Letter to the Editor

Optimized Methods for Biofilm Analysis in *Yersinia pestis*[*]

FANG Nan[1], GAO He[1,2], WANG Li[1], QU Shi[1], ZHANG Yi Quan[1], YANG Rui Fu[1], and ZHOU Dong Sheng[1,#]

Transmission of *Yesinia pestis* relies primarily on the bite of flea vectors, which is closely related to the biofilm formation of this pathogen[1]. *Y. pestis* synthesizes heavily the attached biofilms, a population of bacterial colonies embedded in self-produced exopolysaccharide matrix, in the flea proventriculus[1]. The biofilm formation leads to the blockage of fleas, making the fleas feel hungry and repeatedly attempt to feed, and thus the plague bacilli pump into the host body during these futile feeding attempts[1]. Detection of biofilm formation is an important phenotypic measurement for the potential of flea-borne transmission of *Y. pestis*. Various methods are currently used for detecting *Yersinia* biofilms[2-5]. However, considerable variations exist for the same method used in the different existing reports, and the experimental protocols are often very brief. The purpose of this study is to optimize the commonly used biofilm detection methods in *Y. pestis*. The wild-type (WT) *Y. pestis* used is the biovar *Microtus* strain 201[6]. The base pairs 146 to 468 of biofilm-required gene *hmsS*[7] were replaced with the kanamycin resistance cassette[8], generating the *hmsS* mutant Δ*hmsS*. The recombinant plasmid pACYC184, which contained the cloned fragment composed of the *hmsS* coding region together with its 372 bp upstream region and its 233 bp downstream region, was introduced into Δ*hmsS*, yielding the complemented mutant strain *C-hmsS*[8]. The incubation temperature of 26 °C was employed for the *Y. pestis* cultivation. A two-round passage design was employed to prepare the *Y. pestis* liquid seeds: firstly, the *Y. pestis* glyceric stock was inoculated into 18 mL of Bacto[TM] Brain Heart Infusion (BHI) or Luria-Bertani (LB) broth for growing with shaking at 230 r/min for 48 h to enter the stationary growth phase; secondly, the resulting cell culture was 20-fold diluted into 18 mL of corresponding fresh BHI or LB, and grew at 230 r/min to reach the mid-exponential phase (an OD_{620} value of 1.0 to 1.2).

Crystal violet (CV) staining of biofilms The BHI seeds were incubated at 4 °C for 8 to 12 h for cold shock, 20-fold diluted into fresh BHI, transferred into the 24-well tissue culture plates with 1 mL of cultures in each well, and grew at 100 or 230 r/min for 24 or 48 h. The media containing the planktonic cells were removed for determining the OD_{620} values for the normalization to avoid the effect of growth rate or cell density. The tube with the adherent biofilms was gently washed three times with 2 mL of H_2O, and then incubated at 80 °C for 15 min for fixing the attached cells. The attached cells were stained with 2 mL of 0.1% crystal violet for 15 min, and then the tube was washed three times with 2 mL of H_2O. Bound dye in the tube was dissolved with 2 mL of dimethylsulfoxide (DMSO). The OD_{570} values were recorded, and the relative biofilm formation was calculated with the formula: $250 \times OD_{570}/OD_{620}$.

We compared the effects of cold-shocked and 26 °C-grown *Y. pestis* (WT) seeds for inoculation; the cold shock led to much steadier attachment of biofilms, and in contrast the attached biofilms made by the 26 °C-grown seeds fell off from the walls easily during the washing process (data not shown). Therefore, the cold-shocked seeds were routinely employed for the inoculation. After the inoculation, the bacterial strains WT, Δ*hmsS*, and *C-hmsS* were incubated in the plates at various rotational speeds (100 and 230 rpm) for different times (24 and 48 h). As shown in Figure 1a, the bacterial growth at 230 rpm for 24 h showed the most stable results for all three strains: compared to WT and *C-hmsS* with the similar levels of biofilm production, Δ*hmsS* stained almost no crystal violet (the *hmsS* deletion abolished the biofilm production[7]). Incubation with a longer time of 48 h or at a lower rotational speed

