**Introduction**

*Staphylococci* are among the most common causes of bacterial infection in the community and pose a major danger to human health. *S. aureus* is the most well-known of the species which produce hospital- and community-acquired infections, with methicillin-resistant *S. aureus* presenting a serious public health threat [1]. *S. epidermidis* is the sister species of *S. aureus* which often causes infection in immunocompromised individuals or those following damage to the epithelium. Both of them produce biofilm to protect themselves from host immune system and enhance their resistance to antibiotic chemotherapy [2]. The key component of the biofilm extracellular matrix in *Staphylococci* is polysaccharide intercellular adhesion (PIA) [3], an essential factor in biofilm formation composed of homopolymer of β-1,6-linked N-acetylglucosamine (GlcNAc). The production of PIA depends on the expression of the *ica*ADBC operon, and TcaR and IcaR are a weak and strong negative regulator of transcription of the *ica* locus, respectively [4].

The transcription regulator TcaR is a member of the MarR family, and is involved in teicoplanin and methicillin resistance in *Staphylococci* [5]. The MarR family proteins function as regulators of protein expression and these regulated proteins confer resistance to multiple antibiotics, household disinfectants, organic solvent virulence factors, and oxidative stress agents [6,7,8,9,10,11]. The crystal structures of a number of MarR family proteins have been reported, including MarR from *Escherichia coli* [12], OhrR from *Bacillus subtilis* [13], MexR from *Pseudomonas aeruginosa* [14], MarR from *Xanthomonas campestris* [15], SlyA from *Salmonella typhimurium* [16], AoxR from *Streptococcus pneumonia* [10] and TcaR, which is studied in this work, from *S. epidermidis* [17]. These structures revealed that MarR family proteins are all homodimers. The overall structure of each monomer could be divided into two functional domains, one is the dimerization domain and the other is the winged helix-turn-helix (wHTH) DNA binding domain. The N and the C-terminal α-helices (α1, 5, 6) of one monomer interdigitate with those of the other monomer to produce dimerization interaction. In addition, the wHTH DNA binding domain is composed of α2-α3-α4-[βA-W1]-βB which adopts the winged-helix-fold, and the amino acid sequences of this domain are highly conserved.

As the MarR-type proteins can act as positive, negative, or bifunctional regulators, TcaR also acts as a multi-functional regulator. It is not only as a regulatory factor to affect the transcription of *icaADBC* [4], the first regulator reported for cell wall-anchored proteins (Spa and SasF), but also as the regulator of *sarS* [18,19]. We previously described the 3D structures of TcaR in its apo form and in complex with salicylate as well as several
aminoglycoside and β-lactam antibiotics [17]. In this research, comparison of the native TcaR structures and those in complexes indicated that the regulation mechanism involves a large conformational shift in the DNA binding lobe. Several antimicrobial compounds inhibited TcaR-ssDNA interaction and further induced biofilm formation in S. epidermidis. In the present study, we found that TcaR could interact with ssDNA and the result demonstrated that TcaR shows a stronger preference toward GC-rich ssDNA than to dsDNA by using EMSA, CD, and Biacore experiments. However, the detailed mechanism of the interaction between TcaR and ssDNA still remains to be elucidated. In order to investigate the regulation mechanism of the ssDNA binding ability of TcaR, we applied electron microscopy (EM) technique to reveal TcaR-ssDNA complex. Furthermore, we clarified the role of TcaR-ssDNA interaction by in vitro replication assay and in vivo plaque assay. Taken together, these results provide an in-depth investigation on the multiple functions of TcaR in S. epidermidis.

**Results and Discussion**

**Strong TcaR Binding to ssDNA Oligomers Revealed by EMSA**

TcaR is known to bind and regulate the ica promoter [4]. We previously identified that TcaR most strongly interacts with IcaR DNA1 (a 33-mer pseudo-palindromic sequence containing consensus sequence TTNNAA) compared with other designed IcaR DNA fragments [17]. However, when using the sense strand of IcaR DNA1 (IcaR DNA1S) and the antisense strand of IcaR DNA1 (IcaR DNA1A) (Figure 1A) as controls in electrophoretic mobility shift assays (EMSA), the result demonstrated that TcaR shows a stronger preferences toward ssDNA fragments (IcaR DNA1S and DNA1A) (Figure 1B). To determine the type and length of the TcaR-binding site on ssDNA, a series of GC-rich and AT-rich ssDNA segments were designed (Figure 1A) [20,21]. Their TcaR binding ability was tested using EMSA. As shown in Figure 1C, TcaR does not significantly interact with 17-mer GC-rich (GC17) and AT-rich (AT17) ssDNA oligomers, but shows strong interaction with 33-mer GC-rich (GC33) and AT-rich (AT33) ssDNA sequences with a preference toward the 33-mer ssDNA sequence with a molar ratio of 1:1. Thus, we suggest that TcaR prefers binding to the 33-mer ssDNA.

