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Human Hexa-Histidine-Tagged Single-Chain Variable Fragments for Bioimaging of Bacterial Infections

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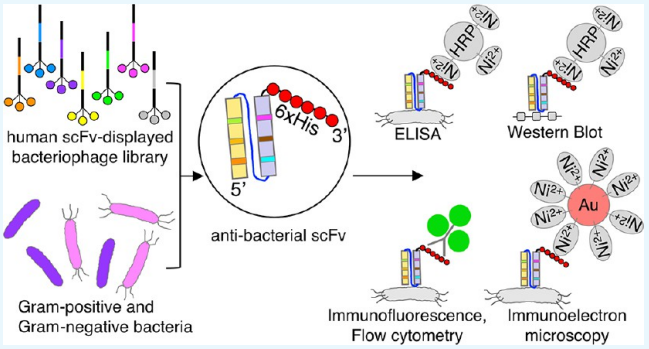
ABSTRACT: The single-chain variable fragment (scFv) of monoclonal antibodies is a promising recombinant nanostructure for various medical applications, including bioimaging and targeted therapy. While numerous scFv antibodies against eukaryotic cell surface proteins (especially cancer biomarkers) have been generated and engineered to suit various purposes, only a few specific scFv against bacterial cell surfaces have been developed, especially those of human origin. Recent incidents of emerging multidrug-resistant pathogenic bacteria and the realization of the importance of a balanced microbiota on the health of the host has led to more interests in the development of recombinant antibacterial antibodies as a detection probe or targeted therapy for bacterial infections. This study reports the generation of two specific human antibacterial scFv using phage display antibody technology. The recombinant scFv fragments of about 30 kDa and a diameter of 5 nm were produced and purified from engineered *Escherichia coli* that can enhance cytosolic disulfide bond formation. As a proof of principle, *Propionibacterium acnes* and *Pseudomonas aeruginosa* were used as model Gram-positive and Gram-negative bacteria, respectively. Specificity at the strain and species level to both planktonic and biofilm forms of these bacteria were demonstrated in various assay formats, namely, ELISA, flow cytometry, western blot, immunofluorescence, and electron microscopy via the hexa-histidine tag. This recombinant scFv generation platform can be applied for other bacteria, and since the scFv obtained has a benefit of being a human origin, it could be conveniently engineered for various therapeutic or theranostic applications with minimized adverse immunoreaction.



INTRODUCTION

Phage display antibody technology, one of the most popular methods for the generation of recombinant antibodies, has recently been awarded the Nobel Prize in Chemistry for the year 2018.[1] The key advantage of this technology relies on the simplicity but powerful affinity selection (bio-panning) procedure and the direct linkage between the displayed antibody and its encoding gene within the virion genome.[2] Recombinant antibodies against a desired target can be identified directly from diverse repertoires of antibody genes, generating high-affinity binding sites without the constraint imposed by classical methods for generating either polyclonal or monoclonal antibodies.[3] Since this method does not depend on an animal's immune system, antibodies to a wide variety of antigens, including molecules that cannot stimulate the immune system of animals, such as small molecule haptens and self-antigens have been successfully isolated using this technology.[4] Various forms of antigen-binding fragments, including Fab and scFv have been cloned and displayed on the filamentous bacteriophage M13.[5] ScFv is a nanostructure that consists of light and heavy chain variable domains of immunoglobulins of mammals, linked with a peptide linker $(G_4S)_3$. One scFv fragment has a molecular weight of about 30 kDa and a diameter of 5 nm, which is about one-fifth the size of the parental IgG molecule.[6,7] Once the sequence of scFv against any target has been identified, it can be further engineered into various formats including whole immunoglobulin (IgG) monoclonal antibodies and bispecific antibodies or fabricated into various nanoparticles for both therapeutic and diagnostic purposes.[8]

Most of the research and development on recombinant antibodies mainly focuses on cancer. Several hundred monoclonal antibodies have been approved or are in clinical trial stages for the treatment of cancer and autoimmune diseases.[9] Until now, only a handful of publications regarding the generation of recombinant antibodies against infectious micro-organisms have been published.[10,11] The emergence of multidrug-resistant pathogenic bacteria as well as the concern

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for a balanced microbiota have led to more interest in generating recombinant antibodies against pathogenic bacteria.[12]

The model Gram-negative bacteria in this study, *P. aeruginosa*, is one of the WHO prioritized pathogens for which research and development of new antibiotics are critically needed.[13] Some research groups used molecular targets, such as purified recombinant proteins of *P. aeruginosa*,[14−18] whereas others used whole bacterial cells in suspension as targets for affinity selection.[19,20] All carried out affinity selection using peptide display phage libraries, and from this, anti-*P. aeruginosa* peptides were identified. On the other hand, a study group from MedImmune Ltd., UK,[21,22] used *P. aeruginosa* whole cells as a target for affinity selection against a human scFv library derived from patients who had recently recovered from *P. aeruginosa* infections and identified a scFv against *P. aeruginosa* exopolysaccharide residue (Psl). Another study group used the recombinant exotoxin A of *P. aeruginosa* to do affinity selection against a human scFv phage display library based on 60 Thai blood donors and isolated anti-exotoxin A scFv clones.[23,24] To the best of our knowledge, only the anti-Psl scFv of MedImmune Ltd. group has been developed into an anti-pseudomonal drug candidate.

The model Gram-positive bacteria of this study, *P. acnes*, have been used as an affinity selection target by one study group.[25] They generated a scFv display phage library from human peripheral blood mononuclear cells, which were immunized *in vitro* with heat-killed *P. acnes* and three anti-*P. acnes* scFv clones were identified, but there was no known further development from those clones.

Biopharmaceuticals could become a reliable resource to fight back multidrug- and pandrug-resistant bacteria, which has been a global concern. In 2017, multidrug-resistant *P. aeruginosa* caused an estimated 32,600 infections among hospitalized patients and 2700 estimated deaths in the US alone. It has been estimated that human deaths attributable to antimicrobial resistance (AMR) will surpass that of cancer in 2050 and the global GDP loss related to AMR will be trillions of USD.[26,27] Recently, cartography of opportunistic pathogens and antibiotic resistance genes in a tertiary hospital environment, from genomic and metagenomic analysis, has been reported.[28,29] Therefore, obtaining scFv nanostructures against living bacteria, which could be further engineered to be used as alternative diagnostic and therapeutic strategies to combat deadly bacterial infections, is highly attractive.

This study reports an efficient method for the generation of specific human scFvs against both Gram-positive and Gram-negative bacteria using phage display technology.

RESULTS AND DISCUSSION

Two opportunistic bacteria with different cell surface structures were used as model bacteria in this study because they have important clinical relevance and are easier to handle with a standard biosafety level in a general laboratory. The procedures presented in this study could be adopted for the generation of scFvs to other important pathogenic bacteria with no current effective treatment.

Affinity Selection against *P. acnes* and *P. aeruginosa*. A summary of the affinity selection results is shown in Table 1. After the first round of selection against *P. acnes* (DMST 14916), 844 colonies of TG1 strain *E. coli* infected with eluted virions were obtained. Then, 576 colonies were manually picked and were subjected to monoclonal phage ELISA. Three

Table 1. Summary of Affinity Selection Results against *P. acnes* and *P. aeruginosa*

affinity selection step	*P. acnes* DMST 14916	*P. aeruginosa* DMST 37186
rounds of selection	1	3
colonies obtained	844 colonies	2.2×10^4 colonies
colonies picked up	576 colonies	96 colonies
positive clones at monoclonal phage ELISA	3 clones	61 clones
positive clones at scFv ELISA expressed in HB2151 *E. coli*	1 out of 2 clones tested	4 out of 15 clones tested
clones sent for DNA sequencing	3 clones	4 clones
DNA sequencing result	3 different scFv sequences	all 4 clones have same scFv sequence
scFv clone identity	yPac1A8, yPac1E4, yPac1E7	yPgi3G4

virion clones, eventually designated as yPac1A8, yPac1E4, and yPac1E7, which showed a two-fold higher OD signal against *P. acnes* than the negative control (1% BSA), were selected for binding re-confirmation by phage ELISA and DNA sequence analysis. The result revealed that they possessed unique scFv sequences; however, the clone yPac1E7 had an amber stop codon (TAG) within its open reading frame. Binding confirmation by scFv ELISA of two soluble scFv clones (yPac1A8 and yPac1E4) produced from the non-suppressor strain of *E. coli* (HB2151) proved that only clone yPac1A8 showed good binding (OD value was two times higher than 1% BSA control, data not shown). Therefore, yPac1A8 was selected for expression in pET-21d (+) for further analysis.

