病毒崩具有毒殺帶有 NDM-1 肺炎克雷白氏菌與抑制生物膜形成之作用

VirusBom-mediated killing and inhibition of biofilm formation on NDM-1 positive *Klebsiella pneumoniae*

中文摘要

快速於世界各地散播的 **NDM-1 (New Delhi Metallo-β-lactamase)**陽性病原菌已對公共衛生與臨床治療造成極大困擾與威脅，如何有效避免此類病原菌的散播為首要議題。日前，於臺灣大學所發展出來的病毒崩(**VirusBom**)可以快速且有效地殺死不同的病原菌，包含病毒與細菌，公衛防治上有其應用之潛能，因此，我們想進一步探討是否病毒崩可以有效殺死與避免 **NDM-1** 陽性病原菌之散播。在目前最新的研究結果指出，病毒崩可以直接殺死從台灣所分離出來的 **NDM-1** 陽性的肺炎克雷白氏菌，當 **300ppm** 的病毒崩在室溫下與此細菌直接反應，可以在短短一分鐘內快速殺死它。電子顯微鏡與螢光染色分析顯示病毒崩之毒殺機制是經由改變細胞壁結構而導致死亡。有趣的是當此細菌處在含有不同濃度病毒崩之培養液環境下培養，就算高達 **300ppm** 的病毒崩並不會明顯改變其生長，但卻可以有效地抑制其生物膜(**Biofilm**)的形成，甚至已經形成的生物膜在 **300ppm** 的病毒崩直接處理之後，生物膜可以有效地被瓦解。因此，從目前的結果顯示，病毒崩可以快速殺死帶有 **NDM-1** 的肺炎克雷白氏菌，並可以藉由抑制生物膜的形成，來避免此類細菌的散播與附著，未來有極大的潛能應用於醫院與公共場所之衛生防護。

Abstract

Worldwide emergent carbapenem-resistant NDM-1 (New Delhi Metallo-β-lactamase)-producing pathogens have threaten public health and clinical therapy. How to efficiently control their spreading is an important issue. VirusBom, which is developed by National Taiwan University Center for Advanced Nanomaterials (NTU-CANM), has been shown to possess antivirus and antibacterial effects. Here, we show that VirusBom can rapidly kill NDM-1 positive *Klebsiella pneumoniae*, which is isolated in Taiwan 2010. When resuspending bacteria in 300 ppm or higher VirusBom at room temperature, no survival bacteria are observed after overnight culturing. Analysis by scanning electron microscope and bacterial live-dead staining shows that VirusBom induces death through alteration of cell wall structure. Interestingly, VirusBom, even up to 300 ppm, fails to affect bacteria growth during growing in Luria Broth (LB) medium in microtiter plate-based biofilm attachment assay, but it efficiently inhibits biofilm formation and further induces biofilm collapse. Accordingly, VirusBom has great potential of application in public health through killing pathogen and reducing biofilm formation.

重要性

帶有 NDM-1 質體的細菌的全球性散播已為臨床治療與公共衛生帶來極大的威脅，如何有效避免此種細菌的擴散與進而殺死是具有極大的應用價值。在此篇研究裡面顯示出病毒崩(VirusBom)具由直接毒殺帶由 NDM-1 的肺炎克雷白氏菌，重要的是，此種細菌的生物膜形成能力可有效的被病毒崩所抑制。因此，我們認為病毒崩所擁有的生物膜抑制與毒殺的作用在未來具有極大的發展性。

Significance

Worldwide spread of NDM-1 positive bacteria in recent years causes emergent outbreak within hospital and community in different countries. Development of a new preventive agent is an important issue. In this study, we demonstrate that VirusBom directly kills *Klebsiella pneumoniae* harboring NDM-1 and inhibits its biofilm formation. Since biofilm formation is highly correlated with bacteria survival, antibiotics resistance and nosocomial infection, VirusBom-mediated direct killing and biofilm inhibition will be a new preventive agent against bacterial infection and outbreak.