In order to evaluate the minimal DNA binding length of TcaR, GC-rich fragments of different lengths were designed. As seen in Figure 1D, GC-rich fragments with 33, 29, and 25 bases showed similar binding strength to TcaR; with TcaR forming a large, apparently multimeric complex with GC33, a small complex with GC25, and both small and large complexes with GC29 in EMSA. These results indicated that the minimal observed ssDNA fragment size to allow TcaR binding ranges between 17 to 25-mer; providing useful information for the design of a DNA fragment with precise length suitable for crystal packing. Up to now, only three MarR family protein complex structures have been reported, and the first one is complexed with dsDNA [13,16,22]. The second one is complexed with salicylate [12,17,22,23] and we discovered the third case which is complexed with antibiotics [17]. We have already obtained TcaR-ssDNA crystals and collected X-ray diffraction data to 3.6 Å resolution at Spring-8 (Hyogo, Japan), beamline BL12B2. However, the phase problem is still the main challenge and the works are currently under progress.

Moreover, to investigate whether TcaR preferentially binds to ssDNA or dsDNA, the ability of ssDNA to compete with the TcaR-dsDNA complex was evaluated. For the competition assay, the IcaR DNA1 probe was preincubated with TcaR (dimer) protein to allow formation of the dsDNA-TcaR complex prior to mixing with increasing amounts of single-stranded GC33 DNA. It has been known that ssDNA products have lower migration velocity compared to its dsDNA counterparts in polyacrylamide electrophoresis [24,25]. As shown in Figure 1E, ssDNA, as a competitor, interfered the binding of TcaR to the dsDNA, suggesting a binding preference for ssDNA. To further confirm this result, IcaR DNA1 and GC33 ssDNA oligomers were mixed, and their interaction strengths with TcaR were compared using EMSA (Figure 1F). Findings indicated that increasing the concentration of TcaR produces a ssDNA band shift greater than that for dsDNA, confirming a stronger interaction between TcaR and ssDNA. Moreover, to investigate possible pH effect of ssDNA binding activity of TcaR, a series of buffers with increasing pH were tested for their potential interfere in TcaR-ssDNA binding. As shown in Figure 1G, the EMSA results showed that TcaR had a strongest affinity for GC33 at pH 8.0 and the affinity was reduced by decreasing pH. Consequently, the result indicates that the ssDNA binding activity of TcaR is pH-dependent.

To clarify whether the ssDNA binding site of TcaR is close, or identical, to the dsDNA binding site, a TcaR quadruple mutant (4 positively charged amino acids responsible for DNA binding mutated to alanines to produce R71A/K73A/R93A/K95A) [17] was designed and its ssDNA and dsDNA binding ability tested. As seen in Figure 1H, the mutant failed to interact with either dsDNA or ssDNA. This indicated that these amino acids are essential for binding in both ssDNA and dsDNA.

**Binding of Antibiotics Abolished ssDNA Binding of TcaR**

The MarR protein of E. coli is a multidrug binding transcription regulator. A wide range of compounds, including 2,4-dinitrophenol, plumbagin, menadione, and salicylate, attenuate and inhibit its association with cognate DNA [26]. In our previous study, salicylate and multiple antibiotics interfered with the transcriptional repressor activity of TcaR [17]. These findings prompted the current investigation into the possible effects of antibiotics on the ssDNA binding ability of TcaR. Here, to investigate the possible effect of some drugs on TcaR, four compounds were tested for their potential inhibition on TcaR-ssDNA interaction. These include one beta-lactam antibiotics (ampicillin) that contain a β-lactam nucleus in their molecular structure and act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls, two aminoglycoside antibiotics (kanamycin and gentamicin) that composed of several sugar groups and amino groups, and bacteriostatic antimicrobial (chloramphenicol) which is considered as a prototypical broad-spectrum antibiotic.

As shown in Figure 1I, kanamycin, chloramphenicol and gentamycin interfered with the ssDNA (GC33) binding activity of TcaR at a concentration of 2 μM and this effect was more pronounced at a higher concentration, suggesting that antibiotics inhibit formation of the TcaR-ssDNA complex. Results indicated that ampicillin also antagonized the ssDNA binding activity of TcaR at a concentration of 20 μM. This result is consistent with the observation seen in SPR, as discussed below. Taken together, we believe that diverse kinds of antibiotics may interact with TcaR to regulate its ssDNA binding ability.