For *P. aeruginosa* (DMST 37186), 3 rounds of affinity selection were carried out and 96 colonies were randomly picked for monoclonal phage ELISA. Sixty-one virion clones showed two-fold higher OD values against *P. aeruginosa* than the negative control (1% BSA). After binding confirmation, 15 clones were selected to generate soluble scFv from the non-suppressor strain of *E. coli* (HB2151). Out of 15, 4 clones that showed a high signal by scFv ELISA (data not shown) were sent for DNA sequencing, and the result revealed that all clones possessed the same scFv sequence. This clone, designated yPgi3G4, was further expressed in the pET-21d (+) system.

The affinity selection data confirms the previous observation that a compact non-immunized phage display human scFv library, generated from blood of healthy individuals in the rural regions of Thailand,[4] can be used as a good resource for the generation of specific antibodies against a wide variety of external antigens, including both the Gram-positive and Gram-negative pathogenic bacteria used in this study. It is worth noting that the immobilization of the desired targets is one of the important steps in the affinity selection process of phage display technology. In this report, we have optimized various parameters of the affinity selection procedure.[30] In particular, we have prepared bacterial targets in several ways. These include whole, boiled and sonicated bacterial cells. The effective method which yields positive clones came from using boiled bacterial antigens as described in the materials and methods section.

DNA and Amino Acid Sequence Analysis of Selected Virion Clones. A diagram of recombinant scFv in this study is depicted in Figure 1A. The variable region of the heavy chain (V_H) at the N-terminus is connected to the variable region of the light chain (V_L) by a GGGGSGGGGSGGGGS peptide

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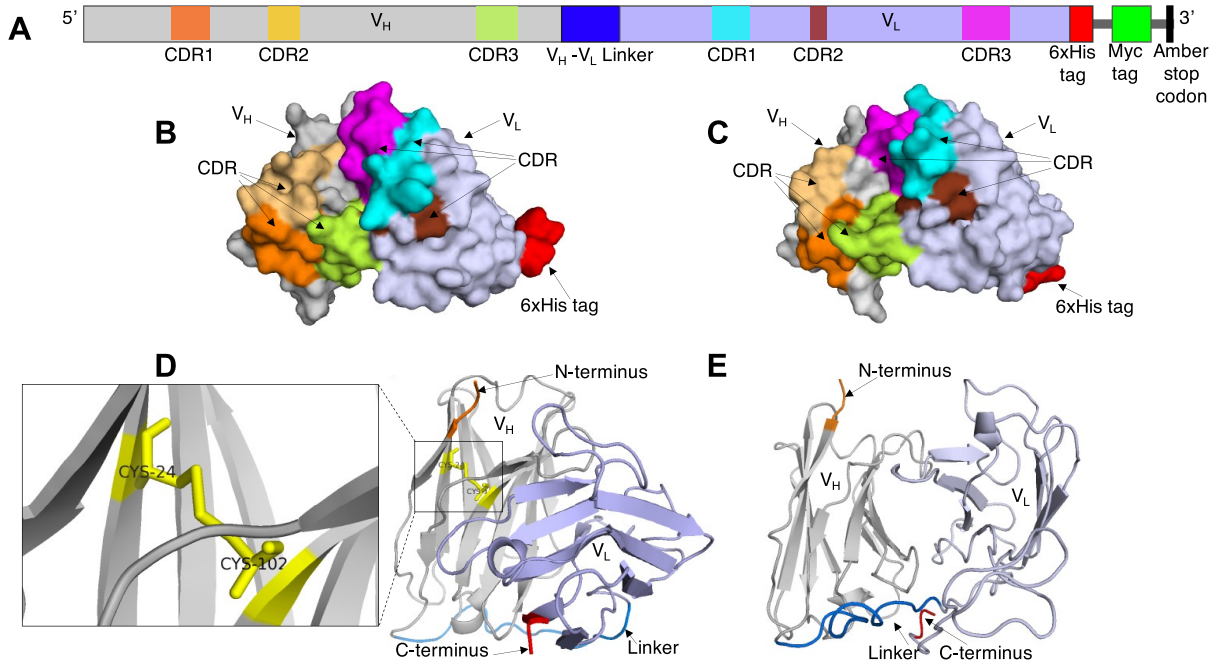

Figure 1. Structural analysis of 6xHis-tagged scFv. (A) Schematic arrangement of scFv primary structure from the N-terminus (5′) to C-terminus (3′): CDR regions of V_H domain, V_H-V_L linker (blue), CDR regions of V_L domain, 6xHis tag (red), Myc tag (green), and Amber stop codon (black). 3D modeling structures based on scFv (PDB ID code 1f3r.1.B) are shown in (B)−(E). Surface view of yPac1A8 scFv (B) and yPgi3G4 scFv (C), with the same color coding as in diagram (A). A ribbon model showing the α-helix and β-sheet of yPac1A8 scFv (D) and yPgi3G4 scFv (E): N-terminus (orange), V_H domain (gray), disulfide bridge (yellow), linker (blue), V_L domain (violet), and C-terminus (red). Only yPac1A8 possesses intra-domain disulfide bridge (shown in inset) between Cysteine 24 and Cysteine 102 residues of the V_H domain.

Table 2. Analysis of DNA and Amino Acid Sequences of Isolated scFv Fragments against Gram-Positive and Gram-Negative Bacteria[a]

scFv clone	germline	identity to germline (%)	amino acid differences from germline	CDR3 sequence
V_H genes				
yPac1A8	Homsap IGHV6-1*01	92.59	13	TRGKYSGFDM
yPac1E4	Homsap IGHV6-1*01	92.59	13	TRGKYSGFDM
yPac1E7	Homsap IGHV6-1*01	91.92	17	VRGQHSAFDM
yPgi3G4	Homsap IGHV3-7*01	93.40	15	ARVLWGFPFDL
V_L genes				
yPac1A8	Homsap IGLV2-18*02	94.44	9	SSFTSTISPSYV
yPac1E4	Homsap IGLV2-14*01	98.96	1	SSYTSSSPNWV
yPac1E7	Homsap IGLV2-18*02	93.75	12	SSYTSSSTWV
yPgi3G4	Homsap IGLV2-8*01	97.57	8	SSYGGSNNLV

[a]Homsap, Homosapien; IGHV, immunoglobulin heavy variable; IGLV, immunoglobulin lambda variable; V-domain, variable domain.

linker sequence. The C-terminus of scFv is linked with a hexa-histidine tag (6 x His tag) followed by a Myc tag. The gene of scFv and the gene III protein sequence of the bacteriophage are intervened by an Amber stop codon (TAG), which is recognized by the non-suppressor strain of *E. coli*, HB2151, as a stop signal.[4] DNA and amino acid sequences of isolated clones in comparison with those of human germline antibody variable region gene sequences are presented in Table 2. Out of the seven existing IGHV (human immunoglobulin heavy variable) gene subgroups, isolated scFv clones from this study belong to subgroups 3 and 6. Clones active against *P. acnes* (yPac1A8, yPac1E4, yPac1E7) are from IGHV subgroup 6 and are derived from the same germline gene and allele IGHV6-1*01 with >91% sequence identity. Their sequences differ from that in the germline by 13−17 amino acids. Three clones share one CDR3 amino acid sequence pattern ("-RG-S-FDM"). The clone active against *P. aeruginosa* (yPgi3G4) is from the IGHV subgroup 3 and derived from germline gene and allele IGHV3-7*01 with >90% sequence identity. Its sequence is different from that in the germline by 15 amino acids.

All isolated scFv clones, either active against *P. acnes* or *P. aeruginosa*, are members of IGLV (human immunoglobulin λ) gene subgroup 2 out of the existing 11 (IGLV1 to 11). The immunoglobulin light chain κ to λ ratio has been estimated to be 1.5−2, and the chance of isolating κ light chain antibodies is higher than that of the λ.[31] However, in our study, all four scFv clones have λ. Two of them (yPac1A8, yPac1E7) are derived from the germline gene and allele IGLV2-18*02 with >93% sequence identity, with 9−12 amino acids difference. Another clone (yPac1E4) originates from the germline gene and allele IGLV2-14*01 with nearly 99% sequence identity and has only one amino acid difference. The last clone (yPgi3G4) is a derivative of the germline gene and allele IGLV2-8*01 with