方法與材料

細菌菌株與培養條件

帶有 NDM-1 的肺炎克雷白氏菌是從一個第一個在台灣疾病管制局(Taiwan CDC)所報導案例的 38 歲台灣男子所分離出來的 (Wu *et al.*)。一般肺炎克雷白氏菌的培養是接種單一菌落在 Luria Broth (LB, Difco)培養液，然後在 37°C 隔夜振盪培養。為了獲取快速生長其階段的細菌，當隔夜培養菌液 1:100 稀釋於新的 LB 培養液中，震盪培養至細菌密度在 600nm 吸光值(OD_{600})約在 0.3 至 0.5 左右，藉由離心方式(8000rpm、10min)收取此階段的細菌，經過無菌的 phosphate buffer saline (PBS，pH7.4)洗滌一次後，回溶在所指定的溶液中進行下階段的實驗。

化學藥品

病毒崩(VirusBom)由臺灣蔓妮藝能有限公司(Money Marketing Corporation, Taiwan)所提供。病毒崩的高濃度原始溶液為 1000ppm，後續在不同溶液中所配製之不同濃度病毒崩皆由此原始溶液序列稀釋得來。病毒崩的原始溶劑以 1%乙醇溶液為基礎。D-glucose 從美國 Sigma 公司所購買。

病毒崩毒殺作用

$1x10^8$ 菌落數(colony-forming unit, CFU)之快速生長期階段的 NDM-1 的肺炎克雷白氏菌回溶在所標示之病毒崩溶液(ppm)或 1%乙醇溶液(控制組，mock)，混合均勻後，立即或在室溫靜置所指定時間之後，利用無菌的 PBS(pH7.4)做序列稀釋，並取出稀釋液均勻塗抹於 LB 固態培養基上，在 37°C 隔夜培養之後，計數所形

成之菌落數，各組所存活之菌落數以 CFU/ml 表示，重覆兩次獨立實驗，計算其平均值與標準差，相對於控制組(mock)之數據，利用 Student's *t* test 計算統計差異。****，P<0.0001; ND (non-detected)表示未偵測到菌落數。

掃描式電子顯微鏡影像分析

如同病毒崩毒殺作用之方法描述，經過 LB(未處理)，1%乙醇溶液(控制組)，或 300 ppm 病毒崩(VB)處理的細菌，經離心收集之後，掃描式電子顯微鏡之分析樣品處理是參照先前報導之操作步驟 (Lembke *et al.*, 2006). 圖像觀察與擷取是利用長庚大學顯微鏡中心所提供的掃描式電子顯微鏡(Hitachi S-3000N，JAPAN)來處理。所呈現的圖片為 10000 倍放大倍數，Scale bar, 5 µm。

細菌存活分析

利用 LIVE/DEAD BacLight Bacterial Viability Kits (Invitrogen)來分析經由病毒崩處理後之細菌是否因為細胞壁結構改變而呈現死亡。參照所提供的方法，離心收下來的細菌經由 SYTO 9 和 propidium iodide (PI) (1:1 混合)染色後，移至 1-1.5% agarose 平面上，利用正立螢光顯微鏡(Leica DM2500)分析，圖像呈現明視野(differential interference contrast, DIC), 與螢光視野(I3 or TX)之圖像，影像擷取與後製是經由 Spot RT3 Slider charge-coupled device (CCD) camera (Diagnostic Instruments Inc.)與 Spot (Diagnostic Instruments Inc.). Scale bar, 5 µm.