**SPR and CD Spectral Analysis of TcaR Bound to ssDNA and dsDNA**

The binding affinity of GC33 and TcaR was determined quantitatively using surface plasmon resonance. Increasing concentrations of TcaR were passed across a flow cell coated with
ssDNA GC33 and the binding response was recorded as changes in response units (RU) after subtraction of the binding response for the reference flow cell. Figure 2A shows a representative sensorgram. Analysis of the sensorgram data indicates $k_a$ for the interaction of TcaR with the ssDNA GC33 is $8.8 \times 10^5 \text{M}^{-1} \text{s}^{-1}$; $k_d$ for the interaction of TcaR with the ssDNA GC33 is $9.5 \times 10^2 \text{s}^{-1}$ (Table 1). To investigate whether TcaR binds to different types and different lengths of DNA molecules, Biacore experiment was used to test the binding of TcaR to DNA fragments of biotin-labeled ssDNA and hairpin DNA duplex (IcaR DNA1). Consistent with previous observations in Fig. 1B, TcaR binds to ssDNA GC33 and ssDNA AT33 with the higher affinity and the association rates of TcaR to IcaR DNA1, and ssDNA GC17, ranging from $4.3 \times 10^5 \text{M}^{-1} \text{s}^{-1}$ to $1.2 \times 10^6 \text{M}^{-1} \text{s}^{-1}$, are much lower (shown in Figure 2B). However, when look at a wider range of DNA sizes, TcaR showed no detectable binding ability to ssDNA 8-mer GC8 but the highest binding ability to ssDNA GC99. The association rate with ssDNA GC99 is 36-fold higher than with ssDNA GC33, along with a 20-fold higher off-rate, suggested that cooperative binding of TcaR may contribute significantly in its ssDNA binding activity (Table 1). Interestingly, the association rate of GC33 ssDNA is two-times higher than IcaR DNA1, but the dissociation rate of IcaR DNA1 is the lowest compared to the DNA fragments tested in this study, suggesting different modes of interaction occurs in ssDNA-TcaR and dsDNA-TcaR complex (Figure 2B).

Furthermore, in order to determine whether other MarR family and TetR family proteins such as SAR2349 from S. aureus and...
IcaR from *S. epidermidis* have the ssDNA binding ability, we conducted a series of SPR experiments to analyze the binding ability of SAR2349 and IcaR proteins to GC33 ssDNA (Figure 2C). The result shows that not all MarR family proteins have this ssDNA binding ability, thus pointing to the specific ssDNA-binding feature of TcaR. In addition, we have previously demonstrated that antibiotics appear to antagonize the ssDNA binding activity of TcaR (Figure 1I). Therefore, a measurement for the effect of kanamycin and ampicillin to the GC33 ssDNA binding affinity of TcaR is conducted using surface plasmon resonance to confirm the result. As seen in Figure 2D, the affinity between TcaR and GC33 ssDNA is shown by a decrease in RU values in the presence of antibiotics. This was especially apparent with kanamycin, which yield the lower binding capacity. Taken together, we demonstrate that TcaR shows a higher binding affinity to ssDNA than to dsDNA, and several antibiotics could regulate the ssDNA binding activity of TcaR.

Conformational changes of TcaR in response to ssDNA were monitored using CD spectroscopy [27]. As shown in Figure 3, the CD spectra of TcaR protein were scanned from 200 to 250 nm in the presence of GC33. With increasing concentrations of GC33 ssDNA, the CD spectrum shows a concomitant decrease in the intensity of the negative peak at 222 and 210 nm, revealing the conformational change of TcaR.

Furthermore, in order to examine whether TcaR shows a binding ability towards much longer ssDNA fragments such as viral ph174-ssDNA, the interaction between them was also examined with increasing concentration (0, 2.5, 10 mM) of viral ssDNA Binding Ability of MarR Family Protein TcaR

Figure 2. SPR sensograms of the binding of TcaR to DNA fragments at 25°C. (A) SPR sensogram of the binding of varying concentrations of TcaR (4, 8, 16, 32, 64 nM) to biotin-labeled GC33 ssDNA (33mer GC-rich single-stranded DNA fragment) captured on SA chip with a ligand density of 120RU. The increase in RUs from the baseline was measured and used to calculate $k_a$ and $k_d$. One RU represents the binding of approximately 1 pg protein/mm². (B) Comparison of SPR derived binding curves for different types of immobilized biotin-labeled DNA fragments interacting with TcaR protein (64 nM). The sensograms represent binding of, from bottom to top: GC8, GC17, IcaR DNA1, AT33 and GC33. The association ($k_a$) and dissociation rate constants ($k_d$) were derived by fitting the sensograms to a Langumir binding rate equations and are tabulated in Table 1. (C) The SPR sensogram of the binding of different DNA binding proteins to the immobilized GC33 ssDNA fragment. The concentration of each target protein is 64 nM, followed by the same condition as used in other Biacore assays. (D) Sensogram of the interaction between the immobilized GC33 ssDNA fragment and the TcaR protein (64 nM) in the presence of 640 nM kanamycin or ampicillin.