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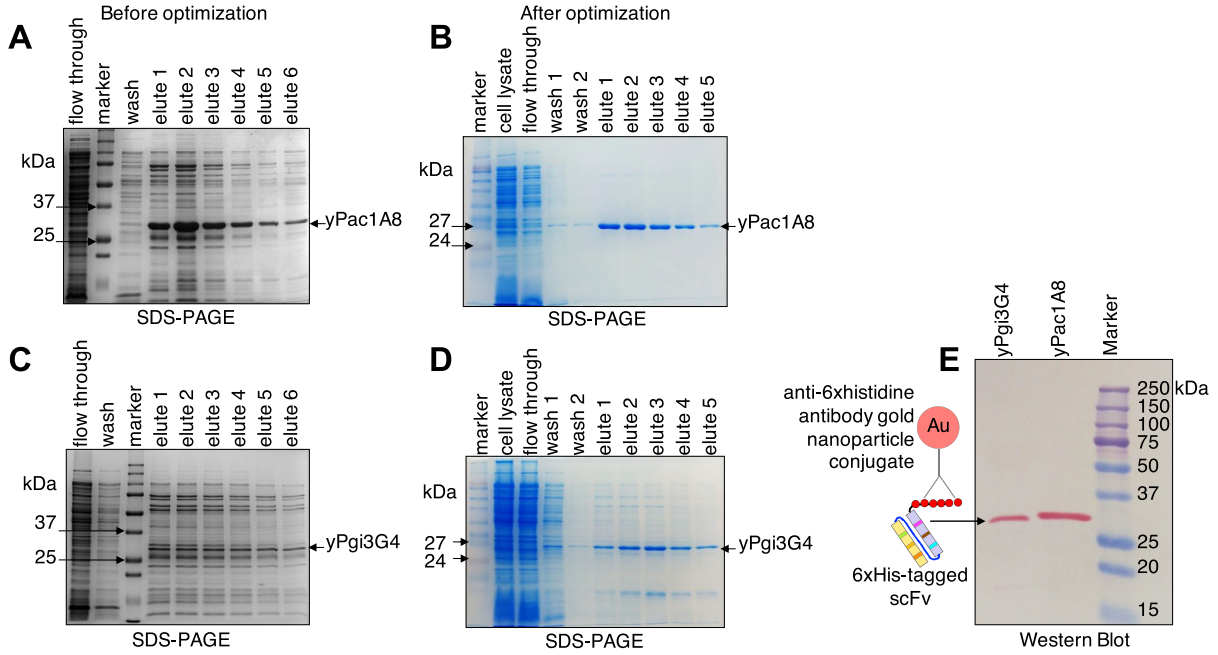

Figure 2. Expression and purification of 6xHis-tagged scFv. SDS-PAGE of 6xHis-tagged scFv samples before and after optimization of expression and purification steps. All gels were Coomassie Brilliant Blue-stained. (A) yPac1A8 scFv before optimization. (B) yPac1A8 scFv after optimization. (C) yPgi3G4 scFv before optimization and (D) yPgi3G4 scFv after optimization. Precision Plus All Blue Prestained Protein Standards (BioRad #1610373, U.S.A.) were used as a molecular weight marker in (A) and (C). Prestained Protein Standards (Enzmart #APC-001, Thailand) were used as a molecular weight marker for (B) and (D). (E) Purified yPac1A8 and yPgi3G4 scFv were resolved by SDS-PAGE and immunoblotted with anti-histidine antibody-gold nanoparticle conjugate. Solid arrow indicates scFv bands at a correct size (about 29 kDa).

>97% sequence identity and has eight amino acids difference. The amino acid sequence of the light chain CDR3 region of all isolated clones starts with "SS" and ends with "V". Sequence analysis of the two scFv genes showed a high number of amino acid differences from germline genes, indicating that the genes were isolated from plasma B cells in the blood after affinity maturation.

The predicted three-dimensional structure of the two scFv clones that were selected for further study, i.e., anti-*P. acnes* clone (yPac1A8) and anti-*P. aeruginosa* clone (yPgi3G4), are illustrated in Figure 1B–E. Anti-*P. acnes* (yPac1A8) scFv was a 29.4 kDa protein of 279 amino acids, while anti-*P. aeruginosa* (yPgi3G4) scFv was a 28.7 kDa protein of 274 amino acids. The surface view of important structures of yPac1A8 and yPgi3G4 is illustrated in Figure 1B and C, respectively. In addition, the α-helix and β-sheet of both clones are shown in Figure 1D and E. The intra-domain disulfide bond of yPac1A8 V_H region was zoomed in and is shown in Figure 1D.

Cloning and Expression of scFv Antibody Fragments. To further characterize the two identified recombinant scFvs against model Gram-positive and Gram-negative bacteria, DNA sequences of yPac1A8 and yPgi3G4 scFvs were subcloned into pET-21d (+), expressed in SHuffle T7 B strain *E. coli*, and purified by one-step Ni^{2+}-NTA agarose affinity chromatography. Optimization of expression and purification processes was carried out, and SDS-PAGE of the purification process of both scFvs, before and after optimization, is demonstrated in Figure 2A–D. The majority of unwanted protein fractions could be removed after optimization. Elution fractions contained protein bands of scFv at the expected molecular weight of approx. 29 kDa. About 3.0 mg of purified yPac1A8 scFv and 2.3 mg of purified yPgi3G4 scFv are routinely obtained from a 1 L Terrific broth by the baffled flask culture system. A discrepancy between the purity of the two scFv clones was observed under the same scFv expression and purification protocols (Figure 2A–E), of which clone yPac1A8, which has a higher scFv yield, appeared purer than clone yPgi3G4.

The two scFv clones were also observed by western blotting (WB) as shown in Figure 2E. About 1 μg of each scFv could be detected by an anti-histidine antibody gold nanoparticle conjugate, indicating that hexa-histidine tag of both scFv fragments could interact with the anti-6xhistidine detection system. Both yPac1A8 and yPgi3G4 scFvs could be observed as bright red bands at the expected size. Although equal amounts of scFv were loaded as estimated by the Bradford standard microtiter plate assay, the yPgi3G4 WB band appeared slightly thinner than that of yPac1A8.

Specific Binding of scFv Antibody by Whole Cell ELISA. To evaluate the binding specificity of the two anti-bacterial scFv antibodies, an ELISA against whole cell bacteria was performed. The binding of yPac1A8 and yPgi3G4 scFv was tested against a panel of Gram-positive bacteria (*Propionibacterium acidipropionici, Propionibacterium freudenreichii, P. acnes*) and Gram-negative bacteria (*Pseudomonas putida, Pseudomonas fluorescens, P. aeruginosa*). Heat-inactivated whole cell bacteria (10^9 cells/mL) were immobilized and incubated with 10 μg/mL of the two scFv clones. Specific binding of yPac1A8 scFv to *P. acnes* and yPgi3G4 scFv to *P. aeruginosa*, respectively, at the species level could be obtained as demonstrated in Figure 3A and Figure S1. Anti-*P. acnes* scFv (yPac1A8) did not bind to five *P. acnes* strains tested, other than the target that was used for affinity selection. Anti-*P. aeruginosa* scFv (yPgi3G4) could bind to three out of four *P. aeruginosa* strains tested, in addition to its affinity selection target.

In this study, successful uses of scFv as a nanostructure for the specific detection of bacterial targets in various formats

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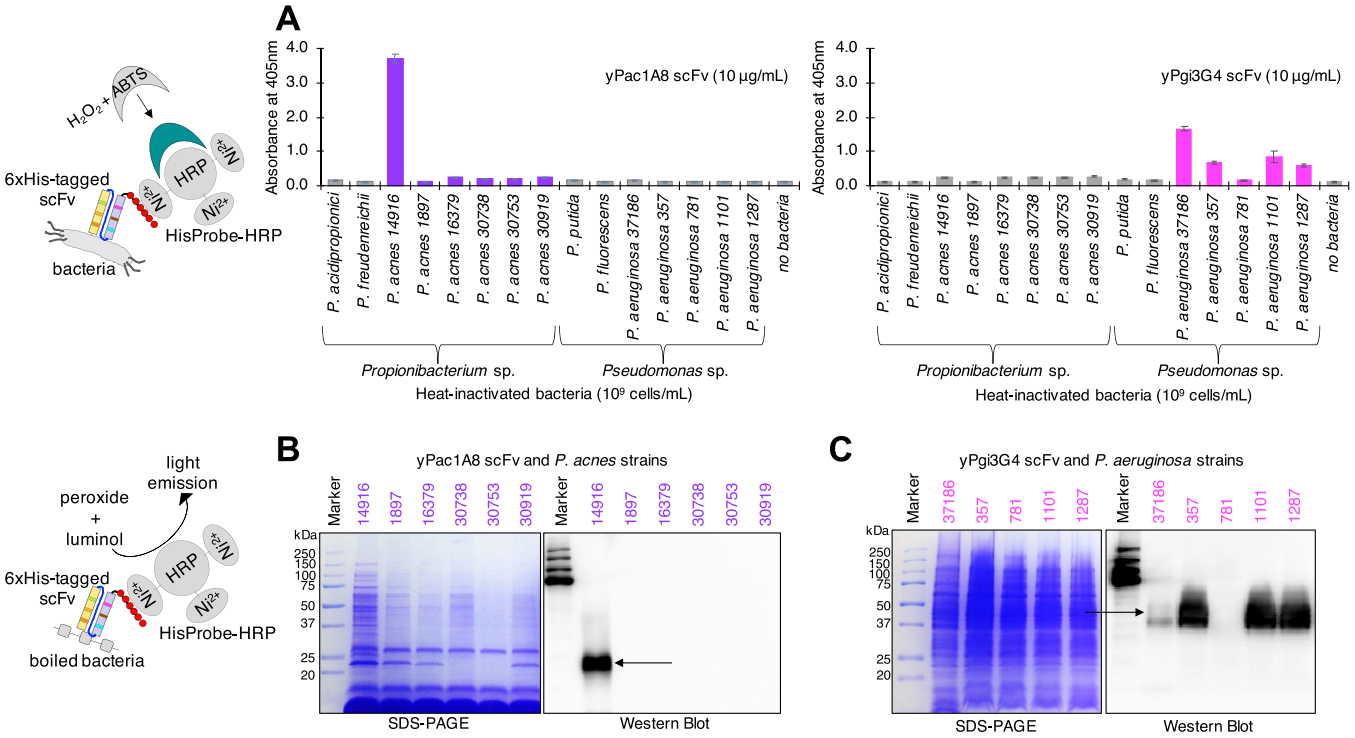