doi: 10.3967/0895-3988.2013.05.012

1. State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, Beijing 100071, China; 2. State Key Laboratory for Infectious Disease Prevention and Control, National Institute for Communicable Disease Control and Prevention, Chinese Centre for Disease Control and Prevention, Beijing 102206, China

of 100 r/min resulted in the unsteady attachment of biofilms, as characterized by unstable CV staining (Figure 1b to 1d). Therefore, the inoculation of cold-shocked bacterial seeds followed by further incubation at 230 rpm for 24 h was recommended for the CV staining assay.

Caenorhabditis elegans biofilm assays To prepare the nematode eggs, overnight culture of biofilm-negative *Escherichia coli* OP50 was spread onto an the NGM (Nematode Growth Medium) agar plates, and grew for 24 h to prepare the OP50 lawns as the standard foods for *C. elegans.* Adult nematode hermaphrodites were placed on the OP50 lawn, and were incubated at 20 °C for about 3 d. The gravid adults and eggs on the lawn were collected in 2 mL of M9 buffer, followed by twice washes with 1 mL of M9 with centrifugation at 1 300×g for 1 min. The washed gravid adults and eggs were re-suspended in 500 µL of various concentrations of sodium hypochlorite (Table 1), and then incubated at room temperature for 10 min with the intermittent up-down reversals to sterilize the residual *E. coli* and meanwhile to lyze the nematode adults from which the eggs would release. A minimum concentration of 7% was needed to fully kill the residual *E. coli*; the yield of nematode eggs enhanced with the increase of concentrations from 1% to 7% (the detecting quantity of nematode eggs were 70±34, 127±30, 213±42, and 228±40 for 1%, 3%, 5%, and 7%, respectively; assays were performed in triplicate), but a concentration of 9% greatly reduced the yield (98±8). Accordingly, 7% sodium hypochlorite was routinely used.

The net eggs were washed twice with 1 mL of M9, and finally re-suspended in 1 mL of M9. For *Yersinia* biofilm measurement, 50 µL of the net egg solution (containing about 200 eggs) was pipetted onto the *Y. pestis* lawn, followed by the incubation at 20 °C for 3 d. The larvae develop from the eggs; meanwhile, *Y. pestis* creates biofilms to primarily cover the larva head, leading to the blocking of larva feeding and thereby the inhibition of larva growth and development. The developmental stages of nematodes on each lawn were scored in order to calculate the percentage of the fourth-stage larvae and immature adults (L4/adult). The nematode eggs were also spotted on a blank NGM agar plate in order to confirm that they were free of contamination of *E. coli* OP50 or other microorganisms in the net egg solution.