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ssDNA ϕX174. In the presence of viral ssDNA ϕX174, the spectral changes were particularly pronounced in the decreased intensity of the negative peak at 222 and 210 nm, with an ultimate diminished negative peak in the 225–230 nm region at high concentration of viral ssDNA ϕX174, which suggests strong interaction between viral ssDNA and TcaR. The results of the CD experiment reported herein provide strong evidence that TcaR exhibits strong binding affinity for viral ssDNA.

TcaR Interacts with Circular Viral ssDNA

In order to further confirm our finding that TcaR forms complex with viral ssDNA, the M13 and ϕX174 phage ssDNA circles were used as probes in EMSA to evaluate TcaR binding. As shown in Figure 4A, TcaR reduced the mobility of the M13 and ϕX174 ssDNA, but S. epidermidis IcaR and S. aureus MarR family protein SAR2349 had no specific interactions with ssDNA. This indicated that TcaR has strong viral ssDNA-binding ability. It is also worth noting that other MarR family protein and TetR family protein do not have such ssDNA binding properties with high affinity, suggesting that the results from TcaR studies are not an artifact due to simple charge interactions between TcaR and ssDNA. Furthermore, since the attempt to obtain the TcaR-ssDNA complex structure was not successful, we resorted to EM analysis to image TcaR-ϕX174 complex and to pursue its 3-D reconstruction. After staining for 4 min with 2.5% uranyl acetate, EM analysis was performed with a Tecnai™ G² Spirit Bio TWIN (FEI CO., The Netherlands) using 120kV acceleration voltage. As seen in Figure 4B–D, EM imaging revealed that no complex was found in the negative control sections, whereas TcaR form a nucleoprotein filament with a circular viral ϕX174-ssDNA fully covered with proteins, suggesting strong and cooperatively interaction between viral ssDNA and TcaR. This is consistent with the EMSA results we observed. We are now testing another EM method as described by Lurz R et al. [28] to confirm the cooperative binding between the TcaR and viral ϕX174-ssDNA.

A distinct group of DNA-binding proteins called the ssDNA-binding proteins (SBP) could specifically bind ssDNA and be used in processes where the double helix is separated, including DNA replication, transcription, and recombination. Because TcaR is known as a MarR family transcription regulator that binds to specific dsDNA sequence with the winged helix-turn-helix (wHTH) DNA binding domain, the ssDNA binding ability of TcaR may not be involved in transcription. In order to clarify the role of TcaR-viral ssDNA interaction, our approach is to examine it with in vitro replication. We used single-primed M13 replication assay to measure the ability of purified TcaR protein to convert a primed single-strand M13 template to the duplex form in a manner that requires processive DNA synthesis. As seen in Figure 5A, M13-based in vitro DNA replication assay showed that the addition of TcaR protein to the reaction mixture, and incubation for up to 30 min, resulted in almost no DNA replication activity compared to controls. This indicated that TcaR markedly inhibits DNA replication and that the mechanism of inhibition, at least in part, involves interaction with viral ssDNA. These results suggest a possible role for TcaR in bacteriophage resistance.

Since 1980, investigators have developed an increasing number of bacteriophage therapies for the treatment, or prophylaxis, of

### Table 1. DNA-binding constants between TcaR dimer and the target DNA determined from SPR kinetic analyses.

| Target DNA     | \(k_a\) (M\(^{-1}\) s\(^{-1}\)) | \(k_d\) (s\(^{-1}\)) | \(K_a\) (M\(^{-1}\)) | Rmax  |
|----------------|---------------------------------|----------------------|----------------------|-------|
| ssDNA (GC33)  | 8.8 \times 10^5                 | 9.5 \times 10^{-3}   | 9.3 \times 10^7      | 507   |
| ssDNA (GC17)  | 1.2 \times 10^5                 | 4.3 \times 10^{-2}   | 2.8 \times 10^6      | 220   |
| ssDNA (GC8)   | -                               | -                    | -                    | -     |
| ssDNA (GC99)  | 1.6 \times 10^6                 | 4.7 \times 10^{-4}   | 3.4 \times 10^2      | 4354  |
| ssDNA (AT33)  | 8.1 \times 10^5                 | 9.3 \times 10^{-3}   | 8.7 \times 10^6      | 476   |
| dsDNA (IcaR DNA1) | 4.3 \times 10^5                 | 5.0 \times 10^{-3}   | 8.6 \times 10^2      | 268   |
| Control dsDNA | -                               | -                    | -                    | -     |

\(K_a\) value obtained from \(k_a\) divided by \(k_d\). doi:10.1371/journal.pone.0045665.t001

