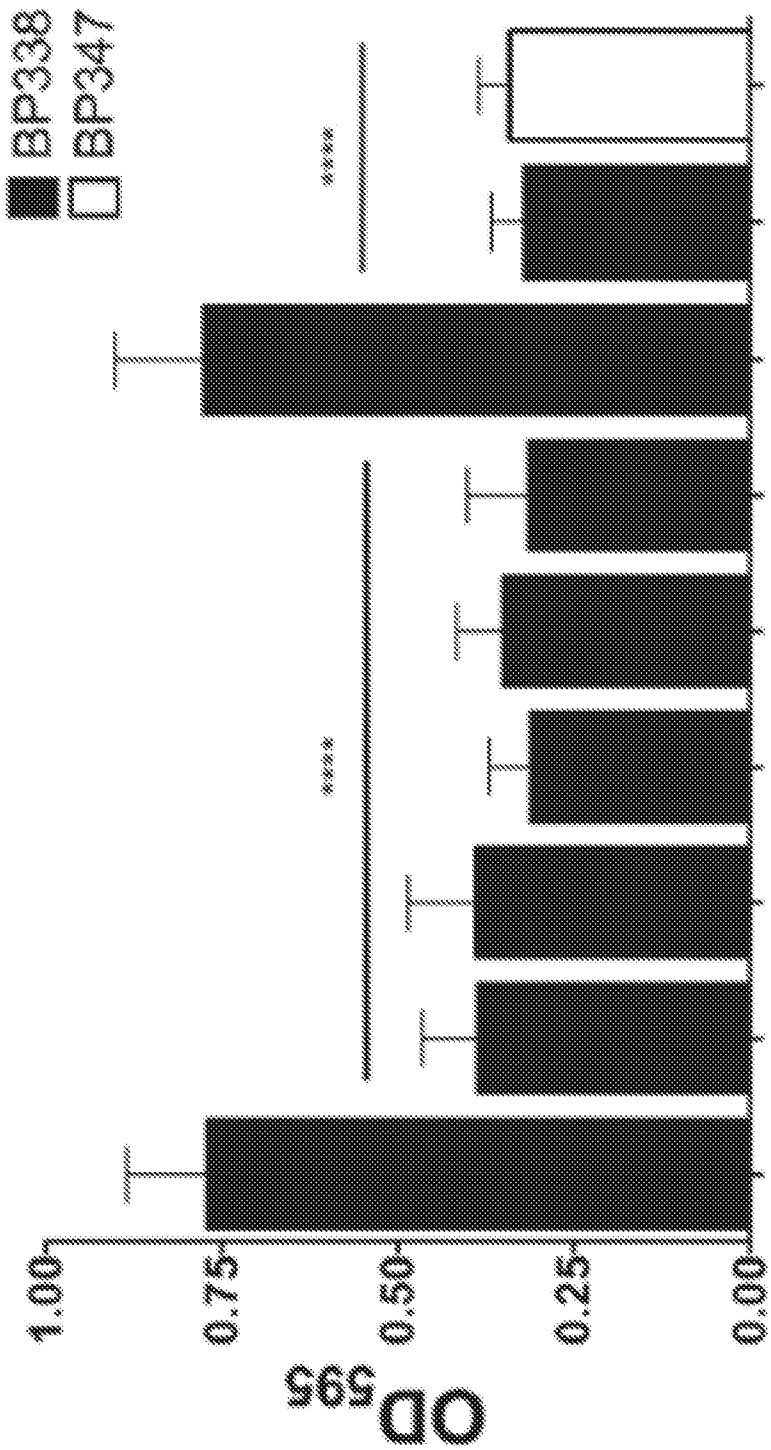
BP338
BP347
OD595
1.00
0.75
0.50
0.25
0.00
****
****
+ ACT
+ ACT ΔH
+ ACT ΔHR1
+ ACT ΔR
+ AC Domain
+ ACT ΔAC
+ iACT

Fig. 8

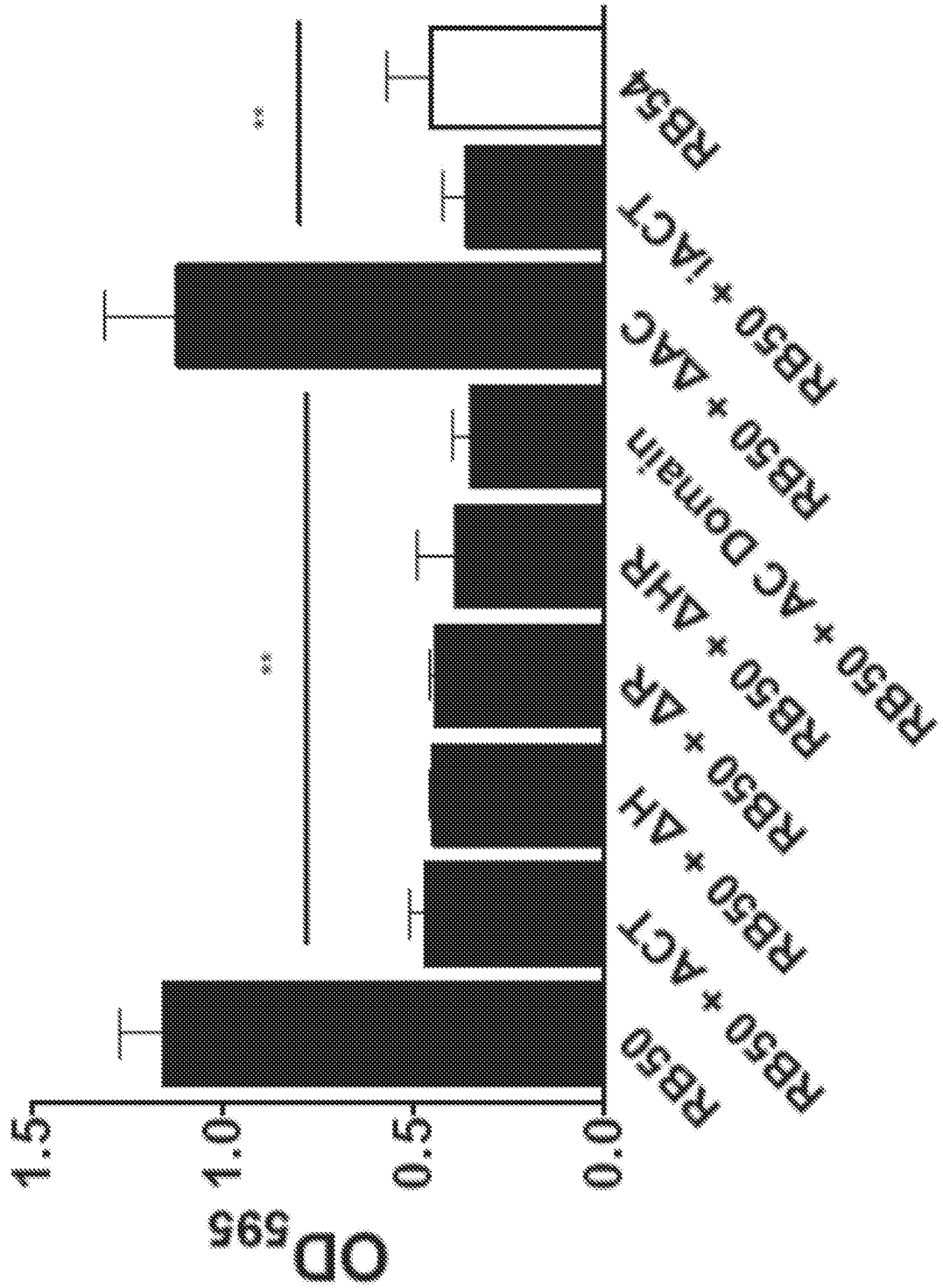
OD595
1.5
1.0
0.5
0.0
RB50
RB50 + ACT
RB50 + AH
RB50 + AR
RB50 + AHR
RB50 + AC Domain
RB50 + AAC
RB50 + iACT
RB54
**
**

Fig. 9

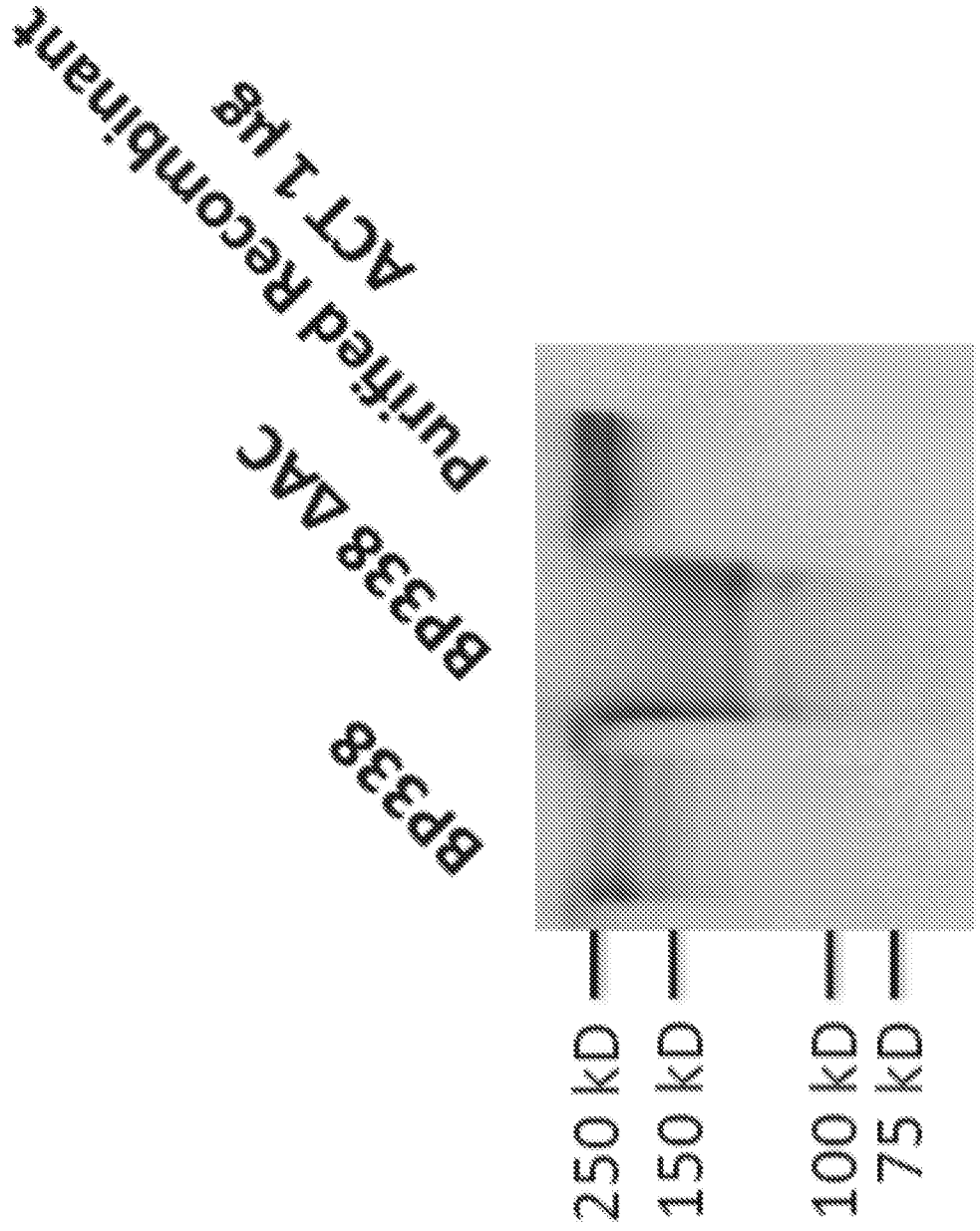
250 kD
150 kD
100 kD
75 kD
BP338
BP338 ΔAC
Purified Recombinant
ACT 1 µg