生物膜貼附實驗

生物膜貼附實驗室根據先前 Lin et al. (Lin *et al.*)的步驟，經過簡單修飾之後來進

行。簡單來說，將隔夜培養的 NDM-1 陽性的肺炎克雷白氏菌以 1:100 稀釋於含有 0.2%葡萄糖(D-glucose, Sigma)的 LB 培養液中，在不同濃度的病毒崩(ppm)或添加等同體積之 1%乙醇溶液(控制組,mock)之下,分裝至 polyvinylchloride (PVC)材質的 96 孔盤中，在 37°C 及 50rpm 之下培養 24 小時，上清液可分裝至另一透明 96 孔盤中 (NUNC, 平底)，利用分光光度計計數在 600nm 的吸光值(OD_{600})，以表示在含有不同濃度病毒崩之培養液之下，細菌之生長密度；取出上清液之各孔，經過三次 PBS (pH7.4 洗滌、室溫烘乾 10 分鐘、最後加入 150 µl 之 1%結晶紫(crystal violet, Sigma)溶液至各孔中，室溫靜置 20 分鐘，去除結晶紫溶液，利用二次水洗滌三次後，室溫烘乾 10 分鐘，擷取影像並利用 200 µl 的 95%乙醇溶液溶解出所染上的結晶紫，校正至 1 毫升體積之後，利用分光光度計計數在 630nm 下之吸光值(OD_{630})以表示生物膜貼附程度，經過三次獨立實驗之後，所計算出來的平均值與標準差，利用 Student's *t* test 計算各實驗組相對於控制組之統計差異。*， $P<0.05$; *， P<0.01。為了了解是否病毒崩具有崩解已形成之生物膜的能力，NDM-1 陽性的肺炎克雷白氏菌如同前面所描述的生物膜貼附實驗步驟，在不含有病毒崩之 0.2%葡萄糖 LB 培養液中，所形成之生物膜，完全未作任何處理之控制組(UT)保持在原先培養液之中；實驗組則是在去掉上清液之後，加入 200µl 之 300ppm 病毒崩溶液或 1%乙醇溶液，靜置培養所標示的時間之後，去除各組之上清液，利用相同的洗滌與染色步驟，進行拍照與定量。從三次獨立實驗之中所得之平均值與標準差，相對於未處理之控制組(UT)，利用 Student's *t*

test 計算各實驗組相對於控制組之統計差異。*， $P<0.05$; *， $P<0.01$。

Materials and Methods

Bacterials strains and culture condition

NDM-1-harboring *K. pneumoniae* is isolated from A 38-year-old Taiwanese man, which is the first case in Taiwan promagated by the Centers for Diseases Control (CDC) (Wu et al.). *K. pneumoniae* is cultured in Luria Broth (LB, Difco) at 37°C with agitation. To collect bacteria in exponential phase (OD600: 0.3~0.5), overnight culture of *K. pneumoniae* is 1:100 diluted in LB medium and cultivated at 37°C with agitation for 2 hours (h). Bacteria are collected by centrifugation at 8000 rpm for 10 minutes (min), washed once with sterile phosphate buffer saline (PBS, pH7.4), and resuspended in indicative media with indicative bacterial numbers (colon-forming units, CFU).

Chemicals

VirusBom is purchased from Money Marketing Corporation in Taiwan. Stock concentration of VirusBom is 1000 ppm constituting 1 gram of VirusBom in 1 liter of 1% ethanol solution with perfume. Indicative working solution is directly diluted in culture medium or 1% ethanol solution. D-glucose is purchased from Sigma (U.S.A)

VirusBom-mediated direct killing

NDM-1 positive *K. pneumoniae* in exponential phase is collected ($1x10^8$ CFU) and resuspended in indicative concentration (ppm) of VirusBom or 1% ethanol solution

(mock), followed by incubation at room temperature for indicative time. The mixture is removed immediately (Immed.) or indicative time (min) after adding the test reagent, serially diluted in sterile PBS (pH7.4), and spreaded onto LB agar plate for culturing at 37°C overnight. The survival bacterial numbers is counted as average of CFU/ml with standard deviation from three independent experiments. ****, $P<0.0001$ in comparison to mock-treated group (Student's *t* test). ND, non-detected.

Scanning electron microscope (SEM) imaging

Bacteria treated with LB, mock or 300 ppm VirusBom (VB) were collected by centrifugation for SEM analysis. Preparation of samples for SEM analysis as previously described (Lembke *et al.*, 2006). Briefly, bacteria were fixed by fixation solution, dehydrated through a series of ethanol, critical-point dried, gold sputtered and examined with a Hitachi S-3000N (Japan). The representative image in magnification of 10000X was shown. Scale bar, 5 µm.