Figure 3. CD spectra of TcaR in various concentrations of GC33 and viral ssDNA ϕX174 (varying from 0 to 125 μM). TcaR concentration was 25 μM in a buffer of 20 mM Tris-HCl, pH 8.0, 150 mM KCl, 0.1 mM MgCl₂, 0.05 mM EDTA, 12.5% Glycerol, 10 mM DTT. The protocols for CD data are described in the Experimental Procedures section. doi:10.1371/journal.pone.0045665.g003
bacterial infectious diseases [29,30,31]. Reports have described that appropriately administered phages can treat lethal infectious diseases caused by gram-negative and gram-positive bacteria, such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Enterococcus faecium*, and *S. aureus* [32,33,34,35]. Antibiotic resistance has become a global public health concern; thus investigators are extensively reevaluating phage therapies to fully exploit their antimicrobial potential [36,37]. However, phages encounter a variety of different antiviral mechanisms during their infection of bacterial cells, such as prevention of phage adsorption and DNA entry, cutting of phage nucleic acids, and abortive infection systems [38]. Most reported antiphage systems have been shown to be relevant to the d ssDNA phage, but not ssDNA, sRNA, or dsRNA phages. To further confirm and clarify the first relationship between the TcaR protein and ssDNA phage resistance, the standard plaque assay was performed in *E. coli* as a model system since little is known about the ssDNA phage infecting *Staphylococci*. As seen in Figure 5B, induction of the TcaR protein in *E. coli* conferred increased host resistance to ssDNA phage (M13 and φX174) infection. However, a TcaR-expressing strain did not demonstrate reduced sensitivity to dsDNA phage Lambda (λ) infection, suggesting that the phage resistance was caused by TcaR-viral ssDNA complex. The observed biological differences point to a remarkable plasticity of TcaR. These findings, thus, may support a hypothesis that TcaR might interfere with viral ssDNA replication and establish a link between TcaR and ssDNA phage resistance.

**Conclusions**

The MarR family transcriptional regulators serve as sensors of changing environments, allowing pathogenic bacteria to survive and persist in a dynamic environment [39]. However, up to now, the knowledge of MarR family protein-nucleic acid interaction has been focused on dsDNA and the MarR family protein-ssDNA interaction ability as well as their contribution to the multiple functions of TcaR is yet to be discovered. Better understanding of these interactions not only will benefit the understanding of many biological mechanisms but also is expected to provide a concept for designing a new therapy for *Staphylococci*. In this work, we present the first attempt to investigate the TcaR-ssDNA interaction. The information of TcaR-ssDNA binding mode and the minimal binding length that we obtained from EMSA analysis and Biacore will be helpful for us to obtain TcaR-ssDNA complexed structure successfully. Moreover, we used in vitro replication assay and plaque assay to elucidate the specific biological role of the ssDNA binding ability of TcaR. Such observations may help us understand the mechanism of antibiotic resistance in the MarR family regulators.

**Materials and Methods**

**Gene Cloning, Protein Production and Purification**

The IcaR and TcaR proteins were expressed in *E. coli* BL21 (DE3) and purified as already described [17,40]. The MarR homologous gene, SAR2349, was amplified directly from the *S. aureus* MRSA252 genome by polymerase chain reaction (PCR) and subsequently cloned into expression vector pET-32. This construct was transferred into *E. coli* of Arctic Express™ (DE3) RIL strain. The His6-tagged wild-type protein was over-expressed in Difico Luria-Bertani (LB) broth containing 50 mg/l ampicillin to an optical density at 600 nm of 0.5–0.6 and then induced with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Cells were grown for 2 days at 16°C. The cells were then harvested by centrifugation at 12,000 g for 30 min and disrupted by Constant...
Cell Disruption System (CONSTANT SYSTEM Ltd, UK) with lysis buffer containing 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 20 mM imidazole. The homogenate was centrifuged at 27,000g for 30 min and the cell-free extract was loaded onto a Ni²⁺-NTA column, which had been previously equilibrated with lysis buffer. The column was washed with lysis buffer, and the His-tagged SAR2349 was subsequently eluted by a linear gradient of imidazole from 10 mM to 500 mM. His-tagged SAR2349 eluted was dialyzed twice against 5 liters of buffer (20 mM Tris-HCl, pH 8.0, and 500 mM NaCl) and then subjected to Thrombin digestion to remove the tag. The mixture was then passed through another Ni²⁺-NTA column, and subsequently untagged SAR2349 protein was eluted by a linear gradient of 10 mM to 500 mM NaCl-containing buffer and then dialyzed twice against 5 liters of buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 2 mM DTT) for storage. The purified SAR2349 protein was finally concentrated by 3 kDa cut-off size membrane of Amicon ultra-15 centrifugal filter units (Millipore, MA, USA) for storage at –80°C.