Fig. 10

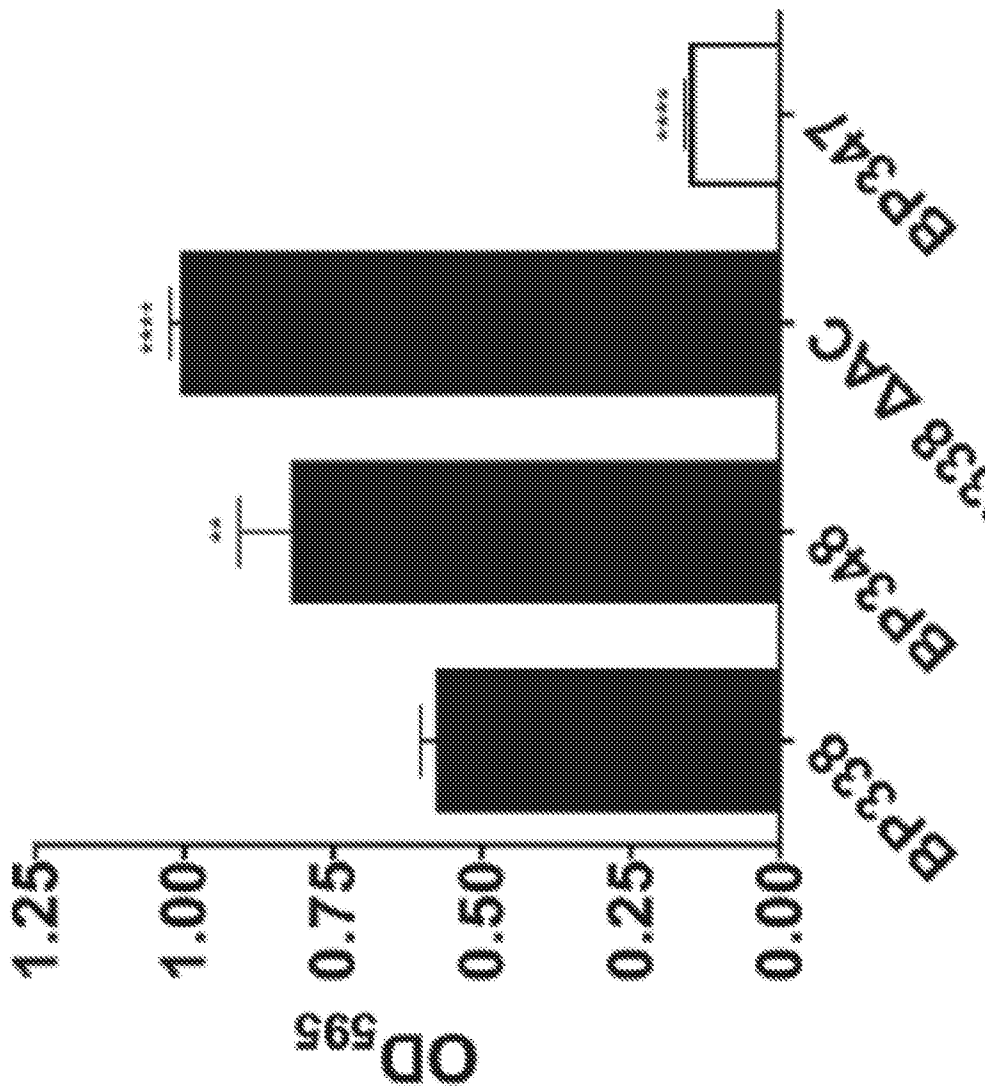

Fig. 11

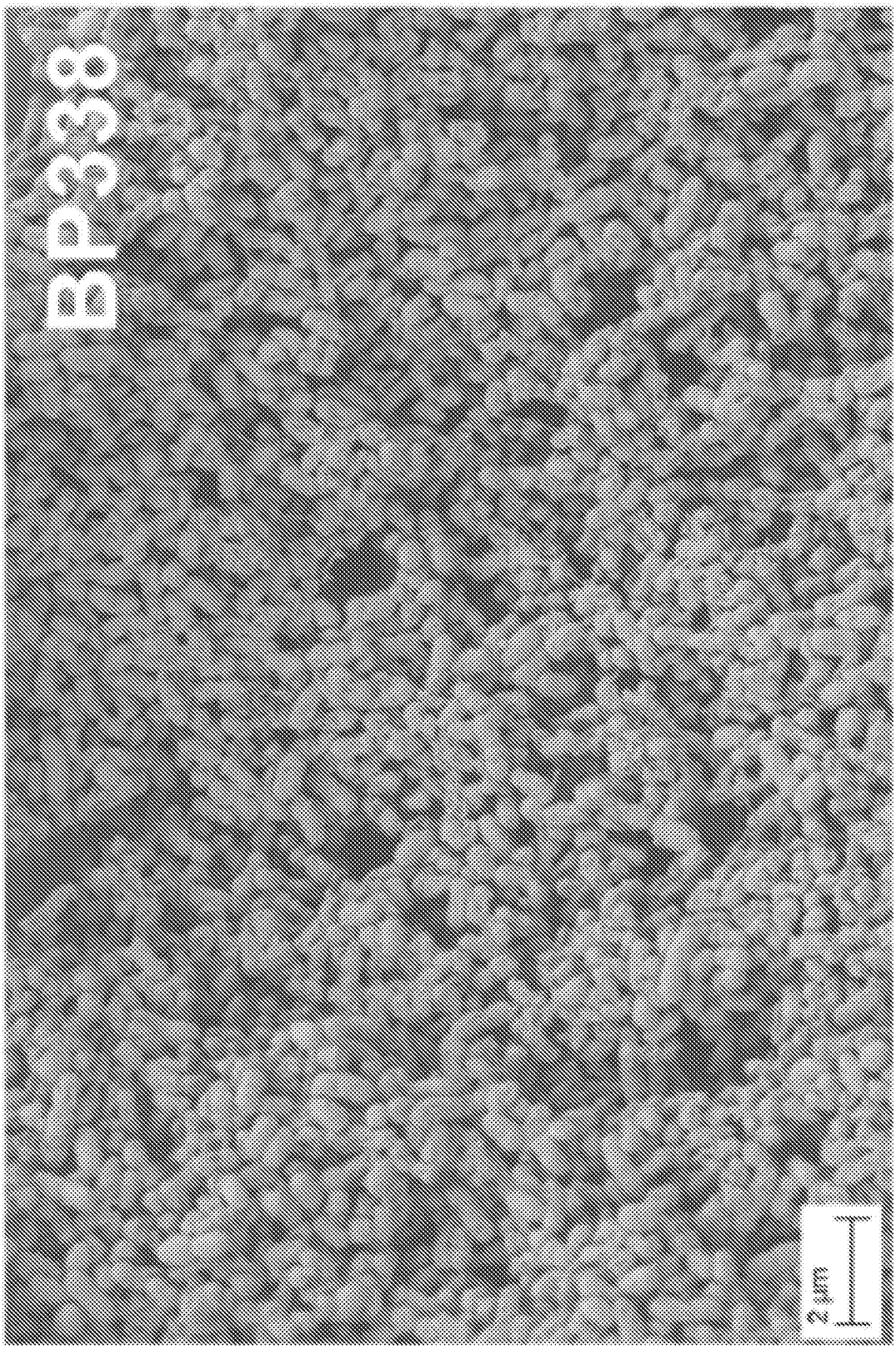

Fig. 12A

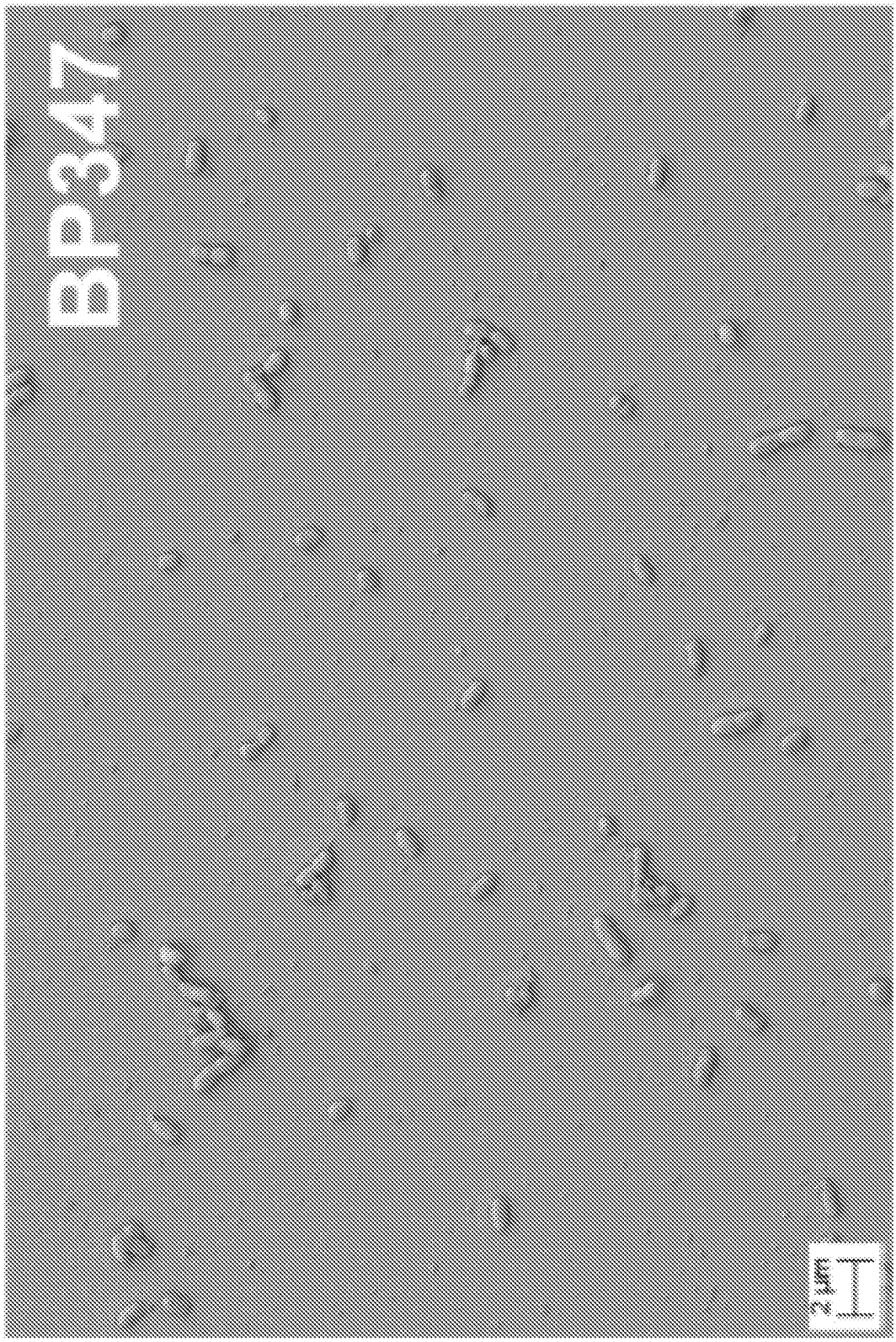

Fig. 12B

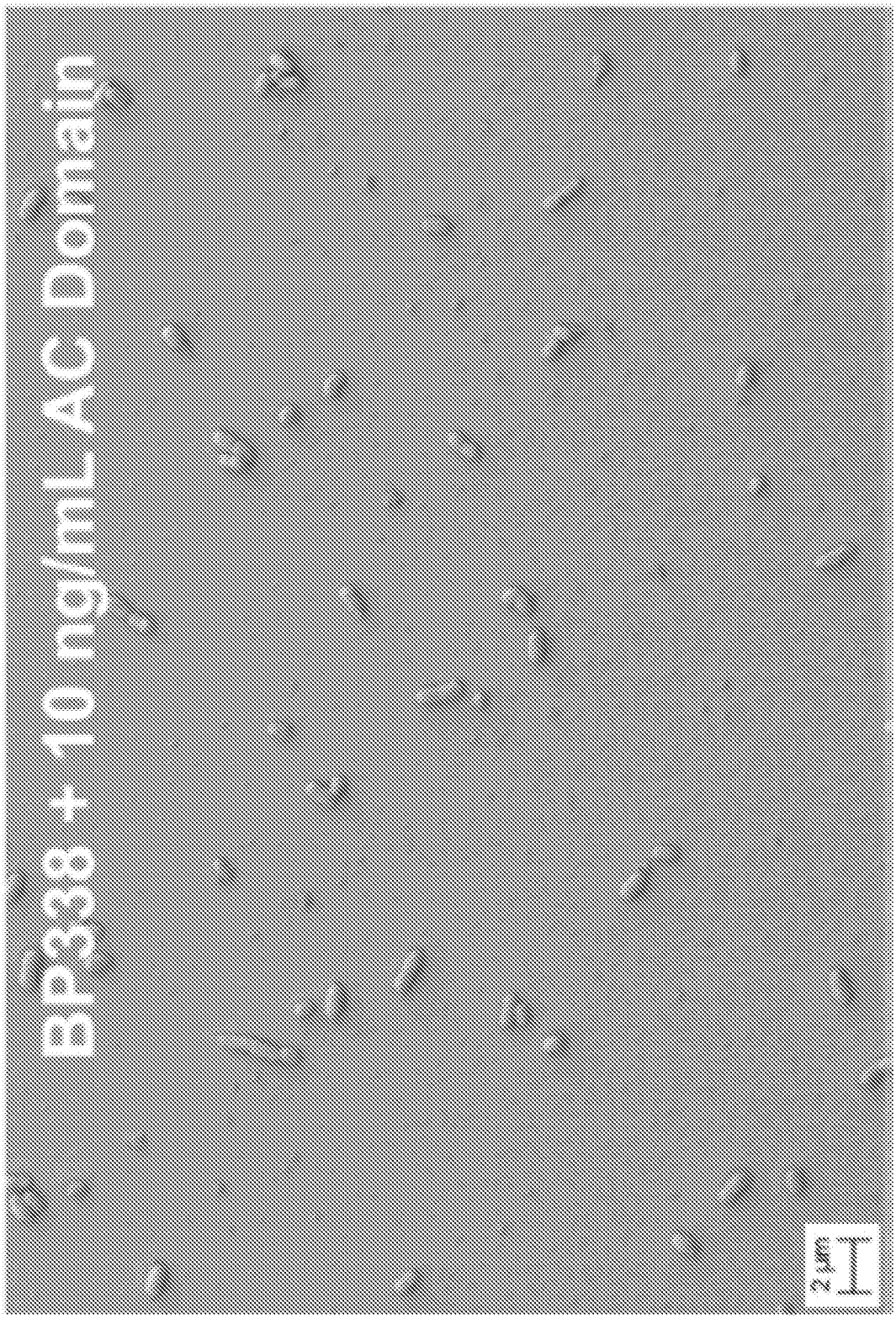

Fig. 12C

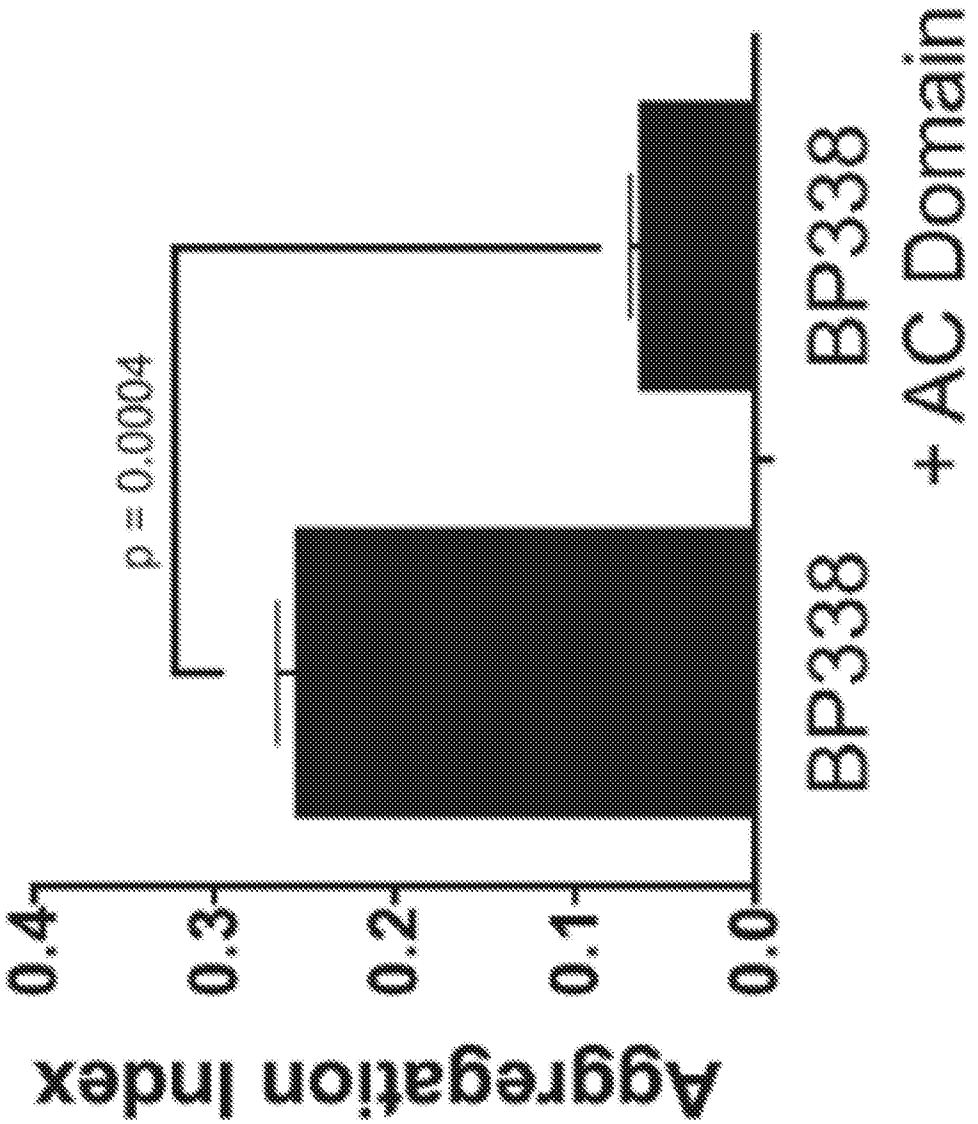
Aggregation Index
0.4
0.3
0.2
0.1
0.0
p = 0.0004
BP338
BP338
+ AC Domain