Bacterial LIVE/DEAD staining

Bacterial viability was next addressed by the use of the LIVE/DEAD (L/D) stain (Invitrogen) containing a mixture of two components: SYTO 9, a green fluorescent nucleic acid stain; and propidium iodide, a red fluorescent nucleic acid stain. SYTO 9 generally stains damaged and intact bacteria whilst propidium iodide penetrates only bacteria with damaged membrane, competing with SYTO 9 when both dyes are

present. Incubated with the right mixture of dyes, intact or 'live' bacterial cells will fluoresce green whilst damaged or death bacterial cells will fluoresce red. Collected bacteria after treatment with LB, mock, or 300 ppm VirusBom (VB) were resuspended in 10 µl of 0.85% sodium chloride containing 0.4 µl L/D staining kit at the ration of 1:1. Stained bacteria were applied on agarose plain surface (1-1.5%) on a slide, and imaged with a Leica DM2500 microscopy under differential interference contrast (DIC), I3 or TX filter sets. The images were captured with a Spot RT3 Slider charge-coupled device (CCD) camera (Diagnostic Instruments Inc.), edited, and merged with Spot (Diagnostic Instruments Inc.). Scale bar, 5 µm.

Biofilm attachemt assay

Following the procedures of Lin et al. (Lin et al.) with minor modification, overnight culture of NDM-1 positive *K. pneumoniae* is 1:100 diluted in 100 µl of LB medium containing 0.2% glucose in the presence of indicative concentration (ppm) of VirusBom or same volume of 1% ethanol solution (mock) and transferred to polyvinylchloride (PVC) microtiter plate for further incubation at 37°C with agitation (50 rpm) for 24 h. To verify bacterial growth density, the resulting supernatant is transferred to a new transparent 96-wel microtiter plate (NUNC, flat) and used for determining the absorbance at 600 nm (OD_{600}) by spectrophotometer. The remained well is washed with PBS (pH7.4) three times, air-dried at room temperature for 10

min, and stained with 150 μl of 1% (w/t) crystal violet (Sigma, U.S.A.) for 20 min. After removing the supernatant, each well is washed with distilled water three times and air-dried for 10 min. The representative of stained biofilm is captured by camera and extracted by 200 μl of 95% ethanol for 10 min. The quantification of biofilm attachment is calculated as average of the absorbance at 630 nm (OD_{630}) with standard deviation from three independent experiments. *, $P<0.05$; **, $P<0.01$ in comparison to mock group (Student's *t* test). To evaluate the effect of VirusBom on disruption of formed biofilm, the biofilm assay is performed in the LB medium containing 0.2 glucose without VirusBom. After removal of supernatant, the resulting biofilm is treated with 200 μl of 300 ppm VirusBom or 1% ethanol solution (mock) for indicative time. The untreated group (UT) is remained in the original medium without any treatment. The biofilm image and quantification is as the previous description. *, $P<0.05$; **, $P<0.01$ in comparison to untreated (UT) group, respectively (Student's *t* test).

圖表說明

圖一，病毒崩對於具有 NDM-1 的肺炎克雷白氏菌的毒殺作用。(A) 帶有 NDM-1 的肺炎克雷白氏菌在 LB 培養液中培養至快速生長期階段，$1x10^8$ 的細菌數經由離心並去掉上清液之後，以 100 µl 之不同濃度(ppm)的病毒崩或 1%乙醇(mock, 控制組)回溶，在室溫培養所標示之時間長度，利用 PBS 序列稀釋之後，塗盤在 LB 培養基上，37°C 隔夜培養，圖表示所長出的菌落。(B) 各組存活活的細菌數以每毫升有多少菌落數(CFU/ml)表示，****， $P<0.0001$，比較各組之中的控制組，利用 Student's *t* test 所統計出來的 P 數值。 ND, non-detected，表示未偵測到菌落數。 (C 和 D) 如同(A)所描述之處理，細菌經離心收集，分別經掃描式電子顯微鏡分析(C)或 LIVE/DEAD BacLight Kits (Invitrogen)染色後，利用正立螢光顯微鏡(Leica, Germany)觀察細菌死活比例(D)。Scale bar, 5 µm。