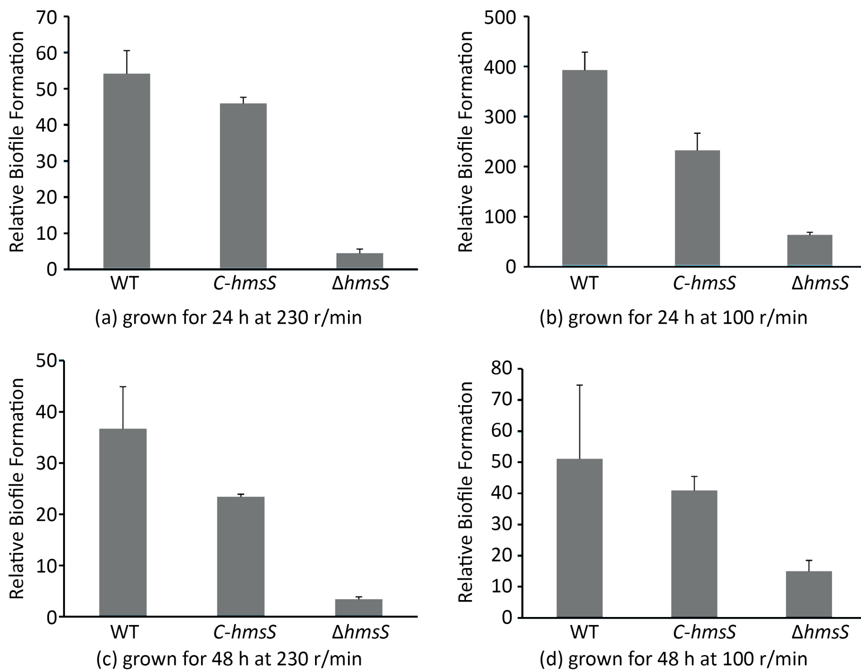

Figure 1. Quantitative detection of biofilms by CV staining. The biofilms of WT, *ΔhmsS*, and *C-hmsS* attached to the liquid-solid interface were directly stained by CV, respectively, to determine the OD_{570} values, which were further normalized with the OD_{620} values determined from the media containing the planktonic cells.

To make the *Y. pestis* lawns for the above use, the *Y. pestis* WT seed culture in BHI or LB was 20-fold diluted, and an aliquot of 200 µL was spread onto an NGM plate and grew for 24 h (Figure 2a); the lawns from the BHI seeds gave a percentage of L4/adult nematodes of nearly zero, indicating that extremely heavy biofilms attached to the larva mouth almost completely inhibited the growth and development of larvae; in contrast, the lawns from the LB seeds gave about 15% of L4/adult nematodes, indicating that a small portion of larva had grown and developed to L4/adult nematodes. Accordingly, the bacterial seeds in the LB broth were selected for further analyses. We further tested various incubation times (18, 24, 48, and 72 h) for the preparation of *Y. pestis* WT lawns (Figure 2b). With the increase of incubation times, more *Yersinia* biofilms were generated and lower percentages of L4/adult nematodes were detected. The appropriate incubation time was 18 to 24 h, since the detected percentages of L4/adult nematodes were about 10 to 30%.

Based on the above investigations, a refined scheme was established with the use of 7% sodium hypochlorite, 24 h incubation of *Y. pestis* lawns on the NGM plates, and LB *Y. pestis* seeds. This scheme was further applied to test the biofilm formation of WT, *ΔhmsS*, and *C-hmsS* (Figure 2c). The three strains produced the L4/adult nematode with the rates of 18.70%, 19.55%, and 98.33%, respectively, confirming that the *hmsS* deletion abolished the biofilm formation, and that the complementation of *ΔhmsS* restored the biofilm-forming ability comparable to that of WT.