**EMSA Assay**

The six oligonucleotide probes used in EMSA experiments were purchased from MBioInc. (Taiwan) (Figure 1A). The viron φX174 and M13 ssDNA were purchased from New England Biolabs (USA). For the preparation of double-stranded IcaR DNA1, equimolar amounts (100 μM each) of complementary oligonucleotides were mixed, fully denatured by heating at 95°C for 5 min in 10 mM Tris-HCl pH 8.0, 20 mM NaCl and allowed to cool gradually to room temperature. Gel shift assays were performed by incubating 1 μM of ssDNA or dsDNA with 1–4 μM purified recombinant proteins under binding conditions (20 mM Tris-HCl, pH 8.0, 150 mM KCl, 0.1 mM MgCl₂, 0.05 mM EDTA, 12.5% Glycerol, 0.1 mM DTT and 1 mg/ml BSA) for 15 min at room temperature with gentle vortex. After incubation, 15 μl of the reaction solution was mixed with 3 μl of the sample loading dye and subsequently electrophoresed on 15% polyacrylamide gels in 1/2 Tris/acetate/EDTA (TAE) at 100 V for 30 min and visualized using SYBR Green I nucleic acid gel stain (Invitrogen). For competition assay, IcaR DNA1 probe fragments of 5k to allow replication complexes to assemble at 30°C to allow replication complexes to assemble at

**Electron Microscopy (EM)**

The TcaR proteins (0.3 μM) were first incubated at 30°C for 15 min in reaction buffer [20 mM Tris-HCl, pH 8.0, 150 mM KCl, 0.1 mM MgCl₂, 0.05 mM EDTA, 12.5% glycerol, 10 mM DTT, 12 μM circular viron φX174 ssDNA (5386 nucleotides in length), 0.2 M ammonium acetate] and then chilled on ice to stop the reaction. The reaction product was diluted 100-fold with EM sample dilution buffer (2 mM MgCl₂, 0.5 mM DTT, 10 mM HEPES pH 7.0). A droplet (4 μl) was placed for 1 min at room temperature on a copper grid (300 mesh, Pelco, USA) coated with fresh carbon. The excess buffer was then carefully blotted away from the edge of the grid with Whatman #1 filter paper (Whatman Inc., USA). After staining for 1 min with 2% uranyl acetate, excess liquid was removed and samples were dried at room temperature. Bio-transmission EM was performed with a Tecnai F20 Bio TWIN (FEI Co., Netherlands) using an acceleration voltage of 200 KV. Images were recorded with a slow scan CCD camera (Gatan MultiScan™ 600, USA) at a resolution of at least 4k×4k pixels.

**Repliation Assay**

For M13 replication assay, 250 μM of single primed M13mp18 ssDNA was incubated with/without 2 μM TcaR protein in the reaction mixture (20 μl) containing 25 unit Klenow fragment, 25 mM NaCl, 7 mM MgCl₂, 1 mM EDTA, and 0.5 mM DTT for 3 min at 30°C to allow replication complexes to assemble at the primer template junction [45,46]. Replication was allowed to proceed by addition of 60 μM dNTP. After incubation at 30°C for 30 min, the reactions were terminated by addition of 10 mM Tris-HCl, 5 mM EDTA, 0.5% SDS, and 50 μg of proteinase K (with
total volume of 20 μl, the culture was incubated at 50°C for 1 h. Conventional electrophoresis was then performed to verify the result of DNA replication (20-cm 0.8% agarose gel, 15 mA, 0.5X TBE buffer). The bands are visualized using SYBR Green I nucleic acid dye.

**Phage Studies**

Host sensitivity to phages was tested using a virulent variant of phage M13, φX174 and ψB and E. coli BL21 (DE3) RIL transformed with engineered pET-16c–TcR plasmid containing lacI and lac operator as host [47,48,49]. Cells were grown in LB media until the optical density (OD600) reached 0.6. TcR protein was then induced by adding a final concentration of 0.1 mM IPTG and used in plaque assays as previously described [50,51]. Plaque assays were performed in triplicate. Plates and agar layers were visualized and counted to quantify the plaque numbers. The plaque count ratio of a non-iptg set to the IPTG set [52]. Error-bars were calculated as one standard deviation.

**Protein Data Bank Accession Codes**

The atomic coordinates and structure factors for the TcR–RNA complex have been deposited in the wwPDB with accession numbers 4EJT.

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**Author Contributions**

Conceived and designed the experiments: YMC, AW. Performed the experiments: YMC CKMC YW. Analyzed the data: YMC YCC MHW AW. Contributed reagents/materials/analysis tools: YMC CKMC YW. Wrote the paper: YMC CKMC AW.

**References**

1. Anderson SD, Guns JG (2008) Cellophane: an extended spectrum anti-methicillin-resistant Staphylococcus aureus cephalosporin. Ann Pharmacother 42: 806–816.