Fig. 13A

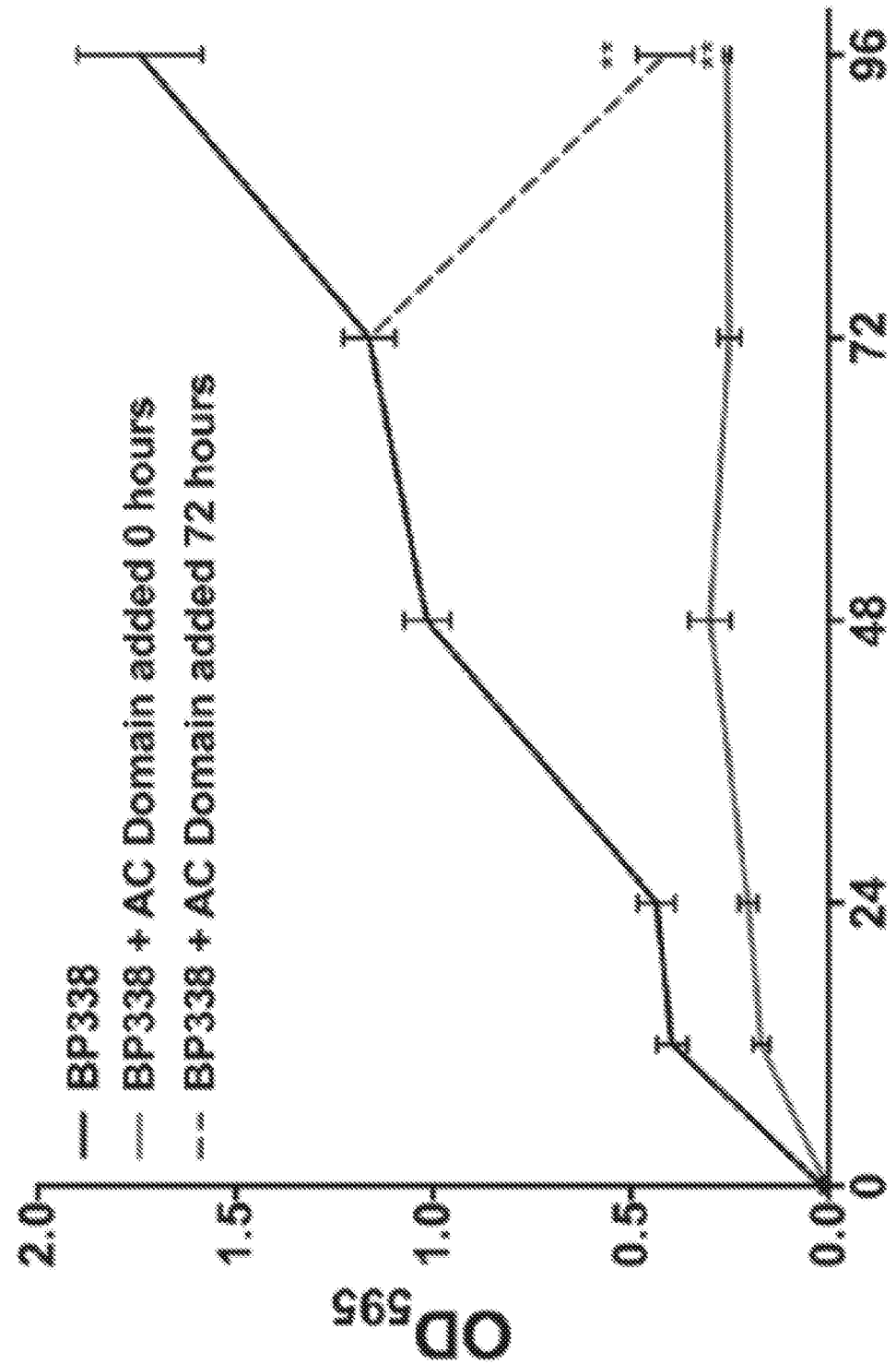
BP338
BP338 + AC Domain added 0 hours
BP338 + AC Domain added 72 hours
OD595
2.0
1.5
1.0
0.5
0.0
0
24
48
72
96
Hours
**
**

Fig. 13B

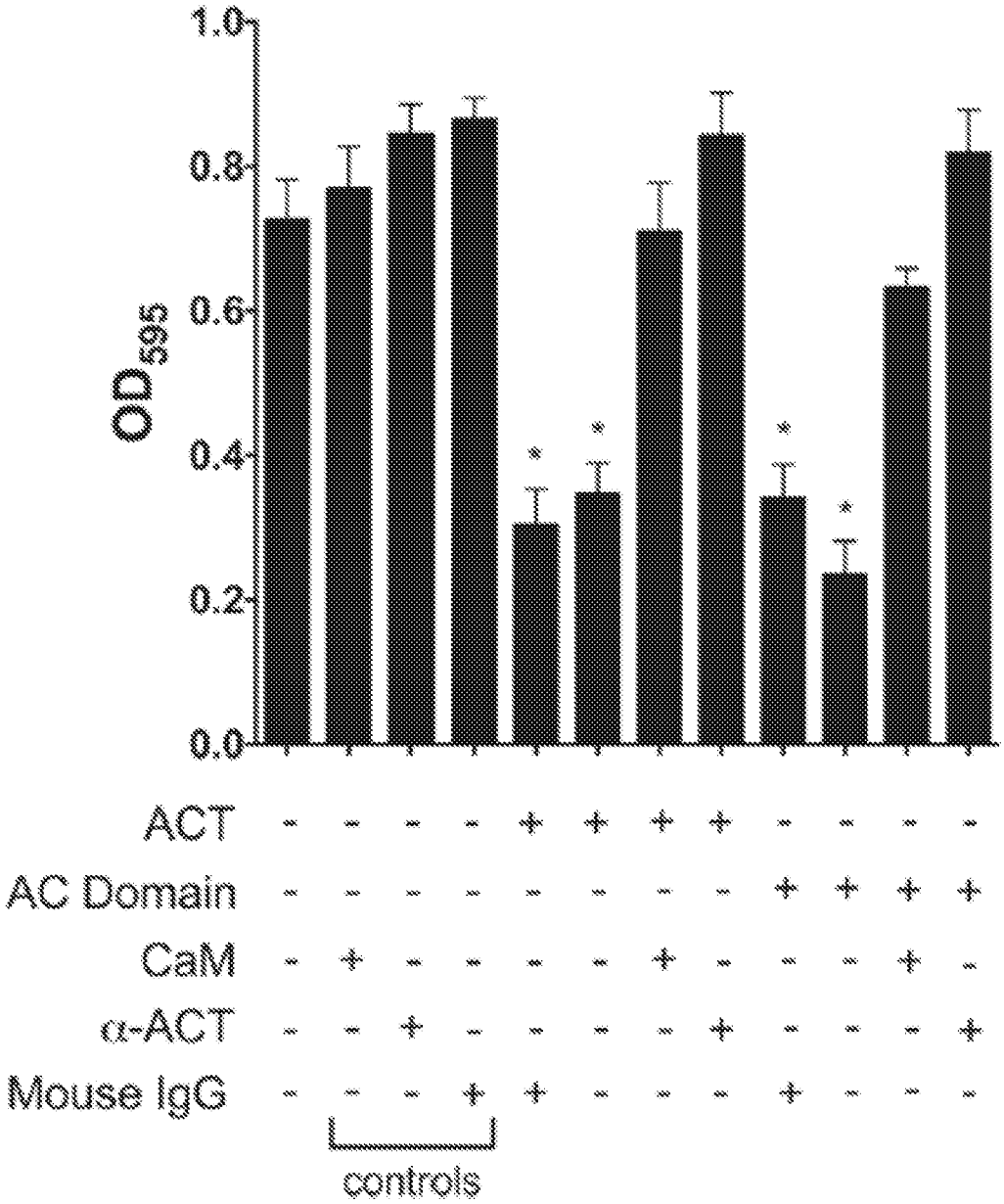
1.0
0.8
0.6
0.4
0.2
0.0
OD595
*
*
*
*
ACT - - - - + + + + - - - -
AC Domain - - - - - - - - + + + +
CaM - + - - - - + - - - + -
α-ACT - - + - - - - + - - - +
Mouse IgG - - - + + - - - + - - -
controls

Fig. 14

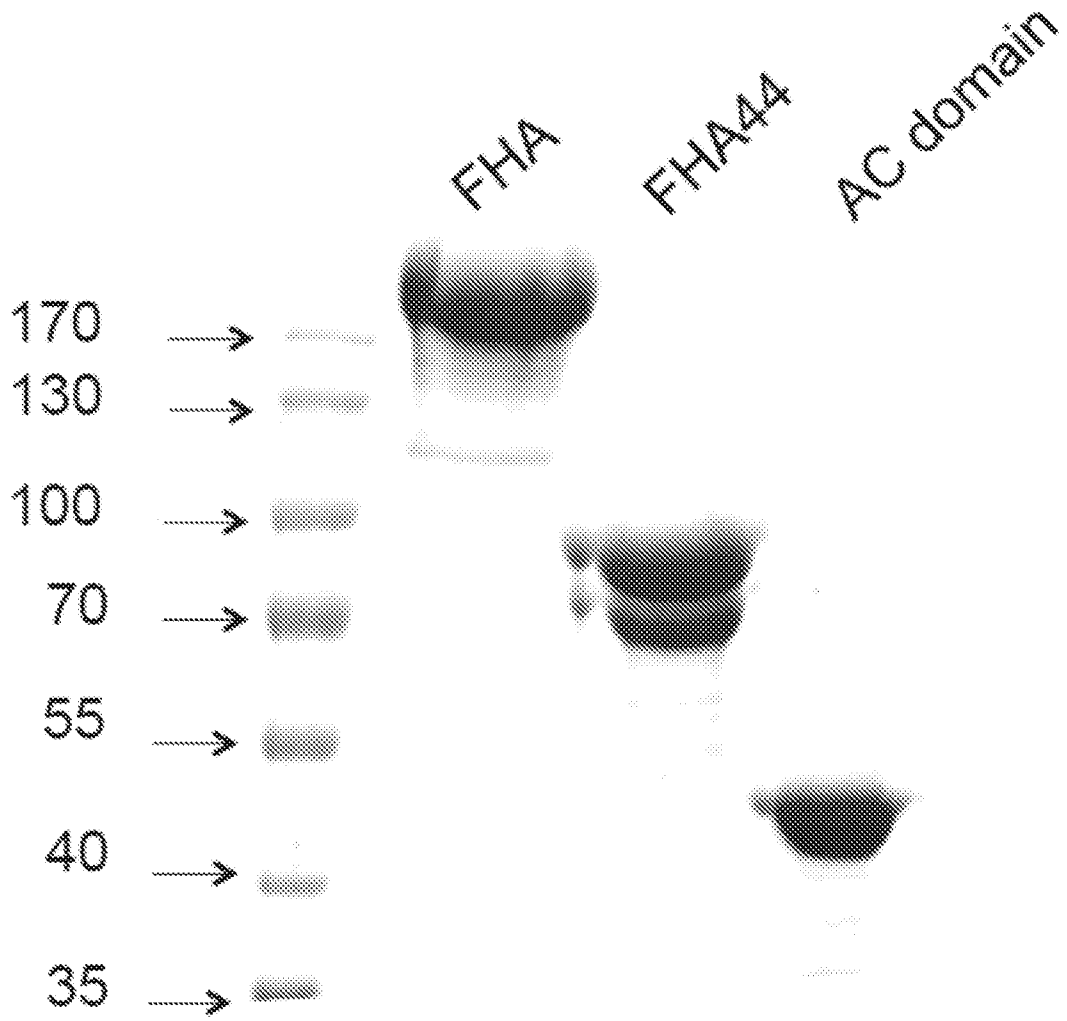

Fig. 15

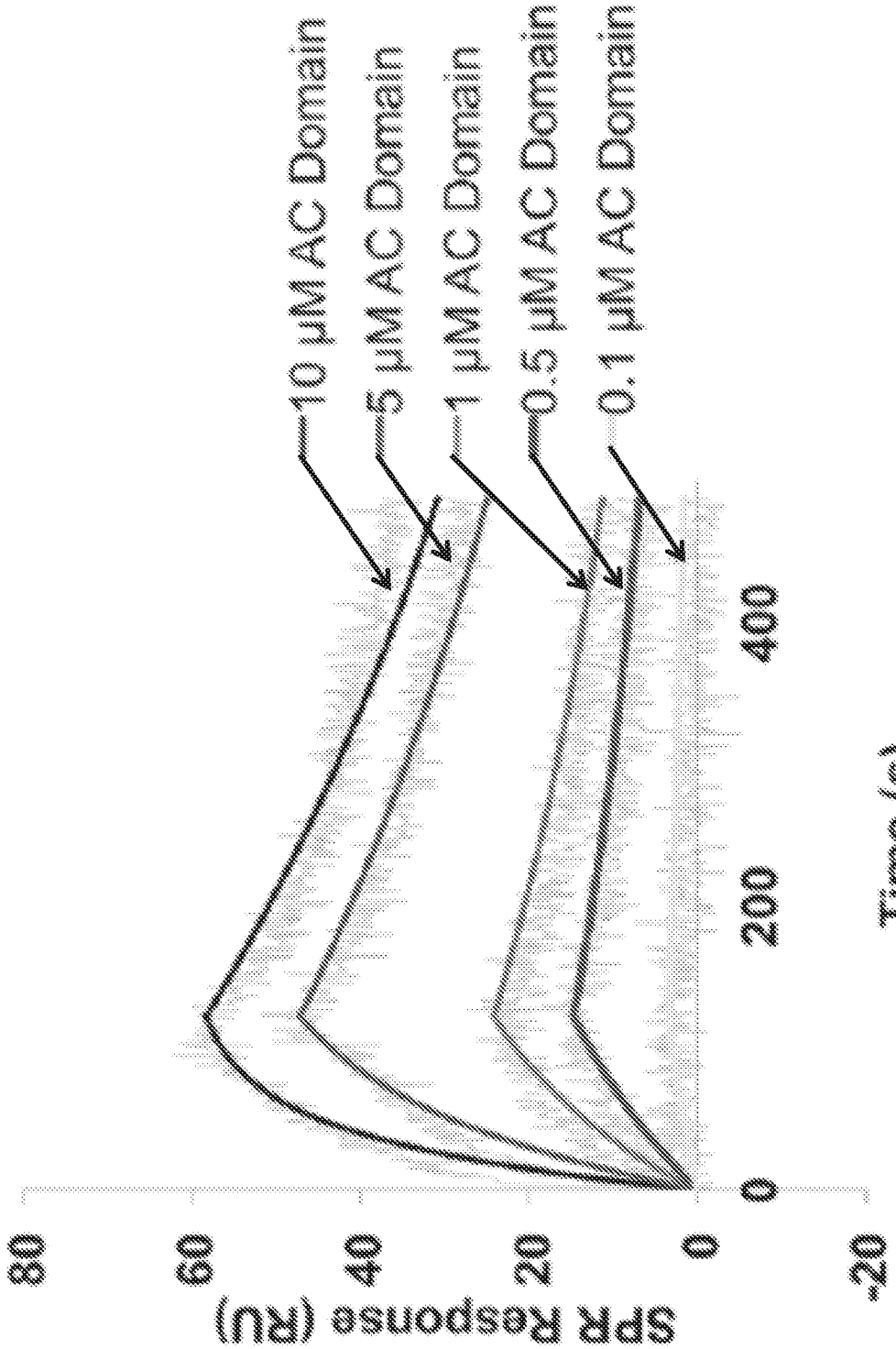
10 μM AC Domain
5 μM AC Domain
1 μM AC Domain
0.5 μM AC Domain
0.1 μM AC Domain
SPR Response (RU)
80
60
40
20
0
-20
0
200
400
Time (s)