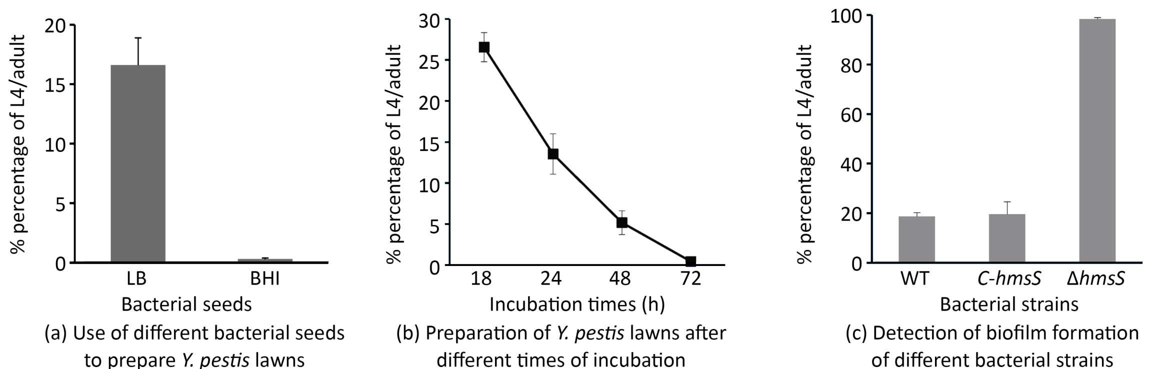

Figure 2. Quantitative detection of biofilms with a nematode model. a) The bacterial seeds in BHI or LB were spread onto the NGM plates and incubated for 24 h to generate the two kinds of *Y. pestis* WT lawns; b) various incubation time intervals (18, 24, 48, and 72 h) were tested for the preparation of *Y. pestis* WT lawns by using the LB seeds; and c) the optimized parameters including 7% sodium hypochlorite, 24 h incubation of *Y. pestis* lawns, and the LB seeds were employed to detect the biofilm formation of WT, *ΔhmsS*, and *C-hmsS*. all assays were performed in triplicate.

Colony morphology assay The degrees of rugose colony morphology of *Y. pestis* grown the agar plates positively reflect the ability to synthesize the biofilm EPS matrix[2]. Different liquid cultures (the *Y. pestis* glyceric stock a, the *Y. pestis* seeds in BHI, and the seeds in LB) were spotted respectively on the BHI or LB plates for the incubation to observe the colony morphology on the plates (Figure 3). For the LB plate spotted with the former two kinds of liquid cultures, expected colony morphologies were observed for WT, *ΔhmsS*, and *C-hmsS*: WT and *C-hmsS* showed almost the same rugose colony morphology, while *ΔhmsS* showed a smooth one (i.e., a destroyed production of biofilm EPS in *ΔhmsS* relative to WT). Owing to the convenience, the incubation of LB plate spotted with the frozen-stored *Y. pestis* glyceric stock was recommended for the colony morphology assay.

In the present study, three different optimized methods were established for the detection of *in vitro* biofilms in *Y. pestis*. Multiple methods are often employed simultaneously for the biofilm detection; the basis of this is that one method can be more convincing with the same results from different methods. In addition, the use of multiple methods enables us to gather different types of data about biofilm or EPS production. The simultaneous use of the above three methods we optimized could be a routine scheme for the detection of the *in vitro* biofilms formed by *Y. pestis* and its very closely related progenitor *Y. pseudotuberculosis*.

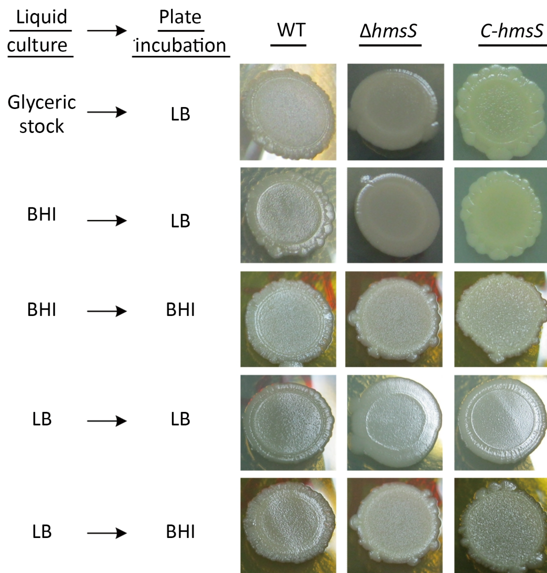

Figure 3. Detection of bioflim EPS through colony morphology assay. Different bacterial liquid cultures were spotted on the BHI or LB plate, followed by the incubation for one week. Assays were performed with at least three agar plates.

*This study was supported by the National Natural Science Foundation of China (Contact Numbers 30930001 and 30900823), and also by the National Basic Research Program of China (Contact Number 2009CB522600).

#Corresponding author: ZHOU Dong Sheng. Tel: 86-10-66948594. E-mail: dongshengzhou1977@gmail.com

Biographical note of the first author: Fang Nan, male, born in 1981, Ph.D, majoring in bacterial gene regulation; Gao He, female, born in 1978, Ph.D, associate professor, majoring in bacterial gene regulation.

Received: March 31, 2012;
Accepted: June 1, 2012

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