2. Stewart PS, Costerton JW (2001) Antibiotic resistance of bacteria in biofilms. Lancet 358: 135–139.

3. Vuong C, Voyich JM, Fischer ER, Braughton KR, Whitney AR, et al. (2004) Phage Studies of Virulence and Resistance in Staphylococcus aureus. PLoS One 9: e5145665.

4. Miller PF, Sulavik MC (1996) Overlaps and parallels in the regulation of the mar regulon. Microbiol Rev 29: 231–262.

5. Brandenberger M, Tschierske M, Giachino P, Wada A, Berger-Bachi B (2000) Insights into the redox-switch mechanism of the MarR/DUF24-type regulator HypR. Nucleic Acids Res. 28: 10448–10454.

6. Alekshun MN, Levy SB (1999) The mar regulon: multiple resistance to antibiotics and other toxic chemicals. Trends Microbiol 7: 410–413.

7. Miller PF, Sulavik MC (1996) Overlaps and parallels in the regulation of intrinsic multiple-antibiotic resistance in Escherichia coli. Mol Microbiol 21: 441–51.

8. Palm GJ, Khanh Chi B, Waack P, Gronau K, Recher D, et al. (2012) Structural insights into the redox-switch mechanism of the MarR/DUF24-type regulator HypR. Nucleic Acids Res. 40: 10451–10460.

9. Guerra AJ, Dann CE, 3rd, Giedroc DP (2011) Crystal structure of the zinc-finger family regulator Mrr of Methanobacterium thermoautotrophicum. J Mol Biol 377: 653–667.

10. Farge G, Hofmarcher T, Kivorotova J, Robouguar R, Hofer A, et al. (2006) The N-terminal domain of TWINKLE contributes to single-stranded DNA binding and DNA helicase activities. Nucleic Acids Res 34: 393–403.

11. Alekshun MN, Levy SB (1999) Alteration of the repressor activity of MarR, the negative regulator of the Escherichia coli marRAB locus, by multiple chemicals in vitro. J Bacteriol 181: 4669–4672.

12. Chang YM, Chen CK-M, Hou M-H (2012) Conformational Changes in DNA upon Ligand Binding Monitored by Circular Dichroism. International Journal of Molecular Sciences 13: 3394–3413.

13. Luz R, Grote M, Dijk J, Reinhardt R, Dobrinski B (1986) Electron microscopic study of DNA complexes with proteins from the Archaeabacterium Sulfolobus solfataricus. EMBO J 5: 3715–3721.

14. Lurz R, Hofmarcher T, Kivorotova J, Robouguar R, Hofer A, et al. (2006) The N-terminal domain of TWINKLE contributes to single-stranded DNA binding and DNA helicase activities. Nucleic Acids Res 34: 393–403.

15. Wiese G, Dray E, Groeser T, San Filippo J, Shi I, et al. (2007) Promotion of homologous recombination and genomic stability by RAD51API via RAD51 recombinase enhancement. Mol Cell 28: 482–490.

16. Pirisi A (2000) Phage therapy–advantages over antibiotics? Lancet 356: 1418.

17. Carlton RM (1999) Phage therapy: past history and future prospects. Arch Intern Med 159: 1999–2006.

18. The Lancet (1983) Phage therapy. Lancet 2: 1287–1288.

19. Merril CR, Scholl D, Adhya SL (2003) The prospect for bacteriophage therapy in Western medicine. Nat Rev Drug Discov 2: 489–497.

20. Reiner LB, Bull JJ (2004) Population and evolutionary dynamics of phage therapy. Nat Rev Microbiol 2: 166–173.

21. Mathey M, Spencer J, Kruger R, Morello L, Wiedenheft B (2008) Bacteriophage therapy–cooked goose or phoenix rising? Curr Opin Biotechnol 19: 608–612.

22. O’Flaherty S, Ross RP, Cofier A (2009) Bacteriophage therapy: advantages over antibiotics? Lancet 356: 1418.

23. Carlton RM (1999) Phage therapy: past history and future prospects. Arch Intern Med 159: 267–274.

24. Summers WC (2001) Bacteriophage therapy. Annu Rev Microbiol 55: 437–451.

25. Merril CR, Scholl D, Adhya SL (2003) The prospect for bacteriophage therapy in Western medicine. Nat Rev Drug Discov 2: 489–497.

26. Mathey M, Spencer J (2008) Bacteriophage therapy–cooked goose or phoenix rising? Curr Opin Biotechnol 19: 608–612.

27. O’Flaherty S, Ross RP, Cofier A (2009) Bacteriophage therapy and their lysins for elimination of infectious bacteria. FEMS Microbiol Rev 33: 801–819.