Figure 3. Cross-reactivity analysis of 6xHis-tagged scFv by whole-cell ELISA and WB. Binding activities of yPac1A8 and yPgi3G4 scFvs were determined by whole-cell ELISA on a plate coated with the indicated bacteria (A): *P. acidipropionici* TISTR 442, *P. freudenreichii* TISTR 446, *P. acnes* DMST 14916, *P. acnes* strains DSM 1897, DSM 16379, DSM 30738, DSM 30753, DSM 30919, *P. putida* TISTR 1522, *P. fluorescens* TISTR 358, *P. aeruginosa* DMST 37186, *P. aeruginosa* strains TISTR 357, TISTR 781, TISTR 1101, TISTR 1287, and wells without immobilized bacteria. The bars represent the average OD values of triplicate samples, and error bars represent the standard error of the mean. A picture of the ELISA plate is shown in the Supporting Information (Figure S1). (B) SDS-PAGE and WB of boiled antigen preparation of *P. acnes* strains, immunoblotted with yPac1A8 scFv: DMST 14916, DSM 1897, DSM 16379, DSM 30738, DSM 30753, and DSM 30919: Coomassie Brilliant Blue-stained SDS-PAGE gel. The *P. acnes* antigen is located between 20 and 25 kDa (arrow pointed) and was detected by yPac1A8 scFv. (C) SDS-PAGE and WB of boiled antigen preparation of *P. aeruginosa* strains, immunoblotted with yPgi3G4 scFv as indicated: DMST 37186, TISTR 357, TISTR 781, TISTR 1101, and TISTR 1287. The *P. aeruginosa* antigen located between 37 and 50 kDa (arrow pointed) was detected by yPgi3G4 scFv. Precision Plus All Blue Prestained Protein Standards (BioRad#1610373, U.S.A.) were used as a molecular weight marker.

have been demonstrated. The key to the success could be because (1) the phage display library was generated from plasma B cells of healthy individuals who might have been exposed to the bacteria and (2) the choice of affinity selection method used in this study. Boiled pathogenic bacterial antigens were used as a target for affinity selection. This is the same type of antigen preparation that is used to immunize animals for generating antibodies via the traditional method. The fact that the target antigen could resist heat denaturation and that the western blot bands were heterogeneous suggested that the epitopes are post-translationally modified, probably by glycosylation or the addition of a short linear epitope on the surface of the protein, but not a discontinuous epitope, which is a segment on the 3D structure of the protein that will not resist heat. Identification of the epitopes of these two scFv antibodies is beyond the scope of this study and will be explored in the next step.

Detection of Bacterial Targets by WB. In addition to ELISA, the target antigens of the two scFv clones were also detected by WB. As shown in Figure 3B, yPac1A8 scFv could detect its target antigen, located between 20 and 25 kDa regions, from denatured *P. acnes* DMST 14916 preparation, but not from other *P. acnes* strains tested. Similarly, as shown in Figure 3C, yPgi3G4 scFv could detect its target antigen, located between 37 and 50 kDa regions, out of the boiled *P. aeruginosa* DMST 37186 preparation. Moreover, yPgi3G4 scFv could detect its target from three other *P. aeruginosa* strains tested (TISTR 357, TISTR 1101, TISTR 1287), but not from TISTR 781. These results are similar to the patterns obtained from ELISA assay. Identification of these bacterial target antigens is beyond the scope of this study and will be carried out in the future.

It has been shown that the level of expression and folding of different scFv fragments varied, depending on the amino acid sequence and intracellular disulfide bond formation.[32] In this report, while there was one intra-domain disulfide in clone yPac1A8 scFv, there was no detectable disulfide bond formation in yPgi3G4; consequently, we observed that the clone yPgi3G4 was less stable and this could be because the folding was disulfide-independent.[33] Even though both scFv antibodies could be expressed at a relatively good yield from *E. coli* SHuffle strain, which favors disulfide bond formation inside the cytoplasm,[34,35] it is possible to improve the stability and hence the binding affinity, by introducing inter-domain disulfide bonds.[36,37]

Checkerboard Titration (CBT) ELISA. In order to determine the optimal concentration of target bacteria and corresponding scFv antibody to be used in the ELISA, a checkerboard titration was performed.[38] This method could be used to estimate the binding affinity of the two recombinant antibodies to their target bacteria,[39] and the results are shown in Figure 4A and C and Figures S2 and S3. For yPac1A8, the

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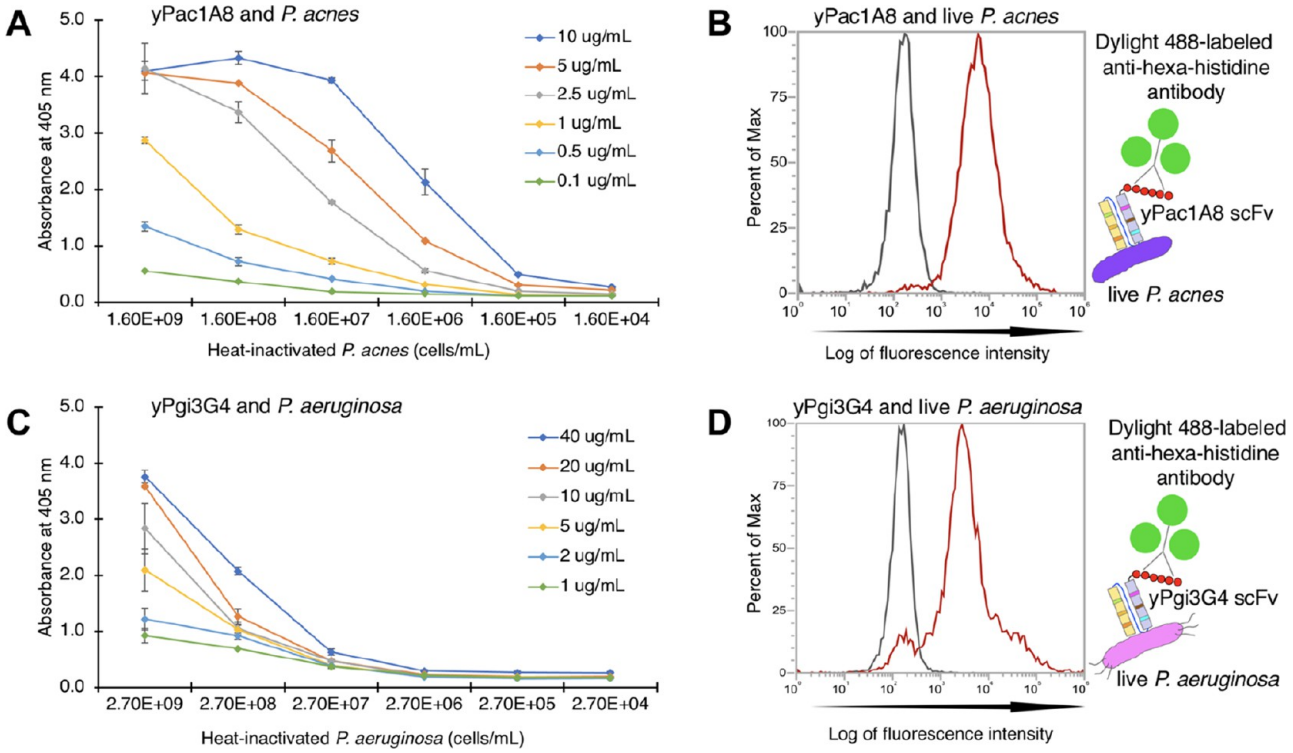

Figure 4. Checkerboard titration and flow cytometry analysis against whole cell bacteria. The limit of detection of yPac1A8 for *P. acnes* DMST 14916 (A) and yPgi3G4 for *P. aeruginosa* DMST 37186 (C) was determined by checkerboard titration of whole-cell ELISA, using a dilution series of scFv and serial dilutions of bacteria as indicated. The lines represent the average absorbance values of duplicate samples, and error bars represent the standard error of the mean. Pictures of ELISA plates are shown in the Supporting Information (Figures S2 and S3). Flow cytometry analysis of yPac1A8 (B) and yPgi3G4 scFv (D) binding to live *P. acnes* DMST 14916 and *P. aeruginosa* DMST 37186, respectively. Reactivity of 6xHis-tagged scFv is indicated by the red line. The secondary antibody alone (control) is indicated by the black line.