圖二，病毒崩抑制具有 NDM-1 的肺炎克雷白氏菌之生物膜形成。(A) 取 LB 隔夜培養的菌液($4x10^6$)，經由離心並去掉上清液之後，以 100µl 含有 0.2% glucose 及不同濃度(ppm)的病毒崩或 1%乙醇(mock,控制組)之 LB 培養液回溶混合之後，分裝至 polyvinylchloride (PVC)材質的 96 孔盤之中，在 37°C 及 50rpm 轉速培養 24 小時，上清液移至新的 96 孔盤用來測定細菌生長密度，經由 PBS 洗過三次並烘乾之後，加入 200µl 之 1%結晶紫(crystal violet)染色 20 分鐘，去掉結晶紫上清液並以二次水洗滌三次，烘乾之後，以照相機擷取影像以顯現生物膜形成之外觀

(下半部份)，之後加入 200μl 之 95%乙醇至各槽中，反應 10 分鐘以利結晶紫之溶解，以二次水校正之 1 毫升之後，利用分光光度計測定 630nm 的吸光值(OD_{630})來定量生物膜(上半部份)，從三次獨立實驗的結果，利用 Student's *t* test 計算 P 數值，*, *P*<0.05; **, P<0.01。(B)圖二 B 之培養 24 小時之後，上清液移至 96 孔盤(NUNC,平底)，利用分光光度計測定 OD600 的吸光值，以表示細菌生長密度。(C)如圖二 A 之方法，帶有 NDM-1 的肺炎克雷白氏菌在含有 0.2% glucose 之 LB 培養液形成生物膜之後，未處理之控制組(UT)為持續培養；病毒崩實驗組則是在去掉上清液之後，加入 1%乙醇(mock，控制組)或 300ppm 的病毒崩(VB)，在所指示的作用時間後，去除上清液，經由如同圖二 B 之步驟洗滌及染色，生物膜形成之外觀如圖所示(下半部份)，生物膜之定量以 OD_{630} 來表示，進行三次獨立實驗，相對於未處理之控制組(UT)，利用 Student's *t* test 計算 P 數值，*P*<0.05; **, P<0.01。

Figure legends

Figure 1. Killing effects of VirusBom on NDM-1-producing *K. pneumoniae*. (A) *K. pneumoniae* harboring NDM-1 in mid-log phase was collected ($1x10^8$) and resuspended in 100 μl of different concentration (ppm) of VriusBom or mock (1% ethanol), followed by incubating at room temperature for indicative time. After serial dilution, the mixture was evenly spreaded onto LB plate for incubation at 37°C overnight. Shown is the representative of plate. (B) The survival bacterial number was counted as CFU/ml with standard deviation from three independent experiments. ****, P<0.0001 in comparison to mock-treated group (Student's *t* test). ND, non-detected. (C) LB, mock, or 300 ppm VirusBom (VB)-treated bacteria were collected by centrifugation and applied to SEM analysis. Scale bar, 5 μm. (D) After treatment, viable bacteria were collected by centrifugation, stained with LIVE/DEAD BacLight Kits (Invitrogen), and observed under fluorescence microscope (Leica, Germany) equipped with DIC, I3 or Tx filter set. Shown is the representative of each condition. Scale bar, 5 μm.

Figure 2. VirusBom inhibits biofilm formation of NDM-1-producing *K. pneumoniae*. (A) Overnight culture of *K. pneumoniae* harboring NDM-1 was collected 1:100 diluted in 100 μl of LB broth containing 0.2% (w/v) glucose in the presence of different concentration (ppm) of VirusBom or mock, and transferred into

96-well polyvinylchloride (PVC) microtiter plate for further incubation with agitation (50 rpm) at 37°C overnight. After washing and staining of 1% (w/v) crystal violet, the representative biofilm was shown (Bottom panel). The efficacy of biofilm attachment was determined as OD_{630} with standard deviation from three independent experiments (Upper panel). (B) The resulting supernatant was used to determine the density of planktonic cells shown as average of OD_{600} with standard deviation. (C) To determine the effects of VirusBom on disruption of formed biofilm cells, *K. pneumoniae* harboring NDM-1 forms biofilm in LB broth containing 0.2% glucose as described previously. After removal of supernatant, the biofilm cells incubate with 300 ppm of VirusBom (VB) or 1% ethanol (mock) for indicative time. The untreated group (UT) was maintained in the original culturing condition without any treatment. The results are shown as OD_{600} (Upper panel) and the representative biofilm cells (Bottom panel). *, $P<0.05$; **, P<0.01 in comparison to mock group (A and B) and untreated (UT) (C) group, respectively (Student's *t* test).