28. Labrie SJ, Samson JE, Moineau S (2010) Bacteriophage resistance mechanisms. Biochim Biophys Acta 1523: 239–242.

29. Chang YM, Chen CK-M, Hou M-H (2012) Conformational Changes in DNA upon Ligand Binding Monitored by Circular Dichroism. International Journal of Molecular Sciences 13: 3394–3413.

30. Luz R, Grote M, Dijk J, Reinhardt R, Dobrinski B (1986) Electron microscopic study of DNA complexes with proteins from the Archaeabacterium Sulfolobus solfataricus. EMBO J 5: 3715–3721.

31. Farge G, Hofmarcher T, Kivorotova J, Robouguar R, Hofer A, et al. (2006) The N-terminal domain of TWINKLE contributes to single-stranded DNA binding and DNA helicase activities. Nucleic Acids Res 34: 393–403.

32. Pirisi A (2000) Phage therapy–advantages over antibiotics? Lancet 356: 1418.

33. Carlton RM (1999) Phage therapy: past history and future prospects. Arch Intern Med 159: 1999–2006.

34. Merril CR, Scholl D, Adhya SL (2003) The prospect for bacteriophage therapy in Western medicine. Nat Rev Drug Discov 2: 489–497.

35. Reiner LB, Bull JJ (2004) Population and evolutionary dynamics of phage therapy. Nat Rev Microbiol 2: 166–173.

36. Mathey M, Spencer J (2008) Bacteriophage therapy–cooked goose or phoenix rising? Curr Opin Biotechnol 19: 608–612.

37. O’Flaherty S, Ross RP, Cofier A (2009) Bacteriophage therapy and their lysins for elimination of infectious bacteria. FEMS Microbiol Rev 33: 801–819.

38. Labrie SJ, Samson JE, Moineau S (2010) Bacteriophage resistance mechanisms. Biochim Biophys Acta 1523: 239–242.
40. Jeng WY, Ko TP, Liu CI, Guo RT, Liu CL, et al. (2008) Crystal structure of IcaR, a repressor of the TetR family implicated in biofilm formation in Staphylococcus epidermidis. Nucleic Acids Res 36: 1567–1577.
41. Hou MH, Lu WJ, Lin HY, Yuann JM (2008) Studies of sequence-specific DNA binding, DNA cleavage, and topoisomerase I inhibition by the dimeric chromomycin A3 complexed with Fe(II). Biochemistry 47: 5493–5502.
42. Huang CY, Hsu YL, Chiang WL, Hou MH (2009) Elucidation of the stability and functional regions of the human coronavirus OC43 nucleocapsid protein. Protein Sci 18: 2209–2216.
43. Hou MH, Lu WJ, Huang CY, Fan RJ, Yuann JM (2009) Effects of polyamines on the DNA-reactive properties of dimeric mithramycin complexed with cobalt(II): implications for anticancer therapy. Biochemistry 48: 4691–4698.
44. Hou MH, Lin SB, Yuann JM, Lin WC, Wang AH, et al. (2001) Effects of polyamines on the thermal stability and formation kinetics of DNA duplexes with abnormal structure. Nucleic Acids Res 29: 5121–5128.
45. Traktman P, Boyle K (2004) Methods for analysis of poxvirus DNA replication. Methods Mol Biol 269: 169–186.
46. Christensen J, Tattersall P (2002) Parovirus initiator protein NS1 and RPA coordinate replication fork progression in a reconstituted DNA replication system. J Virol 76: 6518–6531.
47. Fineran PC, Blower TR, Humpferys DP, Lilley KS, et al. (2009) The phage abortive infection system, TadN, functions as a protein-RNA toxin-antitoxin pair. Proc Natl Acad Sci U S A 106: 894–899.
48. Broun S, Jore MM, Lundgren M, Westra ER, Slijkhuis RJ, et al. (2008) Small CRISPR RNAs guide antiviral defense in prokaryotes. Science 321: 960–964.
49. Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, et al. (2007) CRISPR provides acquired resistance against viruses in prokaryotes. Science 315: 1709–1712.
50. Sitohy M, Chobert JM, Karwowska U, Gozdzicka-Jozefiak A, Haertle T (2006) Inhibition of bacteriophage m13 replication with esterified milk proteins. J Agric Food Chem 54: 3800–3806.
51. Smith HO, Hutchison CA, 3rd, Planckoch G, Venter JC (2003) Generating a synthetic genome by whole genome assembly. phiX174 bacteriophage from synthetic oligonucleotides. Proc Natl Acad Sci U S A 100: 15440–15445.
52. Deveau H, Barrangou R, Garneau JE, Labonte J, Fremaux C, et al. (2008) Phage response to CRISPR-encoded resistance in Streptococcus thermophila. J Bacteriol 190: 1390–1400.