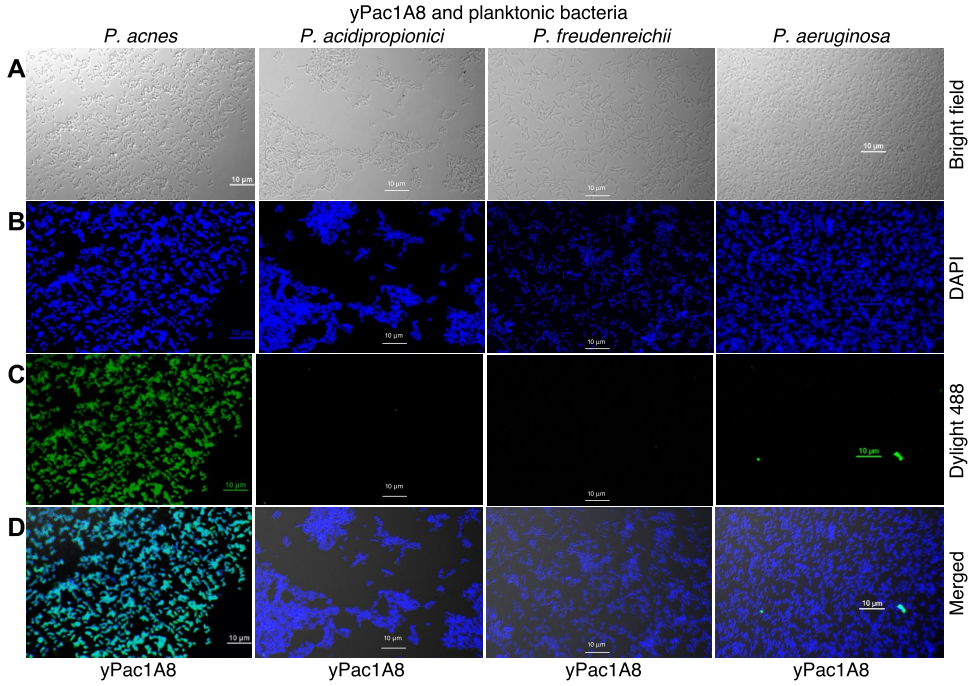

Figure 5. CLSM of yPac1A8 and planktonic bacteria. *P. acnes* DMST 14916, *P. acidipropionici* TISTR 442, *P. freudenreichii* TISTR 446, and *P. aeruginosa* DMST 37186 were incubated with yPac1A8 scFv, detected by Dylight 488-labeled anti-hexa-histidine mouse monoclonal antibody, and counterstained with DAPI. Photos were taken by an Apo TIRF 60x Oil DIC N2 objective of Nikon A1R confocal laser microscope. Scale bars represent 10 μm. In bright field panel (A), individual bacterial cells were seen. In DAPI panel (B), nucleoids of bacteria were stained blue. In Dylight 488 panel (C), only *P. acnes* DMST 14916 was stained green. In merged panel (D), the blue nucleoids and the green cell surfaces of *P. acnes* overlapped. In the case of *P. acidipropionici*, *P. freudenreichii*, and *P. aeruginosa*, only the blue nucleoids were stained.

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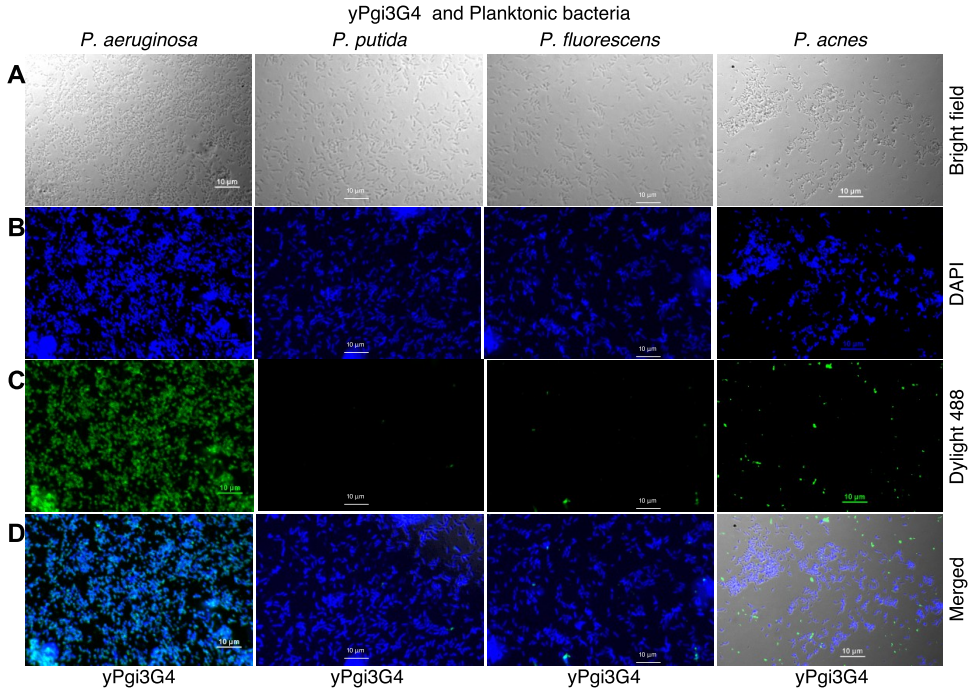

Figure 6. CLSM of yPgi3G4 and planktonic bacteria. Photos of *P. aeruginosa* DMST 37186, *P. putida* TISTR 1522, *P. fluorescens* TISTR 358, and *P. acnes* DMST 14916 were taken at the same specifications as in Figure 5. Scale bars represent 10 μm. In bright field panel (A), bacterial cells are seen. In DAPI panel (B), nucleoids of bacteria are blue. In Dylight 488 panel (C), only *P. aeruginosa* are green. In merged panel (D), the blue nucleoids and the green cell surfaces of *P. aeruginosa* overlapped. In the case of *P. putida*, *P. fluorescens* and *P. acnes*, only the blue nucleoids were stained.

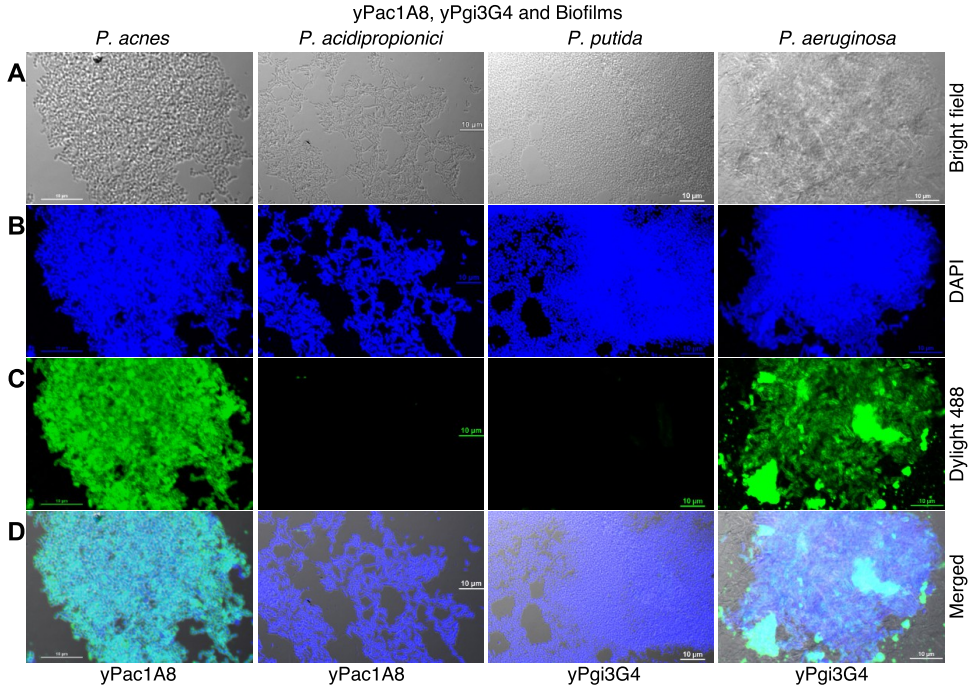

Figure 7. CLSM of biofilms stained by indicated scFv. Biofilms of *P. acnes* DMST 14916, *P. acidipropionici* TISTR 442, *P. putida* TISTR 1522, and *P. aeruginosa* DMST 37186 were stained by yPac1A8 and yPgi3G4 scFvs as indicated. Scale bars represent 10 μm. In bright field panel (A), bacterial cell clusters were seen. In DAPI panel (B), nucleoids of bacteria were stained blue. In Dylight 488 panel (C), *P. acnes* were stained green by yPac1A8 scFv but not *P. acidipropionici*. Likewise, *P. aeruginosa* were stained green by yPgi3G4 scFv but not *P. putida*. In merged panel (D), the blue nucleoids and the green cell surfaces of *P. acnes* and *P. aeruginosa* overlapped. In the case of *P. acidipropionici* and *P. putida*, only the blue nucleoids were stained.