Fig. 16A

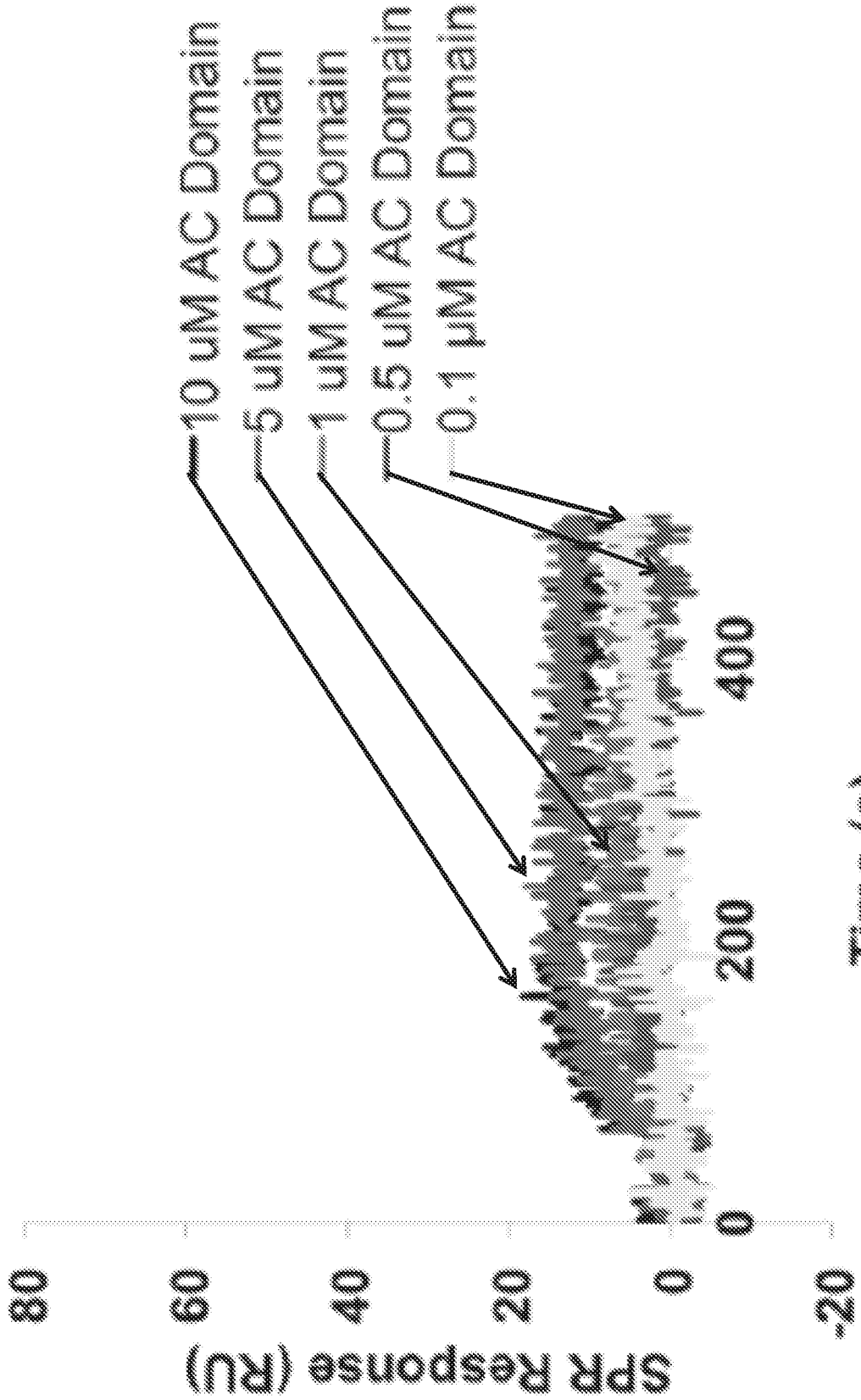
10 uM AC Domain
5 uM AC Domain
1 uM AC Domain
0.5 uM AC Domain
0.1 uM AC Domain
SPR Response (RU)
80
60
40
20
0
-20
0
200
400
Time (s)

Fig. 16B

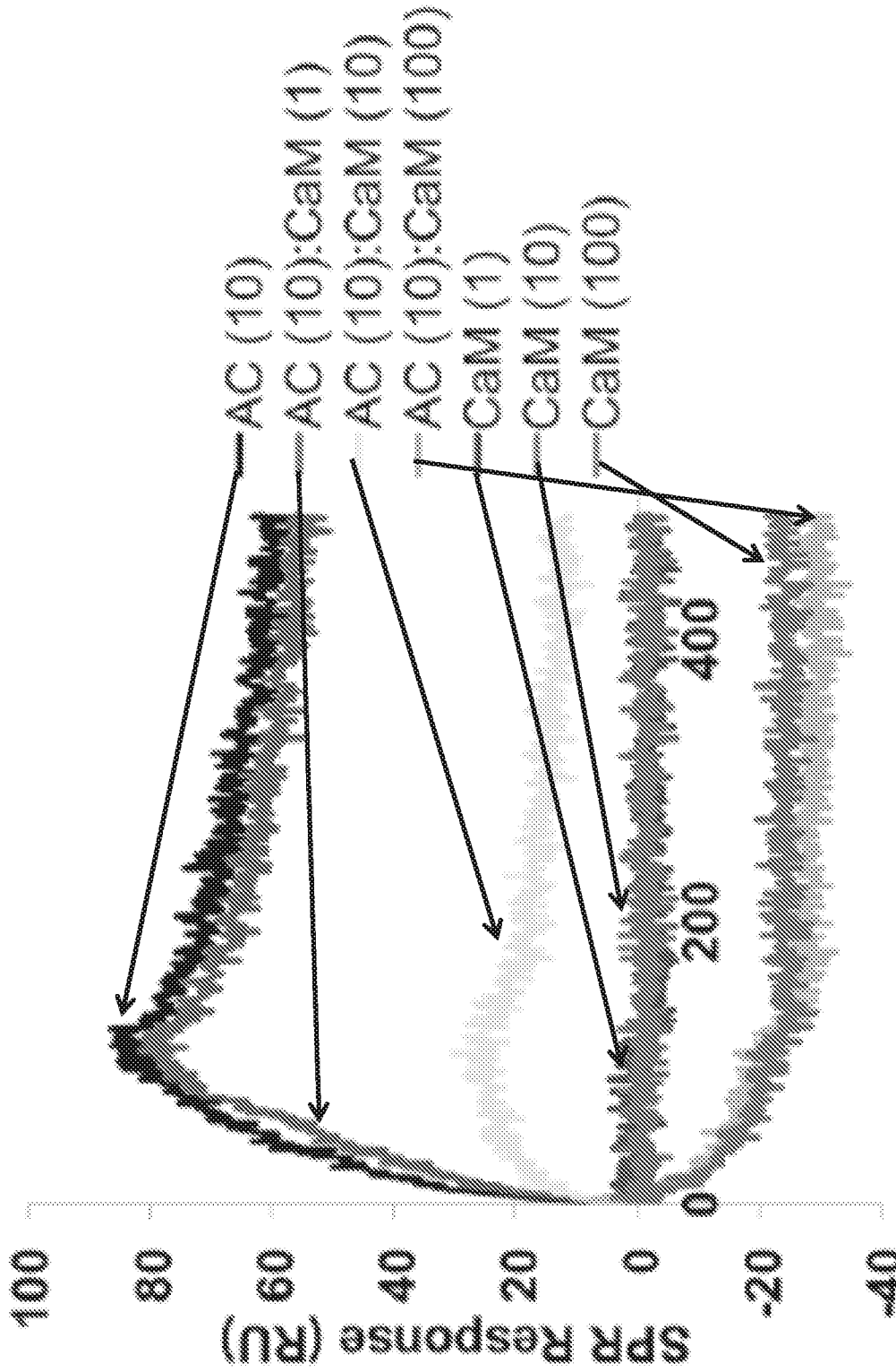
AC (10)
AC (10):CaM (1)
AC (10):CaM (10)
AC (10):CaM (100)
CaM (1)
CaM (10)
CaM (100)
SPR Response (RU)
100
80
60
40
20
0
-20
-40
0
200
400
Time (s)

Fig. 16C

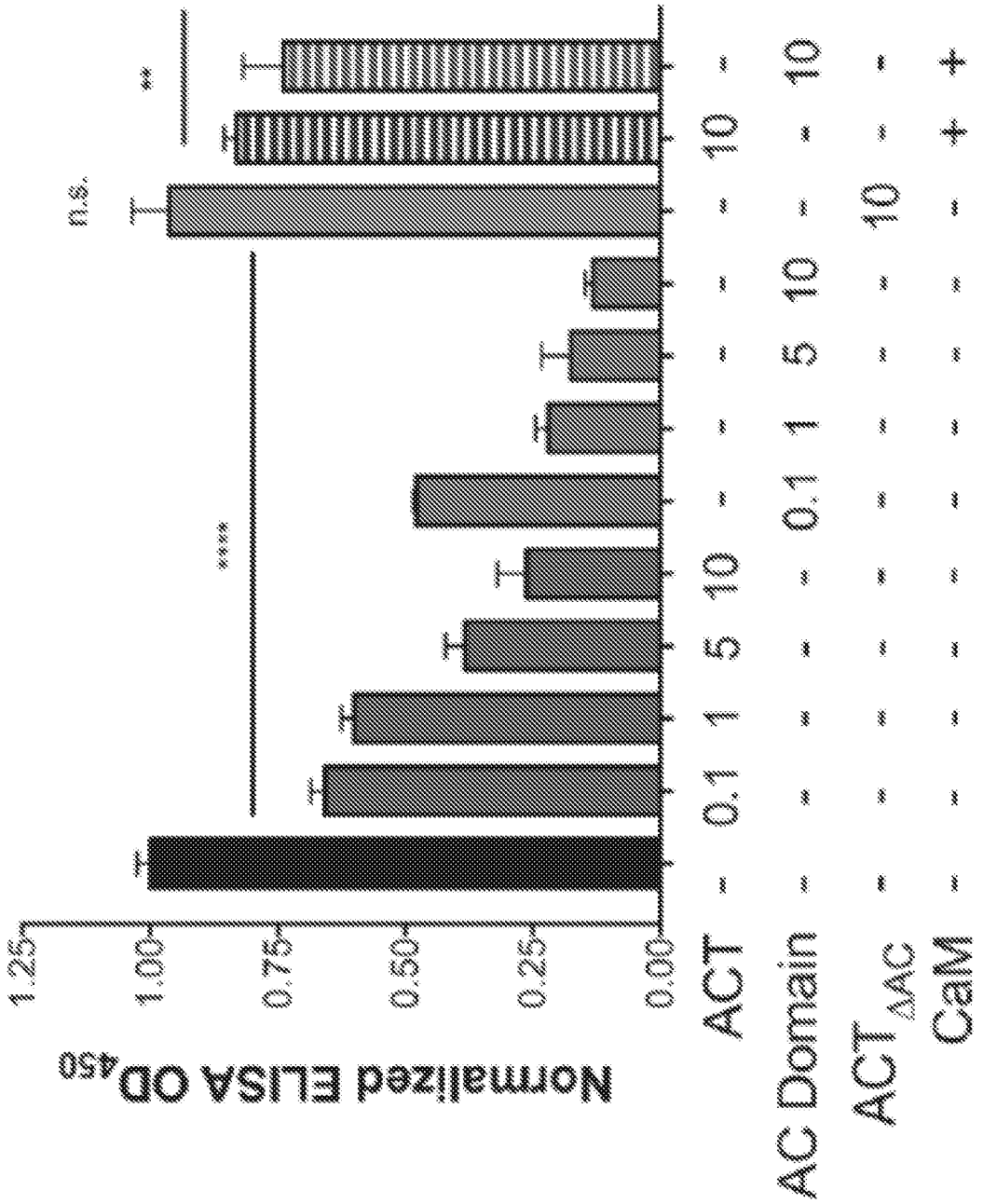
Normalized ELISA OD450
1.25
1.00
0.75
0.50
0.25
0.00
****
n.s.
**
ACT - 0.1 1 5 10 - 0.1 1 5 10 - 10 -
AC Domain - - - - - - - - - - - - -
ACT ΔAC - - - - - - - - - - 10 - -
CaM - - - - - - - - - - - + +