Fig. 1

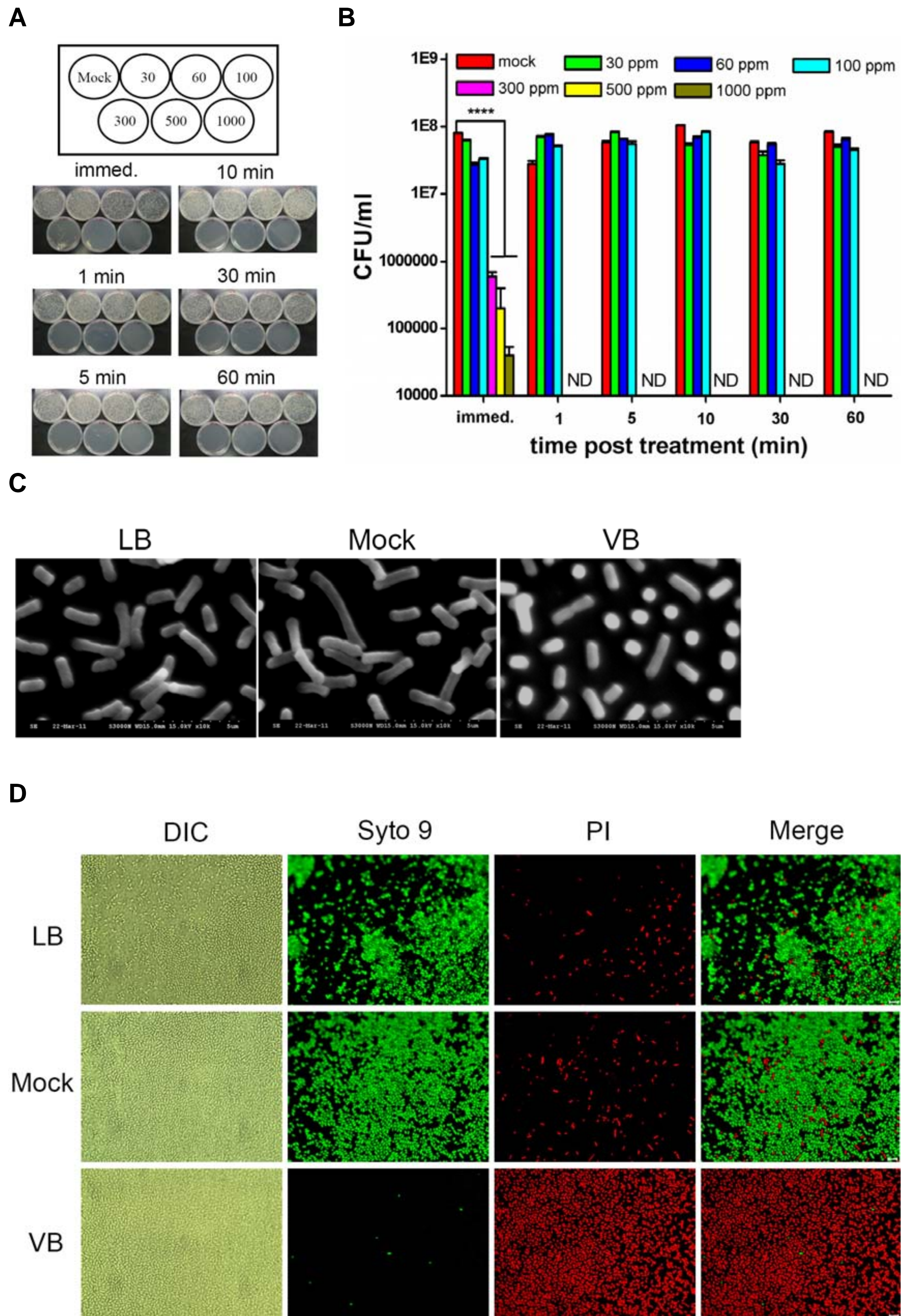
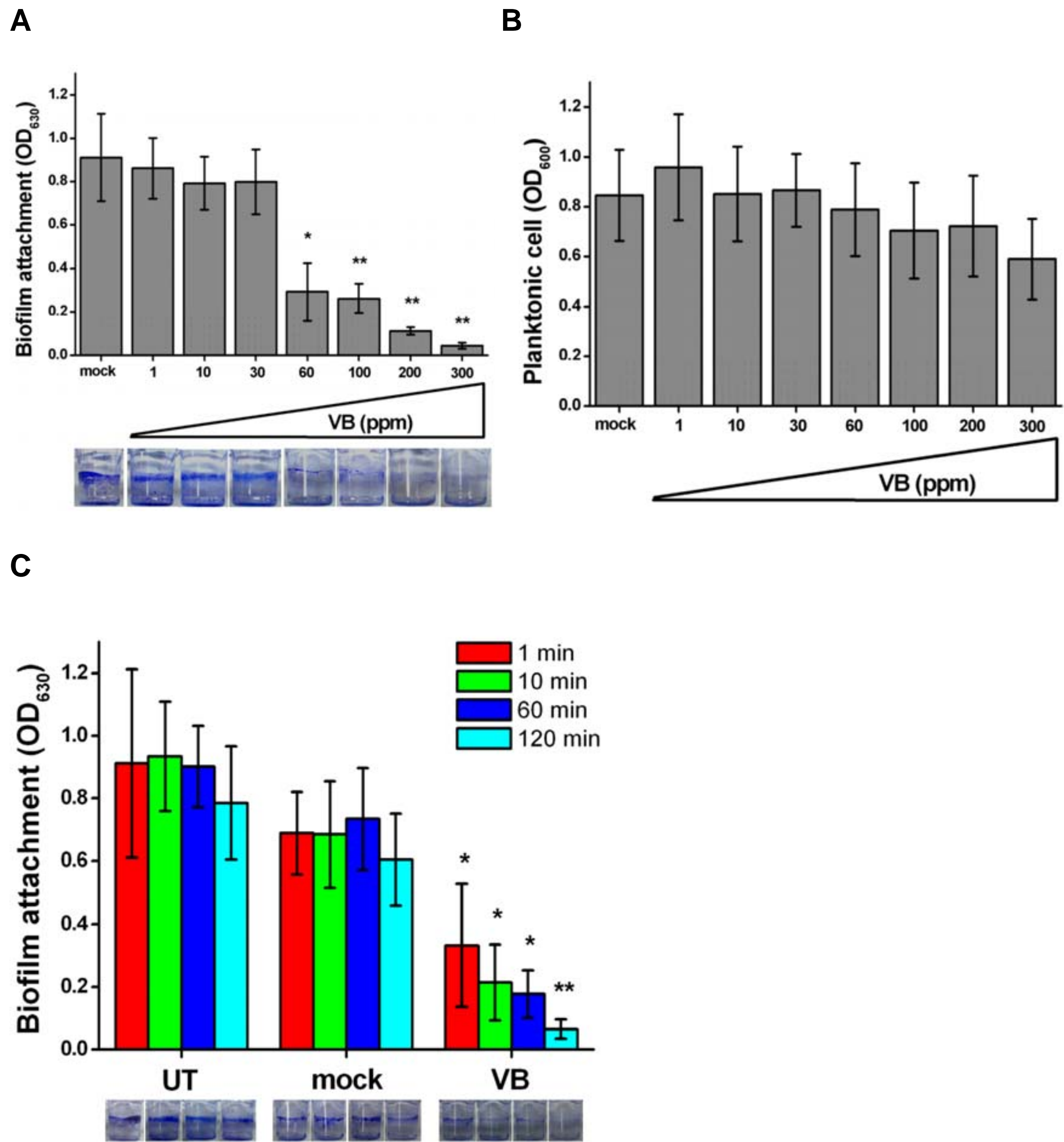


Fig. 2



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