highest scFv concentration tested (10 μg/mL) could detect 1.6 $\times$ 10^5 cells/mL and the lowest scFv concentration tested (0.1 μg/mL) could detect 1.6 $\times$ 10^9 cells/mL. For yPgi3G4, as much as 40 μg/mL scFv could detect 2.7 $\times$ 10^7 cells/mL. These results indicated that the binding affinity of scFv clone yPac1A8 to its target (*P. acnes*) is higher than that of the clone

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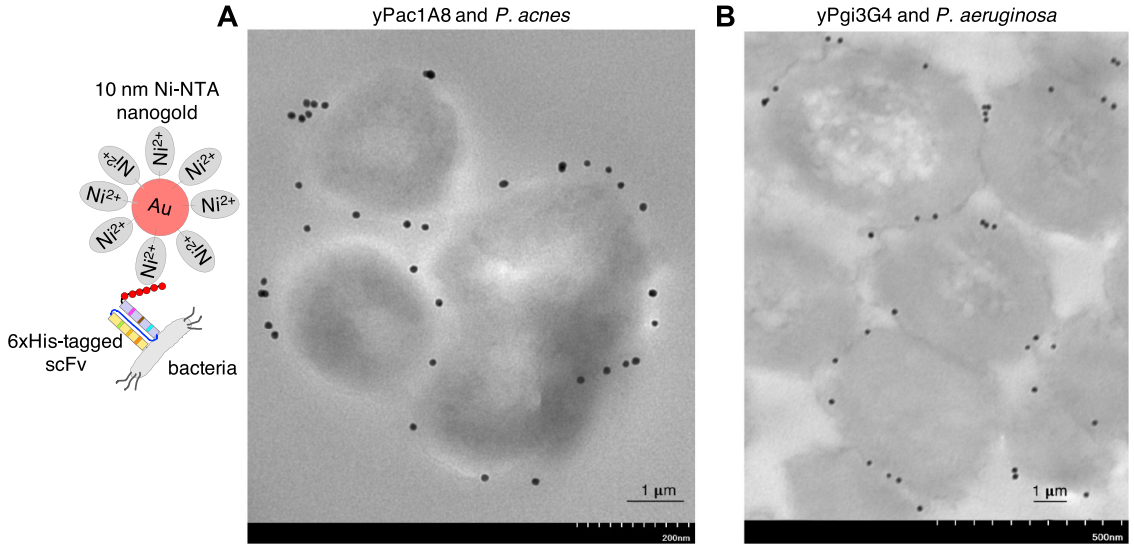

Figure 8. TEM of *P. acnes* and *P. aeruginosa*. Four *P. acnes* DMST 14916 cells (A) and six *P. aeruginosa* DMST 37186 cells (B) were treated with yPac1A8 and yPgi3G4 scFvs, respectively. Photos were taken by the Hitachi Hi-Tech HT7700 transmission electron microscope at x25.0 k magnification with Zoom-1 lens mode at the accelerating voltage of 80.0 kV. Scale bars represent 1.0 μm. The large round structures are bacterial cells, and small dense black dots are 10 nm Ni-NTA nanogold particles.

yPgi3G4 against its target (*P. aeruginosa*). While the apparent binding characteristics of the two isolated antibodies seems sufficient for various assay formats, further improvement of binding affinity or specificity could be performed by different affinity maturation techniques as previously described.[40,41]

Detection of Live Bacteria by Flow Cytometry. To demonstrate the binding of yPac1A8 and yPgi3G4 to living target bacteria, flow cytometry analysis was performed. *P. acnes* and *P. aeruginosa* cells were exposed to their corresponding specific scFv followed by anti-polyhistidine Dylight 488 secondary antibody. Cells stained only with anti-polyhistidine Dylight 488 monoclonal antibody were used as a negative control. From each sample, 10,000 events were collected and around 90% of them were bacteria, which were gated in hierarchy for their Dylight 488 fluorescence signal. About 96.9% and 83.3% of *P. acnes* and *P. aeruginosa* were stained with their corresponding antibodies. Data analysis was performed using the built-in Attune Cytometric Software. The results were presented in a histogram overlay format in Figure 4B and D.

Immunofluorescent Staining of Whole Bacterial Cells and Biofilms. In addition to ELISA, WB, and flow cytometry, the binding of scFv antibodies was also revealed by a confocal laser scanning microscopy (CLSM) technique as illustrated in Figures 5−7. For the species-specific binding of yPac1A8 to *P. acnes*, *P. acidipropionici*, *P. freudenreichii*, and *P. aeruginosa* were used as controls (Figure 5). In the bright field row, bacterial cells were seen dispersed throughout the field. In the DAPI row, the nucleoid regions of bacteria were stained blue. In the Dylight 488 row, only *P. acnes* cells were bound by yPac1A8 hexa-histidine-tagged scFv, which was detected by a Dylight 488-labeled secondary detection agent and the cells appeared green. Some *P. acnes* cells showed a green cell outline along an unstained center suggesting the cell surface localization of the yPac1A8 scFv-ligand. Similar species-specific binding could be observed between the binding of 6xHis-tagged yPgi3G4 against its target bacteria, *P. aeruginosa* cells (Figure 6). *P. putida*, *P. fluorescens*, and *P. acnes* were used as bacterial controls. These results corresponded well with the ELISA as described in the previous section.

Since bacterial biofilms may play a role in pathogenesis and chronic infection,[42] the binding of selected scFvs against target bacteria in biofilms was also investigated. As demonstrated in Figure 7, *P. acnes* and *P. aeruginosa* biofilms cultured on glass coverslips were stained by yPac1A8 and yPgi3G4, respectively. Biofilms of *P. acidipropionici* TISTR 442 and *P. putida* TISTR 1522 were used as controls. In the bright field row, bacterial cell clusters were seen. In the DAPI row, the nucleoid regions of bacteria were stained blue. In the Dylight 488 row, *P. acnes* and *P. aeruginosa* cells were observed as green bacilli with an obvious unstained central halo region. The results indicated that both scFvs could bind to their target bacteria inside a biofilm.

Cell Surface Staining by Electron Microscopy. Electron microscopy was carried out to further investigate the findings obtained by whole cell ELISA, flow cytometry, and CLSM, which suggested that the target antigens are located on the bacterial cell surface. Ni-NTA sensitized 10 nm nanogold particles were used as a detection probe. As expected, nanogold particles were seen as dark dots along the cell outline of *P. acnes* and *P. aeruginosa* under a transmission electron microscope (Figure 8). These results confirmed the cell surface localization of the two anti-bacterial scFv ligands.

The application of anti-bacterial scFvs as nanostructures is demonstrated in different binding assays. In these assays, the 6xHis tag at the C-terminus of the scFv was bound with anti-His antibody or nickel, conjugated with enzyme horseradish peroxidase (HRP), gold nanoparticle, or fluorescent dye. The data from this study indicated that the arrangement of domains comprising V_H-(G_4S)3-V_L-6xHis-Myc, as depicted in Figure 1A, is an effective nanostructure. The predicted 3D structure of both scFv antibodies indicated that the CDR regions of yPac1A8 and yPgi3G4 scFv are located opposite to the 6xHis tag. This conformation seems to be favorable for specific antigen binding activity and for the poly-His tag recognition function to take place without structural constraint.