Fig. 17

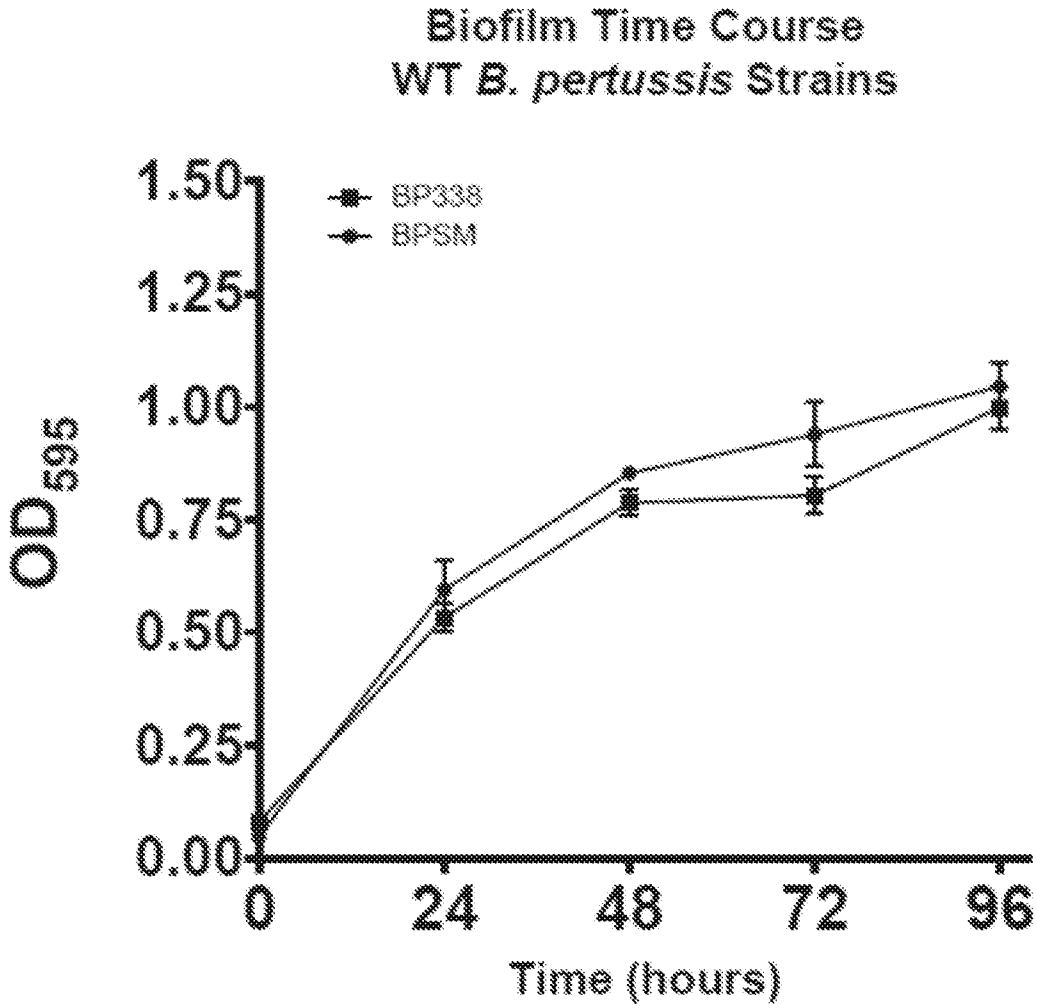
Biofilm Time Course
WT B. pertussis Strains
BP338
BPSM
OD595
1.50
1.25
1.00
0.75
0.50
0.25
0.00
0
24
48
72
96
Time (hours)

Fig. 18A

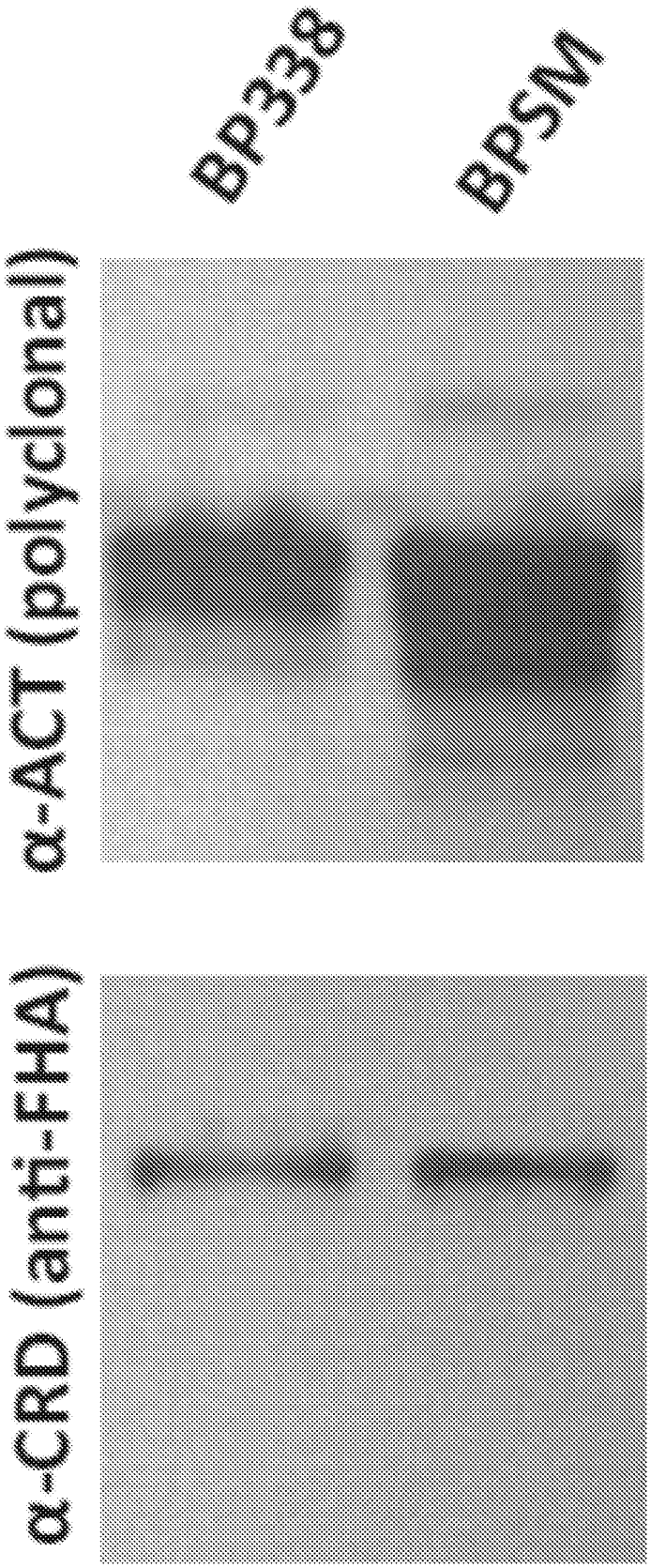

Fig. 18B

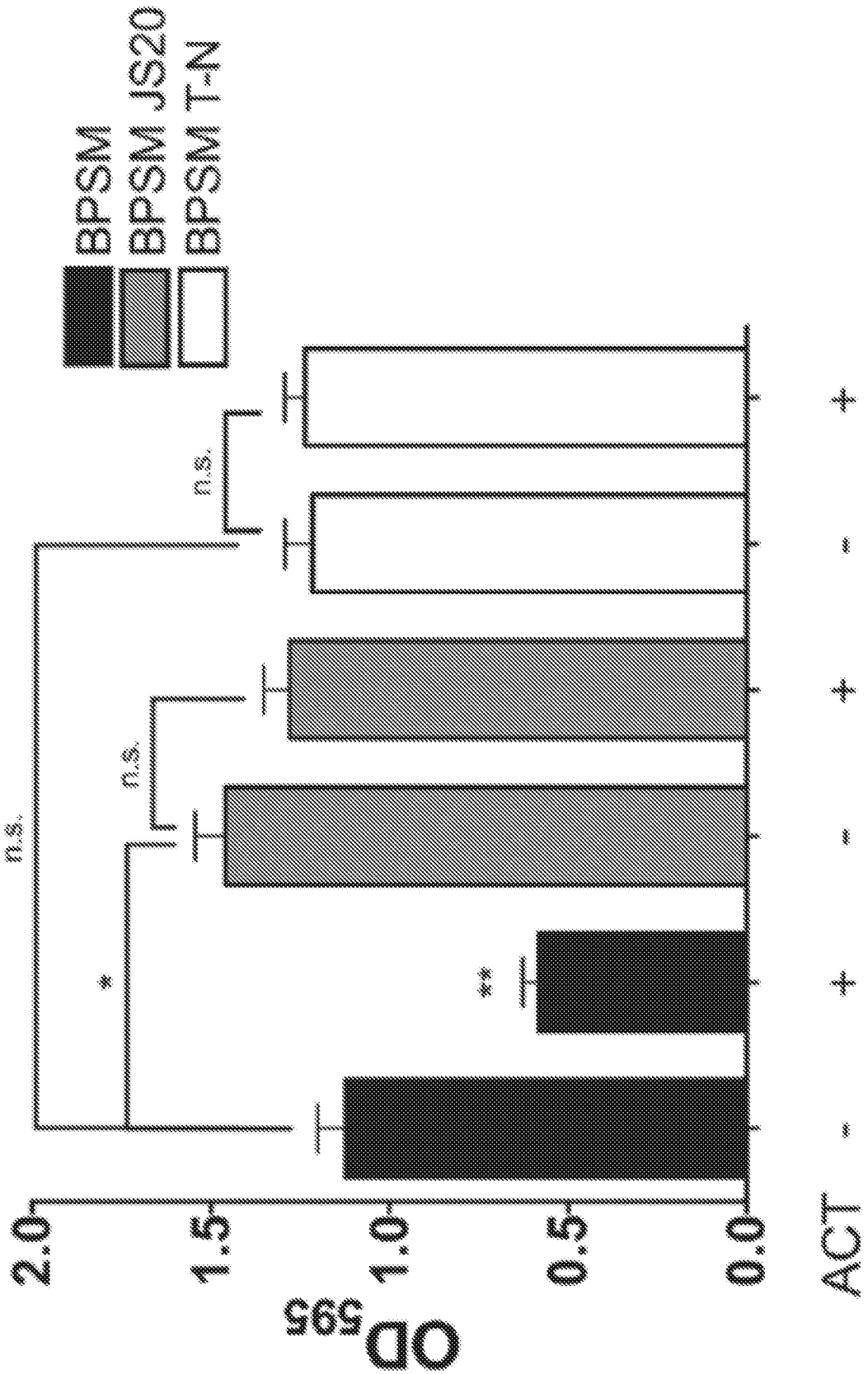

Fig. 19A

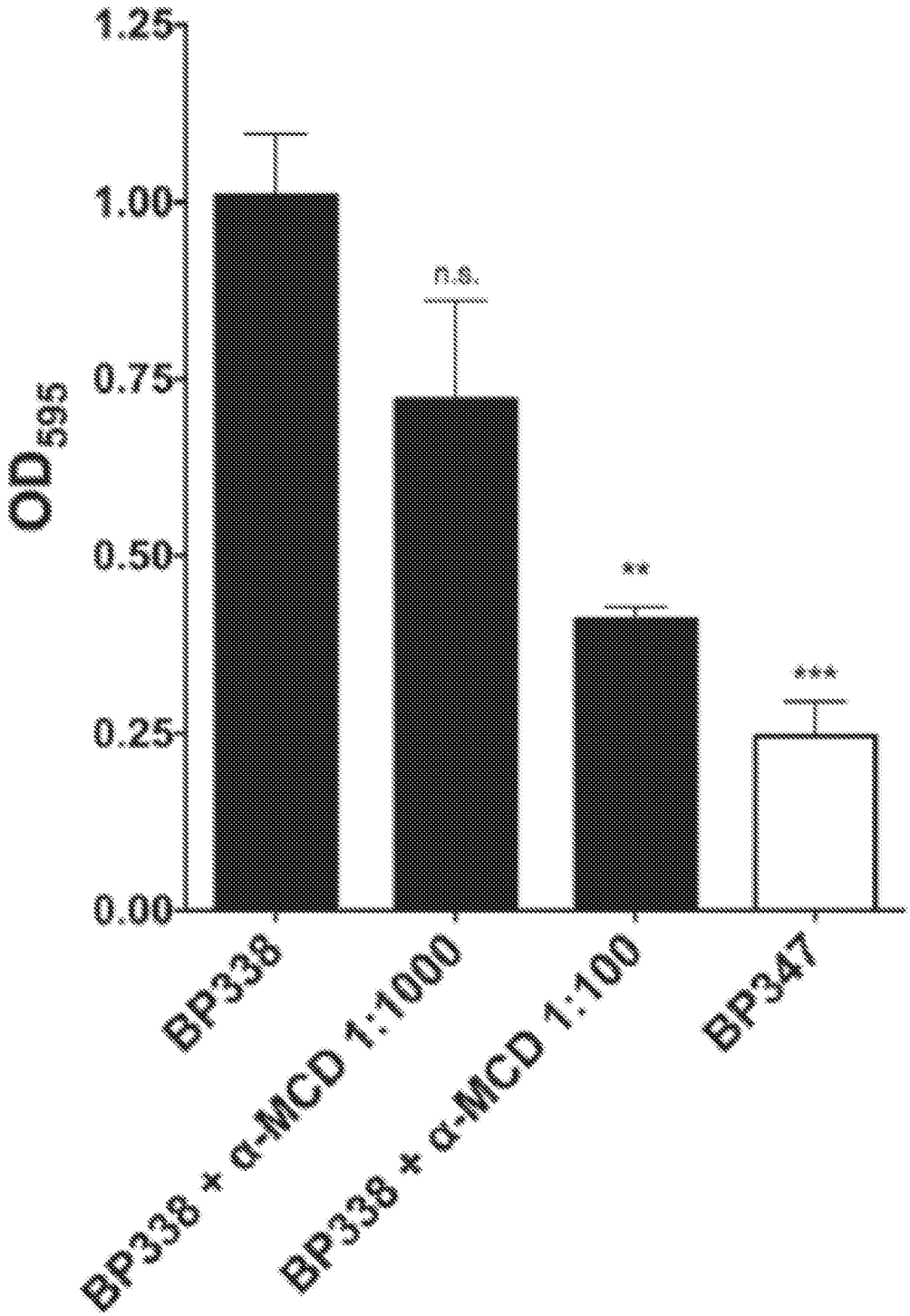
1.25
1.00
0.75
0.50
0.25
0.00
OD595
n.s.
**
***
BP338
BP338 + α-MCD 1:1000
BP338 + α-MCD 1:100
BP347

Fig. 19B

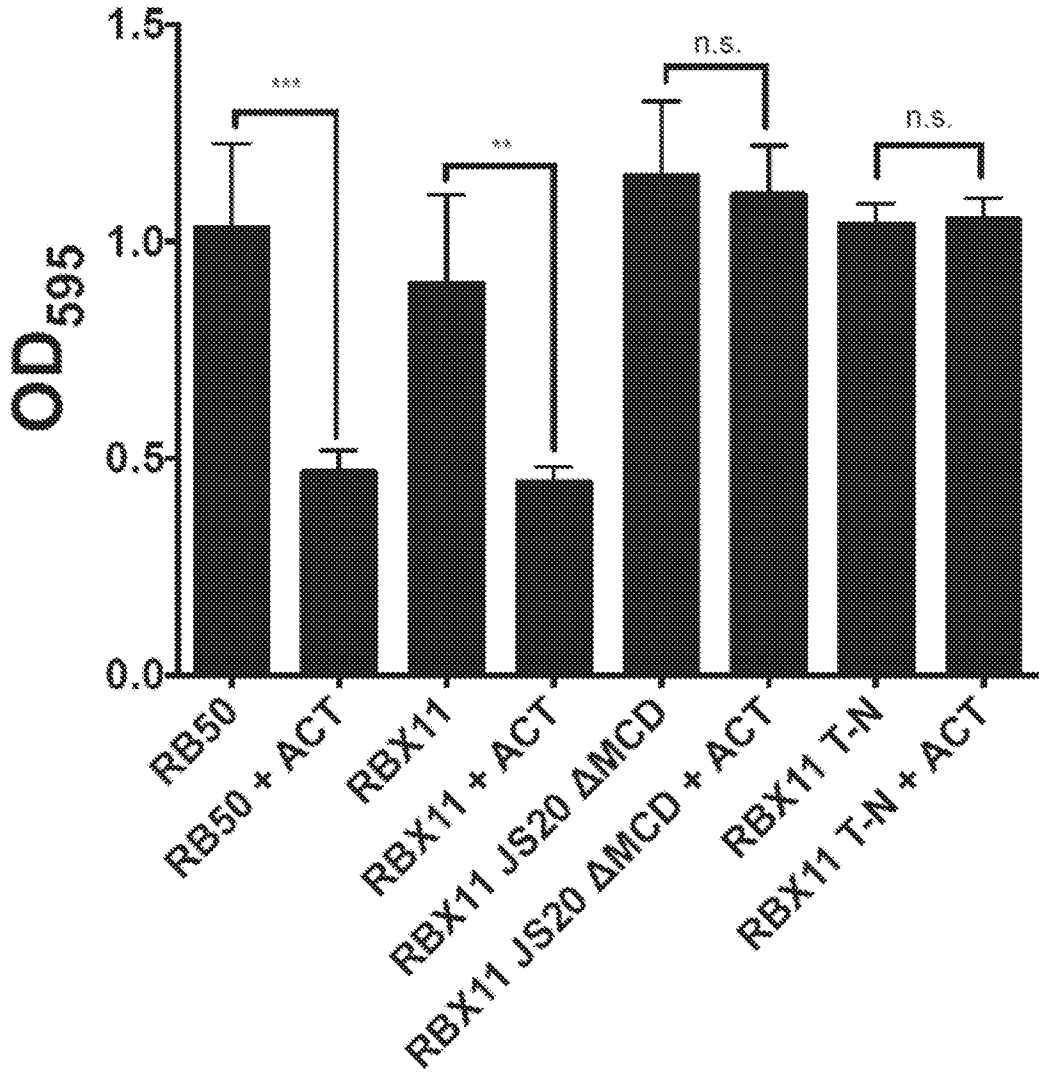
1.5
1.0
0.5
0.0
OD595
***
**
n.s.
n.s.
RB50
RB50 + ACT
RBX11
RBX11 + ACT
RBX11 JS20 ΔMCD
RBX11 JS20 ΔMCD + ACT
RBX11 T-N
RBX11 T-N + ACT

Fig. 20

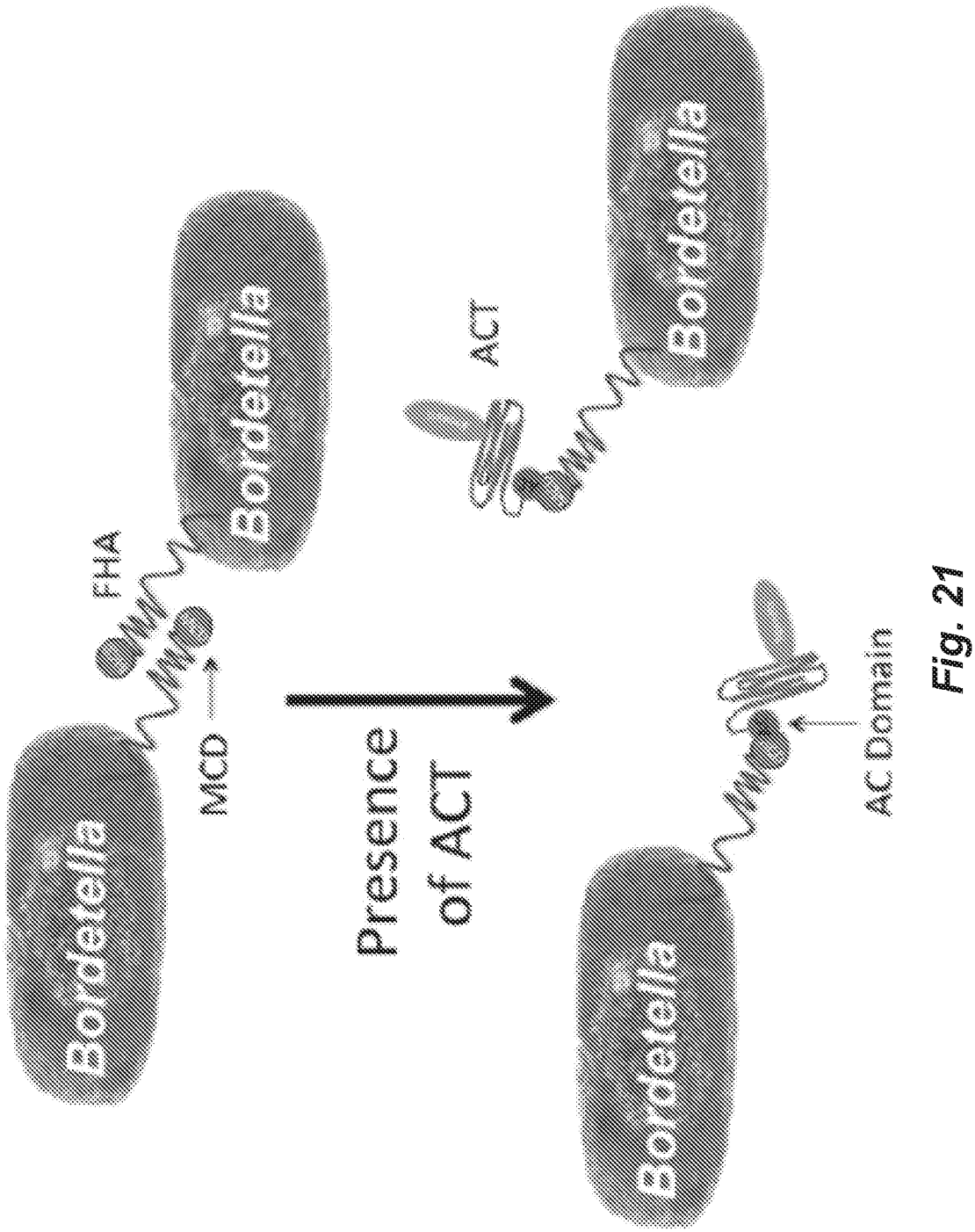
Bordetella
FHA
MCD
Bordetella
Presence
of ACT
ACT
Bordetella
Bordetella
AC Domain

Fig. 21

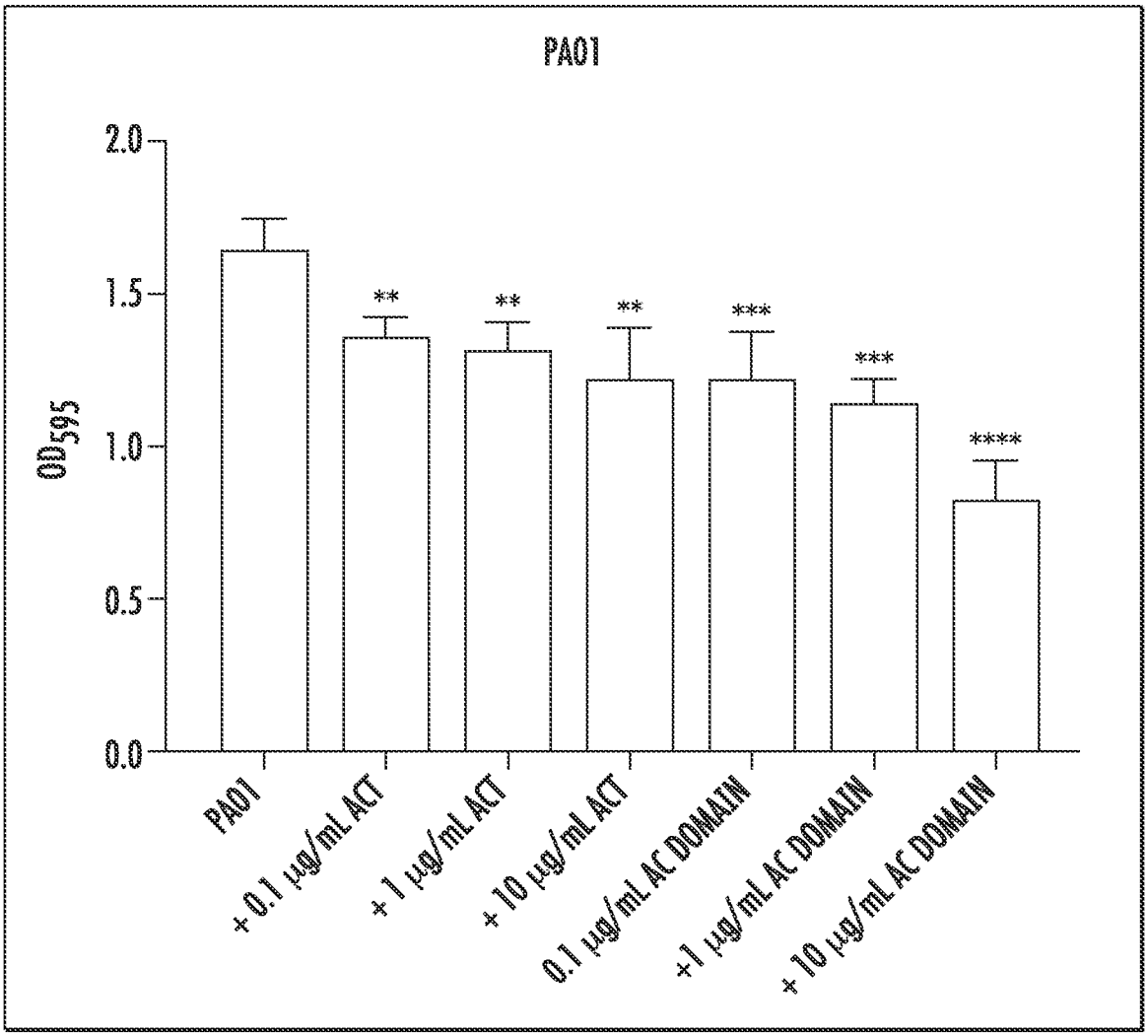
PA01
2.0
1.5
1.0
0.5
0.0
OD595
**
**
**
***
***
****
PA01
+ 0.1 µg/mL ACT
+ 1 µg/mL ACT
+ 10 µg/mL ACT
0.1 µg/mL AC DOMAIN
+1 µg/mL AC DOMAIN
+ 10 µg/mL AC DOMAIN

FIG. 22A

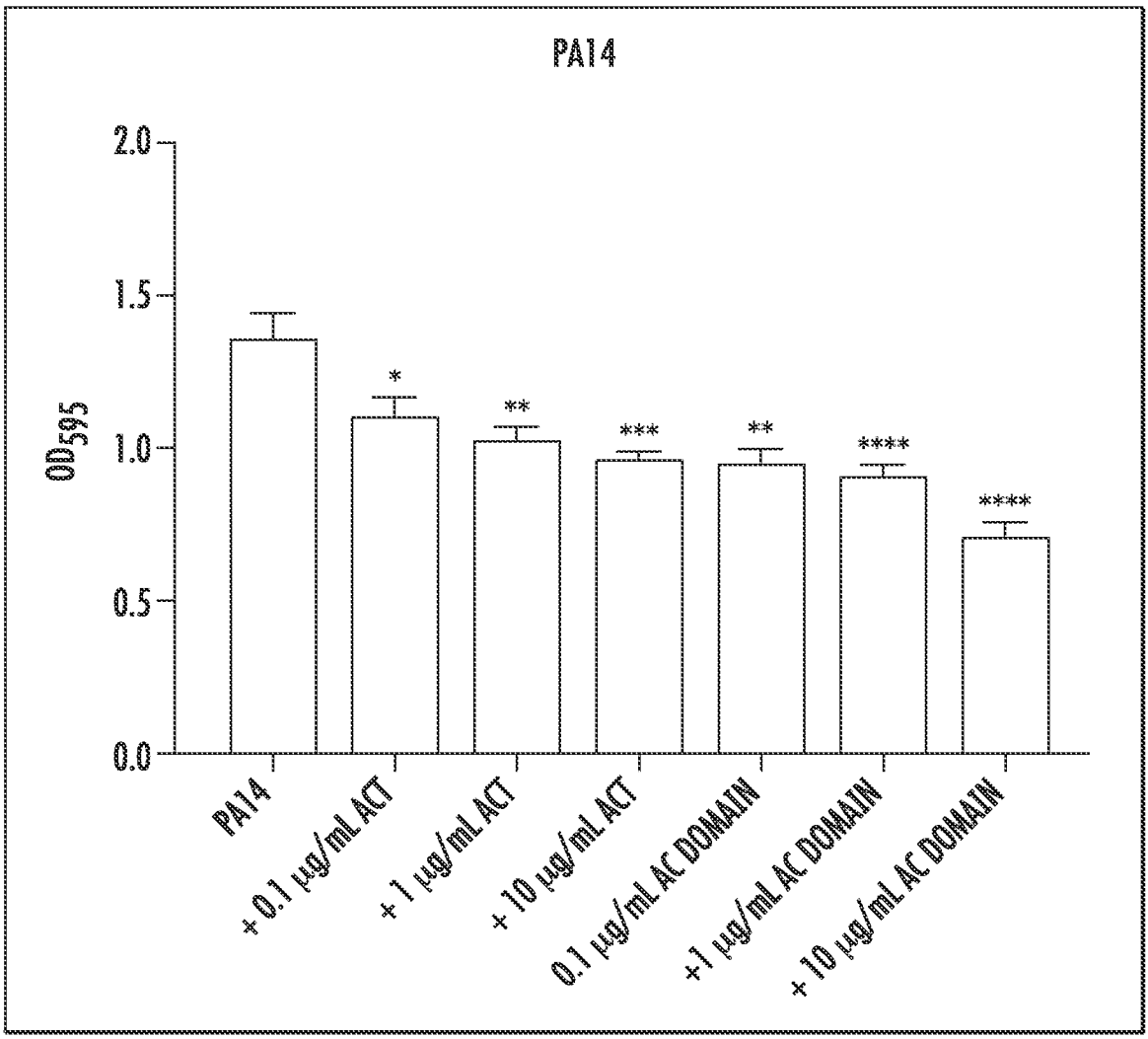
PA14
2.0
1.5
1.0
0.5
0.0
OD595
*
**
***
**
****
****
PA14
+ 0.1 μg/mL ACT
+ 1 μg/mL ACT
+ 10 μg/mL ACT
0.1 μg/mL AC DOMAIN
+1 μg/mL AC DOMAIN
+ 10 μg/mL AC DOMAIN