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The advantage of directional conjugation via the 6xHis-tag over random conjugation is that there is no interference with the antigen binding site. Histidine is strongly involved in the coordinate bond with metal ions and the polyhistidine tag has been used as one of the most popular tags for protein purification by metal chelate affinity chromatography. The Ni^{2+} ion of Ni-NTA binds with two histidine residues of the 6xHis tag.[43] A stable attachment between one scFv and one gold particle is established when three adjacent Ni-NTA groups of the gold nanoparticle bind to the 6x-His tag of scFv. As for metal ions, copper has the highest affinity, and the affinity decreases in the order of nickel, zinc, and cobalt.[44] While nickel is often used for general purposes, the binding affinity between scFv and a detector or carrier can be increased by changing the type of metal ion or by introducing a type of tag, such as using streptavidin-biotin interactions, which are considered the strongest non-covalent natural bond.[45]

CONCLUSIONS AND FUTURE PERSPECTIVES

An efficient method for the identification of scFv nanostructures for the specific detection of two model Gram-positive and Gram-negative bacteria, *P. acnes* and *P. aeruginosa*, by the affinity selection of a non-immunized human phage scFv antibody library against boiled bacterial antigens is reported. The specific binding of 6xHis-tagged scFv fragments to a bacterial cell surface was demonstrated by ELISA, WB, flow cytometry, and confocal fluorescence and electron microscopy. These scFv antibody fragments could be further developed to be used as a point-of-care diagnostic or further engineered and used as novel therapeutic agents in many ways such as conversion into whole immunoglobulin (IgG) molecule for monoclonal antibody therapy, conjugation to nanoparticles loaded with antibiotics, or conjugation with nanoparticles for multiplex biosensor-based detection, which could be very useful for biofilm detection. In addition, genetic engineering can be used to improve the binding affinity or specificity, create bi-specific antibody to enhance the binding spectrum or creating sensitive detection probe by fusing with fluorescent proteins or enzymes such as alkaline phosphatase, horseradish peroxidase, or luciferase. These are examples of interesting research in the next step to be explored.

MATERIALS AND METHODS

Bacterial Strains. Affinity selection targets: *P. acnes* DMST 14916 and *P. aeruginosa* DMST 37186, were kindly provided by Dr. Griangsak Eumkeb, School of Sciences, Suranaree University of Technology, Thailand. The species identity of both strains was confirmed by 16S rRNA gene sequencing at Macrogen, Inc., South Korea. *P. acnes* strains DSM 1897, DSM 16379, DSM 30738, DSM 30753, and DSM 30919 were kindly provided by Professor Dietmar Haltrich, Department of Food Science and Technology, University of Natural Resources and Life Sciences, Vienna. *P. freudenreichii* TISTR 446, *P. fluorescens* TISTR 358, *P. putida* TISTR 1522, and *P. aeruginosa* strains TISTR 357, TISTR 781, and TISTR 1287 were purchased from the Thailand Institute of Scientific and Technological Research. *P. acidipropionici* TISTR 442 and *P. aeruginosa* TISTR 1101 were kindly provided by TISTR for academic research purposes. TG1 strain *E. coli* and HB2151 strain *E. coli* were obtained from the Medical Research Council (MRC) Laboratory, Cambridge, UK. SHuffle T7 B strain *E. coli* (NEB #C3029J, U.S.A.) was purchased from New England Biolabs, U.S.A. The source of materials used is described elsewhere.

Affinity Selection (Biopanning) against *P. acnes* and *P. aeruginosa*. *Antigen Preparation.* *P. aeruginosa* colonies were grown on LB broth and incubated overnight at 250 rpm, 37 °C. *P. acnes* colonies were cultured in BHI broth and incubated for 5 days at 37 °C anaerobically in a GasPak system. Bacterial cells were washed with PBS two times and resuspended in PBS at the OD_{600} of 2.0. Cell suspensions were boiled for 1 h in a water bath, and the protein concentration was determined by Bradford standard microtiter plate assay (BioRad #500-0006, California, U.S.A.).

Affinity Selection Procedure. Affinity selection was carried out according to a previously published protocol.[30] Maxisorp immunotube (Nalgene Nunc International, Denmark) was coated overnight at 4 °C with 25 μg of boiled bacterial antigen in 100 mM $NaHCO_3$ (pH 8.5). The tube was blocked with 2% (w/v) skimmed milk protein in phosphate-buffered saline (PBSM) before incubation with 10^{12} plaque-forming units (pfu) of virion from the Yamo I human phage display scFv library[4] in 2% PBSM. The tube was washed five times with 0.05% (v/v) Tween-20 in PBS (PBST) followed by five more PBS washes. Then, the bound virions were eluted with 1 mg/mL trypsin in PBS followed by 100 mM glycine-HCl (pH 2.0), and neutralization was carried out with 200 mM $NaPO_4$ (pH 7.5). For the second and third round, eluted virions were pooled together, amplified, and purified by PEG precipitation, as previously described.[46]

Amplification of the Individual Virion Clone. Discrete colonies of *E. coli* TG1, infected with eluted virions from affinity selection, were randomly picked and cultured in U-shaped 96-well microplates (Nunclon delta surface, Thermo Fisher Scientific #163320, U.S.A.), containing 100 μL/well of 2×YT medium with 100 μg/mL ampicillin and 1.0% (w/v) glucose (2xYT-AmpGlu). The cultures were super-infected with helper phage KM13 to amplify the isolated virion as previously explained.[47] After incubation at 30 °C with shaking at 250 rpm for 20 h, supernatants containing the virions obtained after the centrifugation of a deep-well plate were used for monoclonal phage ELISA as described in the ELISA section.

DNA and Amino Acid Sequence Analysis of Selected Virion Clones. Phagemids from selected ELISA-positive virion clones were prepared and sent for automated DNA sequence analysis as previously described.[47] Contig alignment of the sequences was performed using Vector NTI software (Thermo Fisher Scientific, U.S.A.), and the aligned sequences were further analyzed with the IgBLAST tool from the National Center for Biotechnology Information[48] and IMGT/V-QUEST tool from the international ImMunoGeneTics information system.[49] Three-dimensional structures of selected scFvs were generated by using the SWISS-MODEL server from the Swiss Institute of Bioinformatics.[50] The template (PDB ID code: 1f3r.1.B) was chosen by sequence identity analysis. Models were built by target-template alignment using ProMod3 and visualized with the program PyMOL, a molecular visualization system from Schrödinger, LLC, U.S.A.[51]

Small-Scale Production of Soluble scFv from E. coli HB2151. Soluble scFv clones were produced from the non-suppressor strain of *E. coli* HB2151, which read the Amber codon between the scFv and geneIII protein gene sequences in the phagemid vector as a stop codon instead of glutamic acid

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in the TG1 strain, as previously described.[41] The culture supernatant containing soluble scFv fragments was used for scFv ELISA as described in the ELISA section.

Production of Hexa-Histidine-Tagged scFv (6xHis-Tagged scFv). *Cloning and Expression of scFv Genes.* The scFv genes against *P. acnes* and *P. aeruginosa* were cloned into pET-21d (+) expression vectors and designated as pET21d +/yPac1A8 and pET21d+/yPgi3G4, respectively, as previously described.[47] The scFv genes were expressed in the cytoplasm of protease deficient *E. coli*, SHuffle T7 B strain (NEB #C3029J, U.S.A.), according to a previous study[52] with some optimization. Briefly, a colony streaked from −80 °C stock of SHuffle *E. coli* C3029 harboring pET21d+/yPac1A8 or pET21d+/yPgi3G4 was inoculated into 5 mL of Terrific Broth (TB: Tryptone 12 g/L, yeast extract 24 g/L, glycerol 4 mL/L, 0.17 M KH_2PO_4, and 0.72 M K_2HPO_4) containing 100 μg/mL ampicillin and incubated overnight at 30 °C with 225 rpm shaking. On the next day, a starter culture was transferred into 500 mL of fresh TB containing 100 μg/mL ampicillin and cultured at 30 °C in a bench-top bioreactor system or in a baffled flask with 225 rpm shaking until the OD_{600} reached about 1.0. Protein expression was induced with 0.85 mM IPTG for 24 h at 25 °C. The culture was cooled on ice for 5 min and centrifuged at 3000*g* for 30 min at 4 °C. The cell pellet was used directly for scFv purification or otherwise stored at −40 °C.

Purification of Hexa-Histidine-Tagged scFv. The 6xHis-tagged scFv was purified by immobilized metal affinity chromatography (IMAC), using a gravity flow column containing a Ni-NTA (Ni^{2+} nitrilotriacetic acid) agarose affinity chromatography matrix, following the company's manual (Qiagen #30230, Germany). The resin was washed with washing buffer (50 mM Tris-HCl, 0.5 M NaCl, 100 mM imidazole, pH 7.5), and scFv was eluted with elution buffer (50 mM Tris-HCl, 0.5 M NaCl, 500 mM imidazole, pH 7.5). Desalting and buffer exchange was performed by dialysis overnight in SnakeSkin Dialysis Tubing with 10 kDa MWCO (Thermo Fisher Scientific #68100, U.S.A.) against cold PBS buffer at 4 °C. The purity of eluted scFvs was checked by SDS-PAGE, and the purified soluble scFv concentration was determined by a Bradford standard microtiter plate assay (BioRad #500-0006, California, U.S.A.). The samples were kept at −80 °C in 250 mg/mL BSA or at −40 °C in 20% glycerol.