FIG. 22B

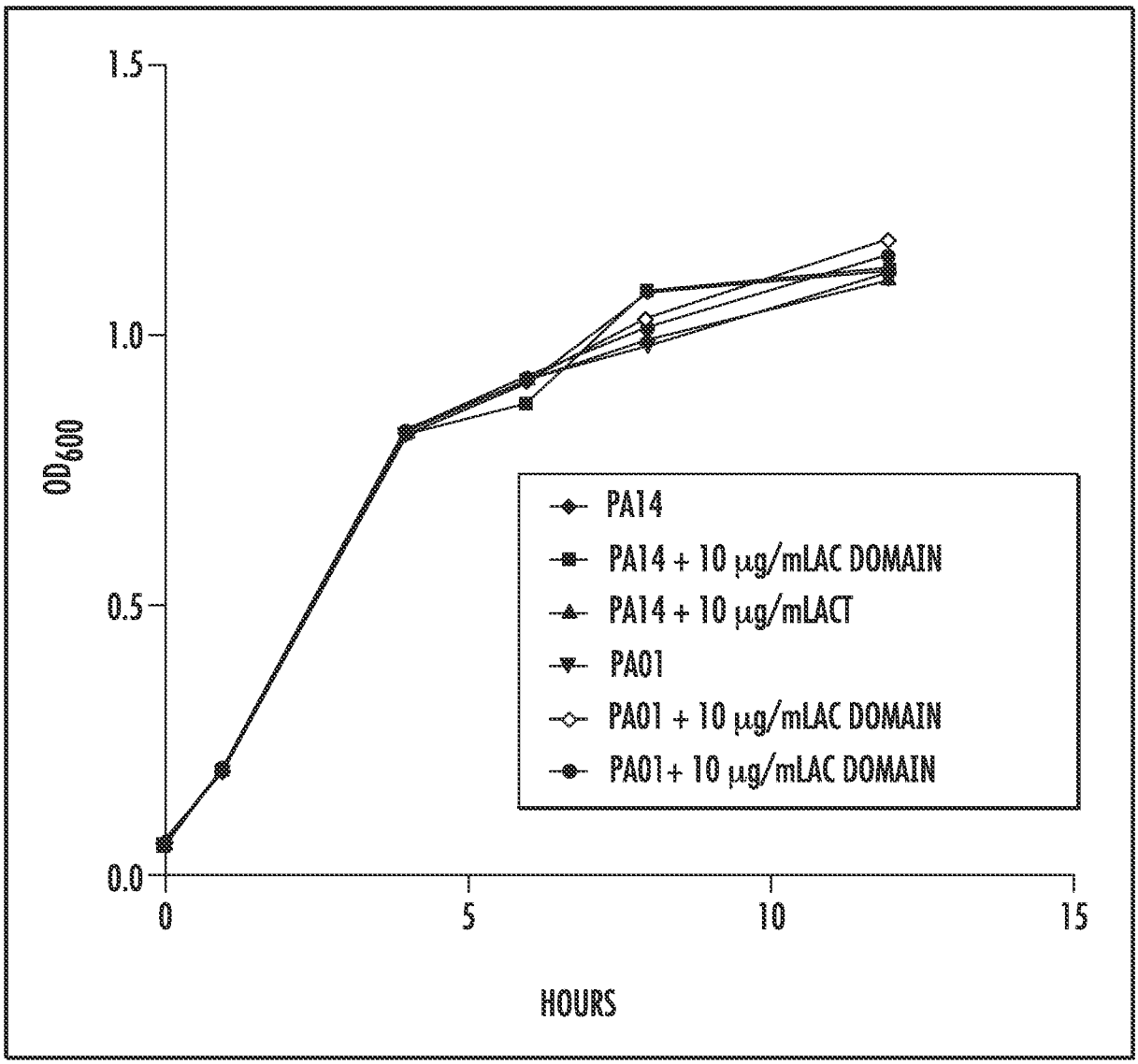
1.5
1.0
0.5
0.0
OD600
0
5
10
15
HOURS
PA14
PA14 + 10 μg/mLAC DOMAIN
PA14 + 10 μg/mLACT
PA01
PA01 + 10 μg/mLAC DOMAIN
PA01+ 10 μg/mLAC DOMAIN

FIG. 22C

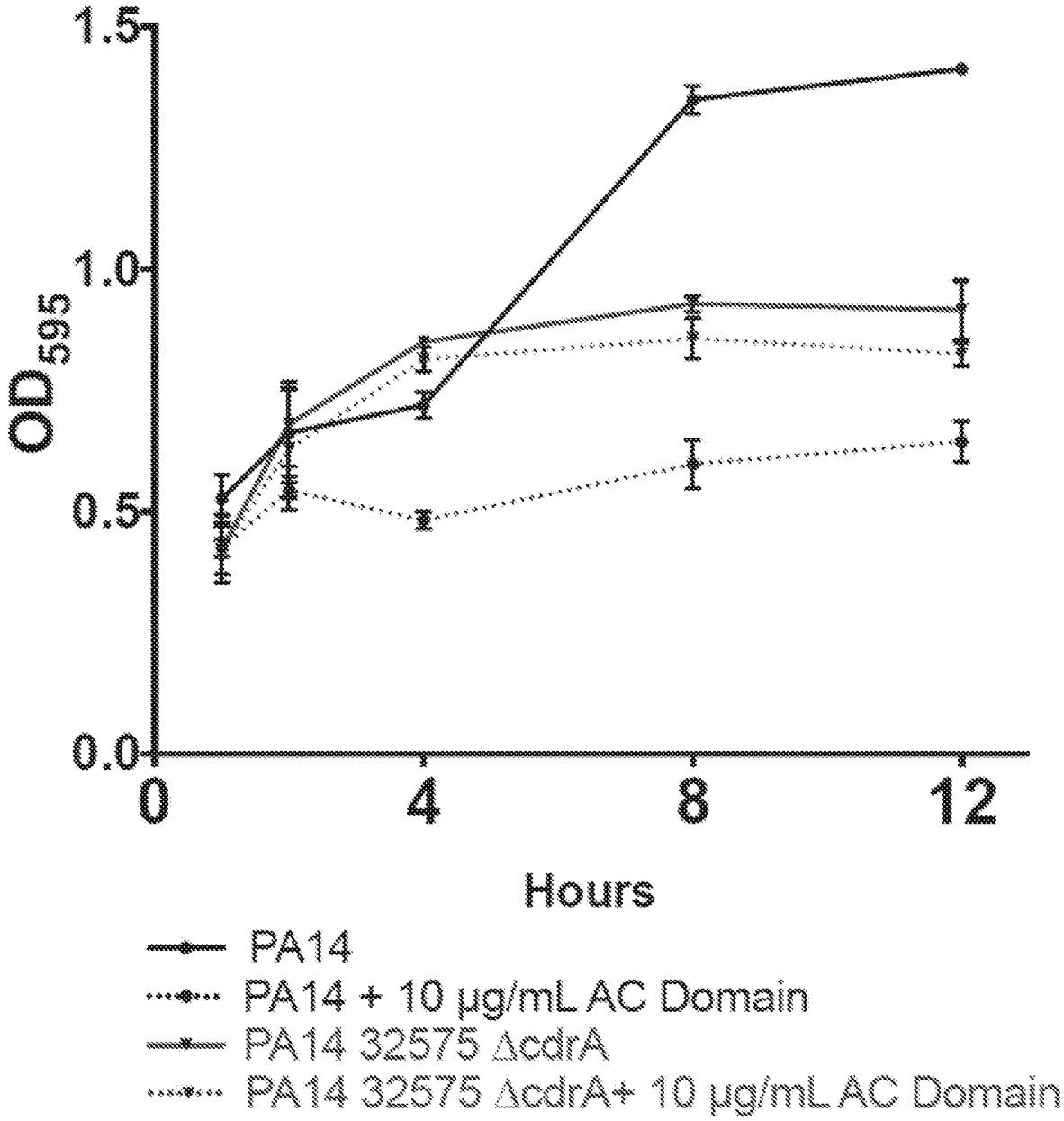
1.5
1.0
0.5
0.0
OD595
0
4
8
12
Hours
PA14
PA14 + 10 µg/mL AC Domain
PA14 32575 ΔcdrA
PA14 32575 ΔcdrA+ 10 µg/mL AC Domain

Fig. 23

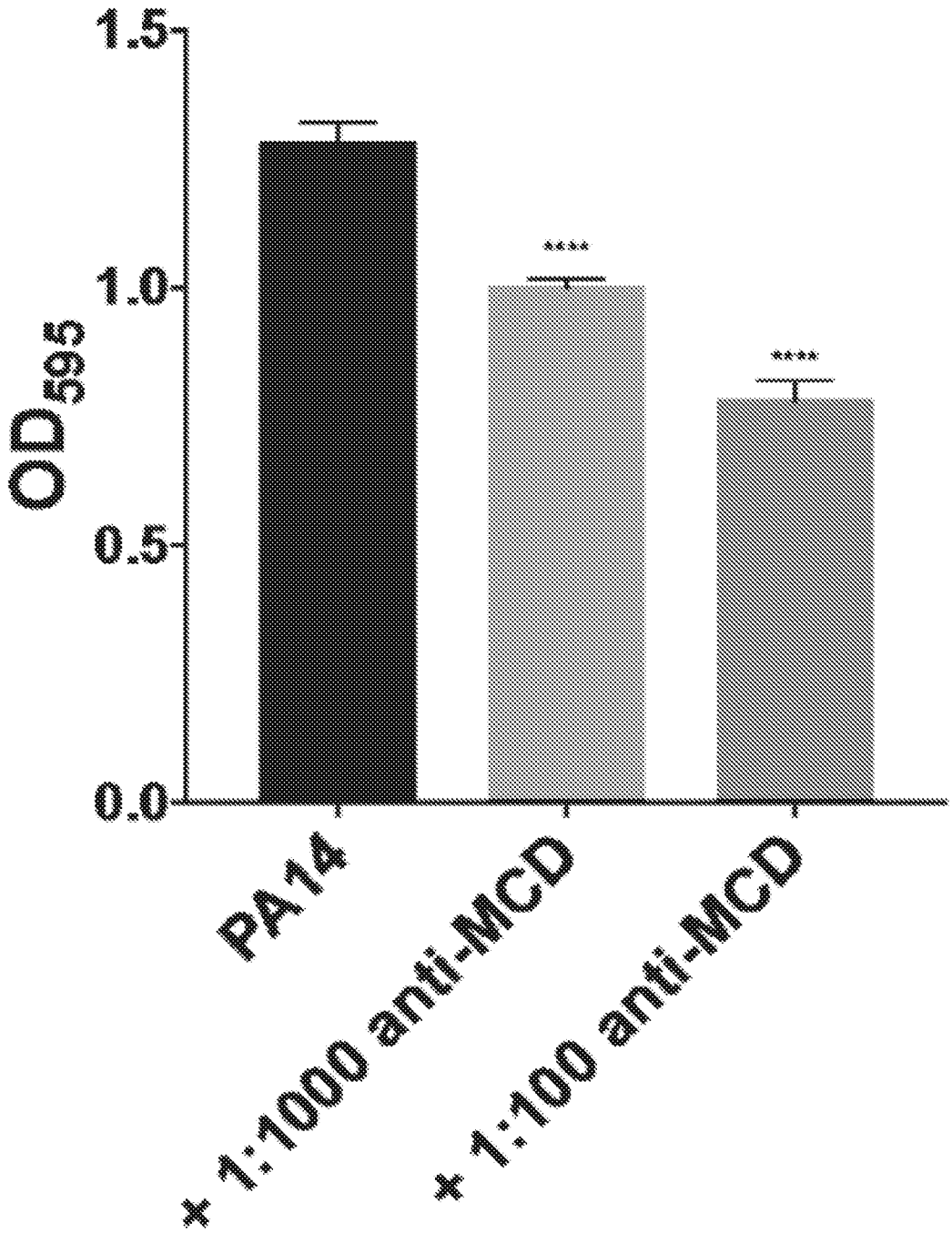

Fig. 24

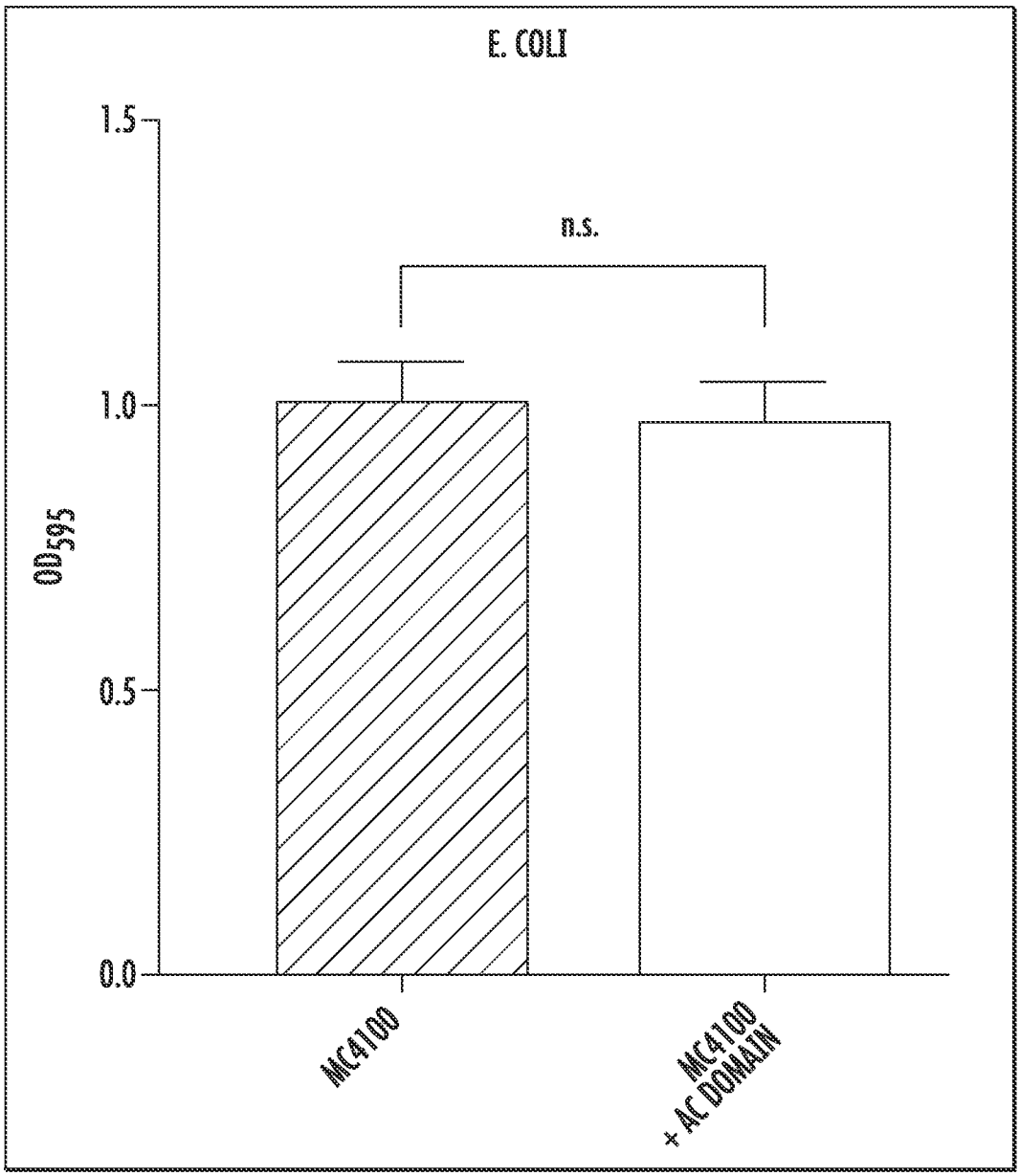

FIG. 25A

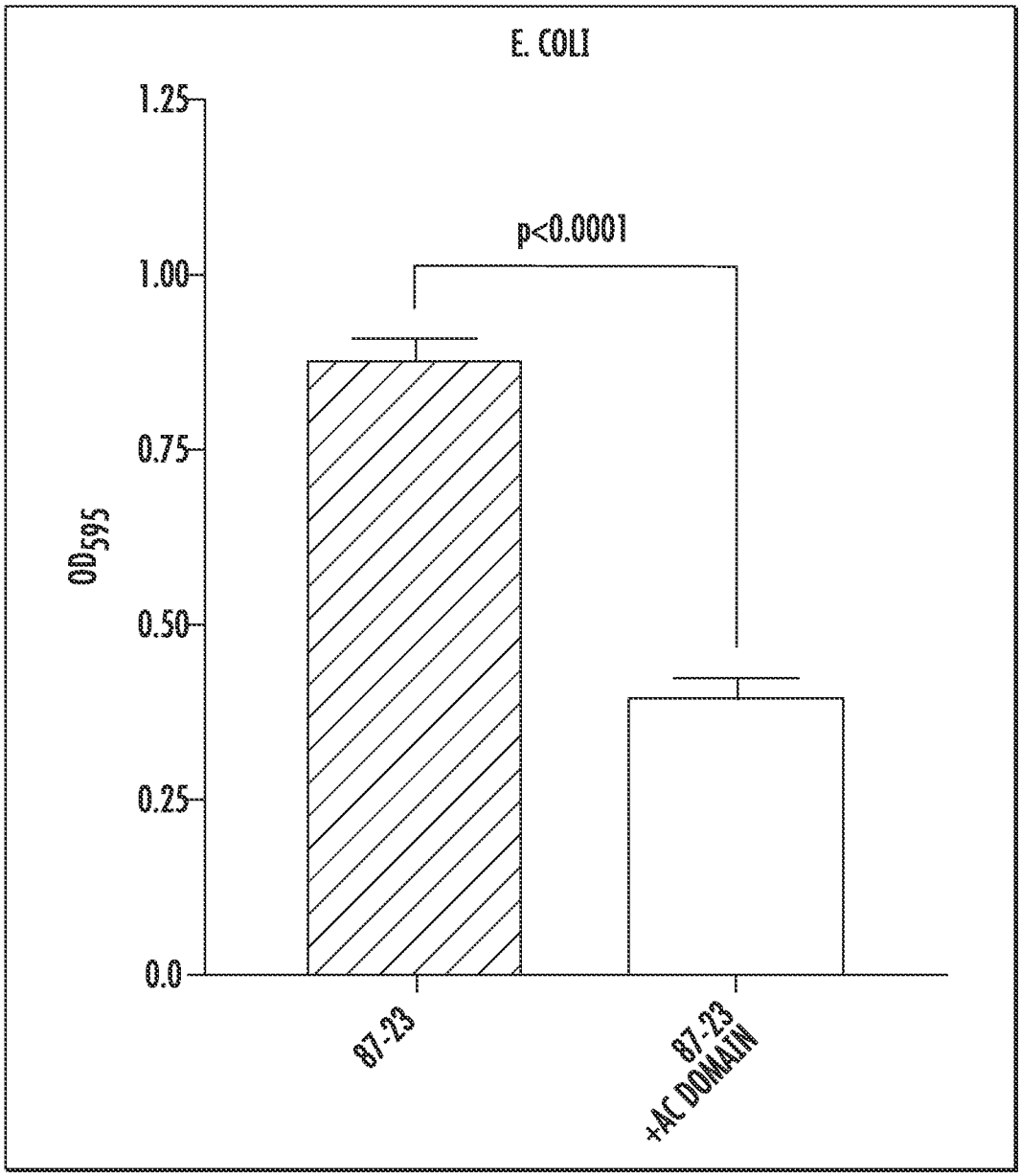

FIG. 25B

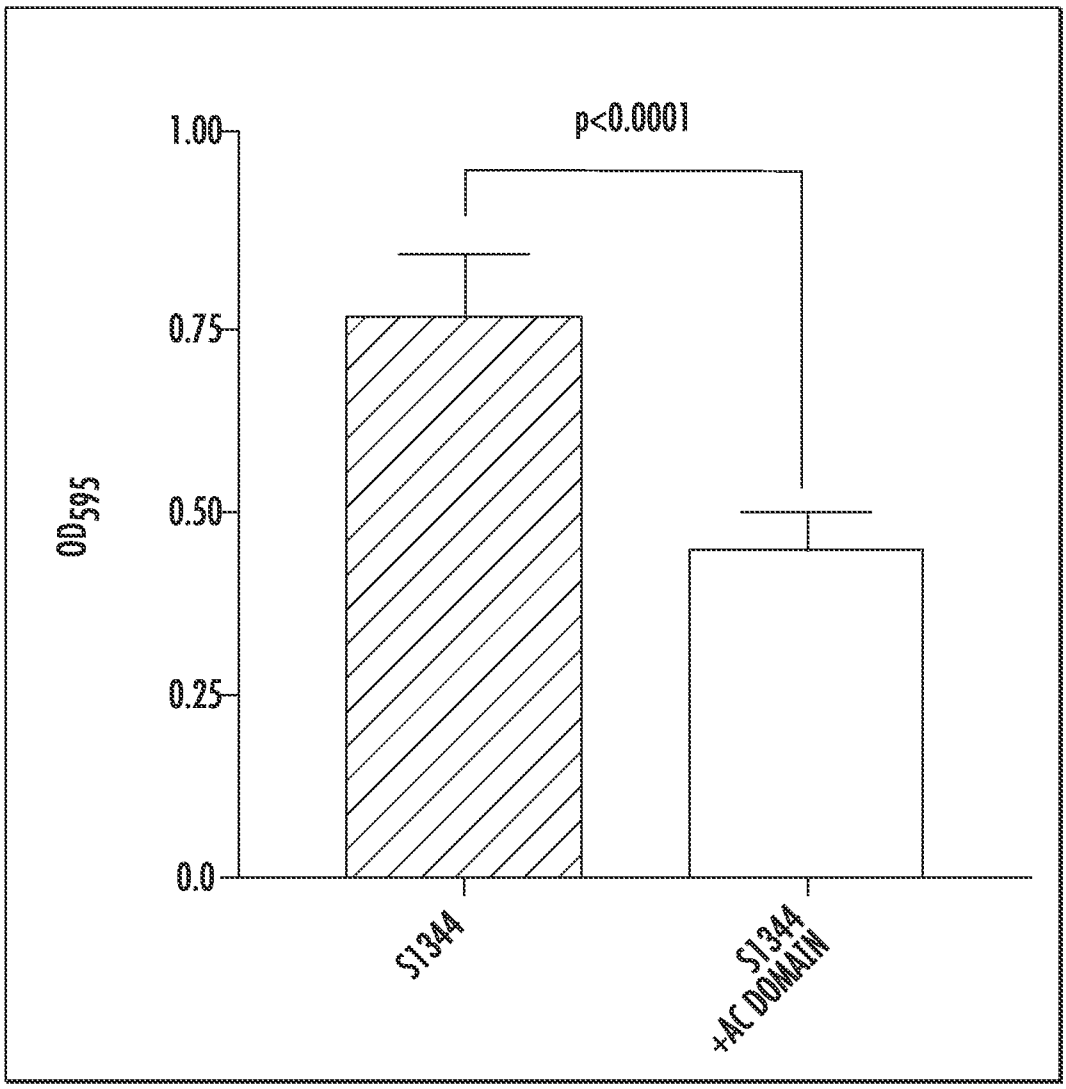

FIG. 25C

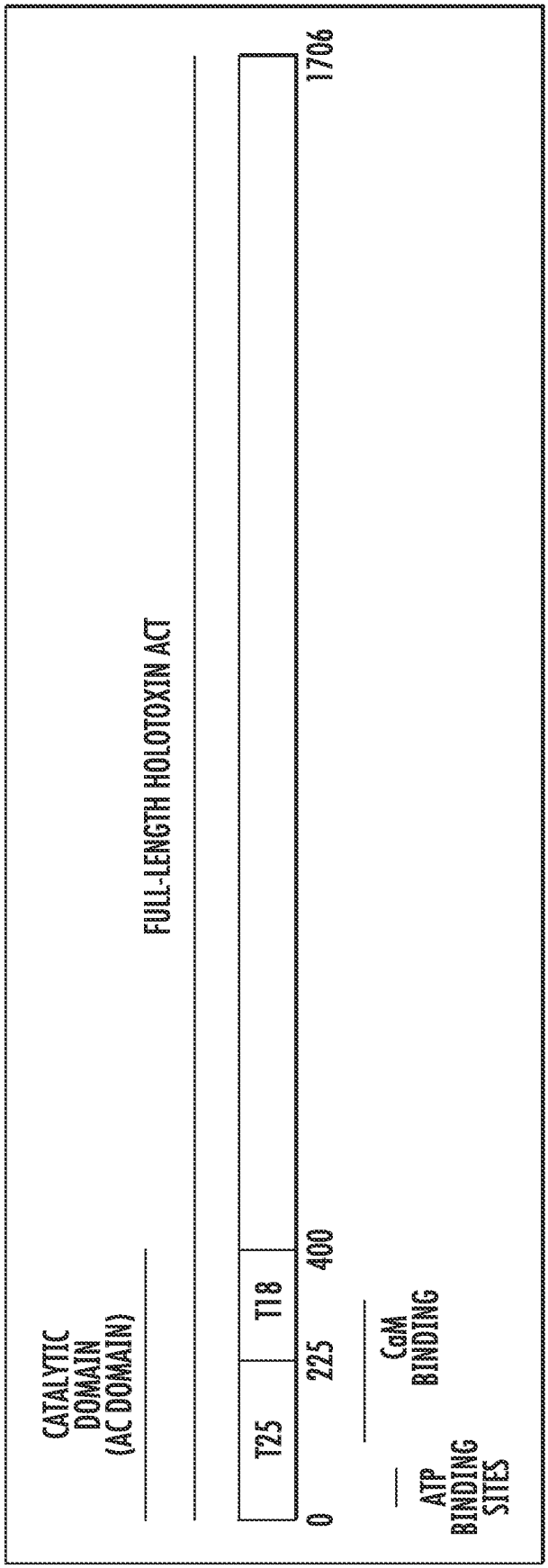
CATALYTIC DOMAIN (AC DOMAIN)
FULL-LENGTH HOLOTOXIN ACT
T25
T18
0
225
400
1706
ATP BINDING SITES
CaM BINDING

FIG. 26A

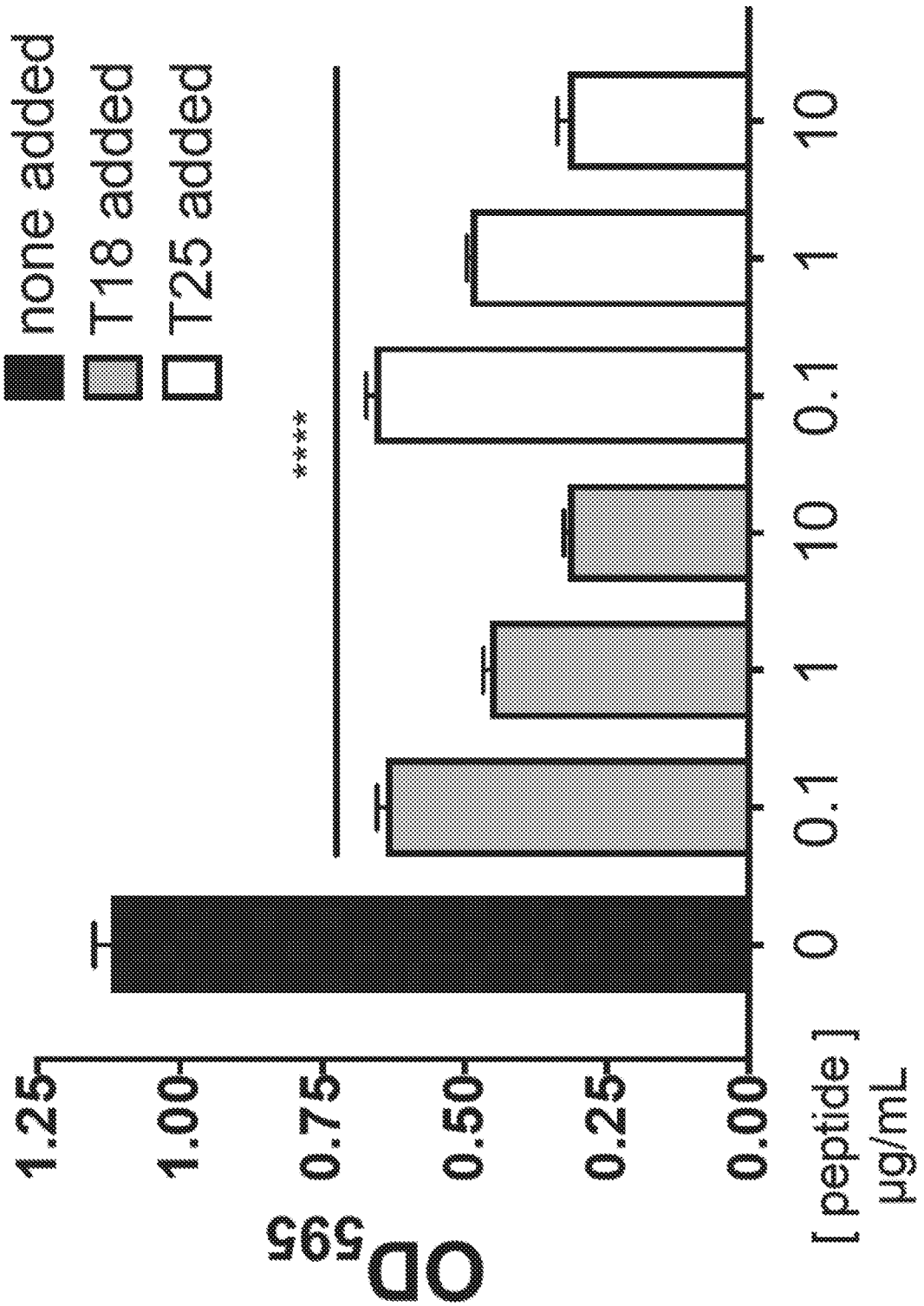
none added
T18 added
T25 added
****
OD595
1.25
1.00
0.75
0.50
0.25
0.00
0
0.1
1
10
0.1
1
10
[peptide]
µg/mL

Fig. 26B