Western Blot Analysis. *WB of Isolated scFv.* Equal amounts (1 μg) of purified scFv (yPac1A8 and yPgi3G4) were heat-treated at 90 °C for 10 min in SDS sample buffer containing β-mercaptoethanol. Then, samples were electrophoresed in 12.5% SDS-PAGE gel for 5 min at 50 V followed by 90 min at 100 V and were transferred to a polyvinylidene difluoride (PVDF) membrane (Cytiva #10600021, U.S.A.) by wet-blotting using a Mini Trans-Blot electrophoretic transfer cell (Biorad #1703930, U.S.A.) at 30 V for 15 h at 4 °C. To detect the scFv, the membrane was incubated with 10 mL of anti-histidine antibody-gold nanoparticle conjugate (Jena Bioscience# PS-110, Germany) for 3 h at room temperature with slight rocking. The scFv bands became visible due to antibody mediated gold nanoparticle accumulation.

WB of Bacterial Targets Using Identified 6xHis-Tagged scFv. About 30 μg of boiled bacterial antigen preparation (*P. acnes* and *P. aeruginosa*) was used as samples. The SDS-PAGE and wet-blotting procedures were the same as above. After blocking with 3% BSA-TBST, the membrane was incubated in 2 μg/mL 6xHis-tagged scFv (yPac1A8 and yPgi3G4) in TBST for 15 h at 4 °C followed by washing three times with TBST (5 min per wash). It was then incubated with 1:5000 diluted HisProbe-HRP (a nickel (Ni^{2+})-activated derivative of horseradish peroxidase) (Thermo Fisher Scientific#15165, U.S.A.) in blocking buffer for 1 h at room temperature with slight rocking followed by washing three times with TBST. Finally, the protein band was detected by chemiluminescence using Amersham ECL Prime Western blotting detection reagent (GE Healthcare #RPN2232, UK). Image analysis was performed by CCD camera-based imaging, using the ChemiDoc XRS Gel Documentation System (Bio-Rad, U.S.A.).

Enzyme-Linked Immunosorbent Assay (ELISA). *Monoclonal Phage ELISA.* For phage ELISA, 5 μg of bacterial antigen in 100 mM $NaHCO_3$ (pH 8.5) was used as a target and 1% (w/v) BSA in PBS as a negative control. The phage ELISA was performed as previously described.[53] Virion clones that showed an OD value of at least two times higher than those of negative controls were selected for confirmation.

scFv ELISA. For scFv ELISA, 2 μg of bacterial antigen in 100 mM $NaHCO_3$ (pH 8.5) was used as a target and 1% (w/v) BSA in PBS as a negative control. The supernatant containing soluble scFv fragments (150 μL/well) was used as a primary detection agent. HisProbe-HRP (Thermo Fisher Scientific #15165, U.S.A.) diluted at 1:5000 in PBS was used as a secondary detection agent. The ELISA procedure was the same as the monoclonal phage ELISA.

scFv ELISA against Whole Cell Bacteria. For *Propionibacterium* species, colonies streaked from −80 °C stock on BHI agar were inoculated into BHI broth and incubated anaerobically for 5 days at 37 °C in the case of *P. acnes* and at 30 °C in the case of *P. acidipropionici* and *P. freudenreichii*. For *Pseudomonas* species, colonies streaked from −80 °C stock on LB agar were inoculated into LB broth and incubated overnight at 250 rpm at 37 °C in the case of *P. aeruginosa* and at 30 °C in the case of *P. fluorescens* and *P. putida*. Broth cultures were washed with PBS two times, and cells were resuspended in PBS. The OD_{600} value of cell suspensions was adjusted to 2.0. Heat inactivation was carried out in a water bath for 30 min at 60 °C. Heat-inactivated bacteria were used for whole cell ELISA.

To check the specificity of 6xHis-tagged scFv, heat-inactivated bacteria (10^9 cells/mL) was used as a target. Purified hexa-histidine-tagged scFv (10 μg/mL) was used as a primary detection agent and HisProbe-HRP as a secondary detection agent. The ELISA procedure was the same as above except that the immobilization step was performed overnight at 37 °C.

Checkerboard Titration (CBT). To optimize the scFv ELISA and to determine the limit of detection of recombinant scFv, the ELISA plate was coated with serial dilutions of heat-inactivated bacterial suspension from rows A to F, where row A had the highest (10^9 cells/mL) bacterial concentration while row F had the lowest (10^4 cells/mL). The ELISA procedure was carried out as described above except that the wells were incubated with six different dilutions of scFv in duplicate format from columns 1 to 12, where columns 1 and 2 had the highest scFv concentration (yPac1A8 scFv 10 μg/mL or yPgi3G4 scFv 40 μg/mL) while columns 11 and 12 had the lowest (yPac1A8 scFv 0.1 μg/mL or yPgi3G4 scFv 1.0 μg/mL).

Flow Cytometry. A bacterial colony was suspended in 900 μL of PBS, and 300 μL of it was diluted with PBS to 1 mL and was centrifuged at 2000*g* for 5 min. The cells were resuspended

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in 1 mL of 1% BSA-0.1% PBST, blocked for 30 min at room temperature, and washed two times with PBS. The cells were incubated with specific 6xHis-tagged scFv (200 μg/mL) for 1 h at room temperature. *P. acnes* cells were exposed to yPac1A8 scFv and *P. aeruginosa* cells to yPgi3G4 scFv. After two PBS washes, the cells were stained with a 1:500 dilution of Dylight 488-labeled anti-hexa-histidine mouse monoclonal antibody (Abcam #ab117512, UK) in PBS for 1 h in a dark place at room temperature. The cells were washed with PBS two times and resuspended in 500 μL of PBS containing 4 μg/mL PI (propidium iodide) solution (Thermo Fisher Scientific #A28993, U.S.A.). Sample reading was performed using an Invitrogen Attune NxT Acoustic Focusing 3-Laser System Cytometer (Thermo Fisher Scientific# P3566, U.S.A.).

Confocal Laser Scanning Microscopy (CLSM). *CLSM of Planktonic Bacteria.* About 1 mL of broth culture was centrifuged (3000*g*, 5 min), washed with PBS two times, and resuspended in PBS. About 5 μL of the suspension was spread into a smear on a glass slide and dried completely at 37 °C. The smear was fixed with 4% PFA (paraformaldehyde) in PBS (pH 7.4) for 30 min, blocked with 1% BSA-300 mM glycine-0.1% PBST for 30 min, treated with 2 μg of 6xHis-tagged scFv for 1 h, incubated with 1:500 dilution of Dylight 488-labeled anti-hexa-histidine mouse monoclonal antibody (Abcam #ab117512, UK) in PBS for 1 h, and counterstained with 300 μM DAPI (4′,6-diamidino-2-phenylindole) for 5 min. The smear was washed three times with PBS between the above steps. The stained smear was covered with slow fade gold mountant (Invitrogen #S36936, U.S.A.) and examined with a confocal microscope (Nikon A1R, Japan).

CLSM of Bacterial Biofilms. Bacterial biofilm was grown by placing autoclaved glass coverslips at the air−liquid interface of broth cultures.[54] The incubation time for *P. acnes* and *P. aeruginosa* biofilms were 5 days anaerobically in the GasPak system and overnight aerobically, respectively. Before staining, the biofilm was washed with PBS three times and dried completely at 37 °C. The staining procedure was the same as above except that 20 μg of 6xHis-tagged scFv was used. One set of biofilms was used for crystal violet staining.

Transmission Electron Microscopy (TEM). The procedure was carried out in a microcentrifuge tube. Bacteria in broth culture were centrifuged at 3000*g* for 5 min, washed with 0.85% NaCl followed by PBS, and fixed with 4% PFA (pH 7.4) in PBS for 30 min at room temperature. After washing with PBS two times, the bacteria were blocked with 1% BSA-300 mM glycine in 0.1% PBST for 30 min at room temperature followed by two PBS washes. Then, 50 μg of 6xHis-tagged scFv in PBS was added and incubated for 60 min at room temperature. After washing two times with PBS, a 1:20 dilution of 10 nm Ni-NTA nanogold (Nanoprobes #2084, U.S.A.) in 0.05% TBST containing 1% BSA, which is the recommended dilution from the manufacturer, was added and incubated for 30 min at room temperature followed by two TBST-BSA washes. Then, the samples were resuspended in PBS, dehydrated by the graded ethanol dehydration method, and polymerized in LR White medium grade resin and accelerator (EMS #14380, U.S.A.) in a BEEM embedding capsule (EMS #69911-01, U.S.A.) by a cold curing method. Finally, ultra-thin sections were examined with a transmission electron microscope (Hitachi Hi-Tech HT7700, Japan).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c05340.

Picture of an ELISA plate for the result shown in Figure 3A (Figure S1) and pictures of ELISA plates for the results shown in Figure 4A and C are provided as Figures S2 and S3 (PDF)

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Author Contributions

T.T.M. performed all experiments and drafted the first draft. M.Y. conceived the study, designed the experiments, analyzed the results, and edited the manuscript. Both authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt; DMST, Department of Medical Sciences Thailand; DSM, German Collection of Microorganisms and Cell Cultures; LR White, London Resin Company's Product; MWCO, molecular weight cutoff

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