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Overexpression of the *tcp* Gene Cluster Using the T7 RNA Polymerase/Promoter System and Natural Transformation-Mediated Genetic Engineering of *Vibrio cholerae*

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Abstract

The human pathogen and aquatic bacterium *Vibrio cholerae* belongs to the group of naturally competent bacteria. This developmental program allows the bacterium to take up free DNA from its surrounding followed by a homologous recombination event, which allows integration of the transforming DNA into the chromosome. Taking advantage of this phenomenon we genetically engineered *V. cholerae* using natural transformation and FLP recombination. More precisely, we adapted the T7 RNA polymerase/promoter system in this organism allowing expression of genes in a T7 RNA polymerase-dependent manner. We naturally transformed *V. cholerae* by adding a T7-specific promoter sequence upstream the toxin-coregulated pilus (*tcp*) gene cluster. In a *V. cholerae* strain, which concomitantly produced the T7 RNA polymerase, this genetic manipulation resulted in the overexpression of downstream genes. The phenotypes of the strain were also in line with the successful production of TCP pili. This provides a proof-of-principle that the T7 RNA polymerase/promoter system is functional in *V. cholerae* and that genetic engineering of this organism by natural transformation is a straightforward and efficient approach.

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Introduction

A plethora of methods exist today to allow genetic engineering of bacteria. Many of those methods have been used for the last 30 years or even longer and straightforward protocols are available today to master those techniques. One prominent example is the seminal book *Molecular Cloning: A Laboratory Manual* published initially in 1982 by Tom Maniatis, Edward Fritsch, and Joseph Sambrook [1] (the currently available 4th edition is authored by Michael R. Green and Joseph Sambrook; http://www.molecularcloning.com). But even though many techniques are available and work well, reducing the time needed for cloning is often desired. Thus, we developed a fast protocol [2–4] to genetically modify our favorite organism, the human pathogen and aquatic bacterium *Vibrio cholerae*, using chitin-induced natural transformation [5,6].

The regulatory network of chitin-induced natural transformation of *V. cholerae* is extremely complicated and brings together the pathways of chitin sensing and degradation, quorum sensing, and carbon catabolite repression (summarized in [7,8] and recently reviewed in [9]). However, the advantage of the DNA uptake process of *V. cholerae* over some other naturally competent Gram-negative bacteria such as *Haemophilus influenzae* and *Neisseria gonorrhoeae* is that *V. cholerae* is not fastidious about the kind of DNA it takes up while it is competent [10]. This makes natural competence and transformation a perfect system for fast and efficient delivery of DNA to the cells. In this study we applied our previously published protocol describing the combination of natural transformation and FLP recombination in *V. cholerae* (TransFLP, [3,4]) in order to integrate DNA sequences in a site-specific manner onto the chromosome. The rational behind this study was to create *V. cholerae* strains in which we were able to artificially express large gene clusters. We reasoned that it would be difficult or even impossible to clone such gene clusters onto plasmids/cosmids thereby expressing the genes *in trans*. Furthermore, as many of the common plasmids seem to be quickly lost from *V. cholerae* without constant selection pressure plasmid-encoded expression was excluded from this study. Consequently, we decided to express our gene cluster of interest by adding a strong and specific promoter *in cis* of the indigenous genes on the chromosome using our TransFLP method (Fig. 1).

To our knowledge the strongest and most exclusive expression system described so far in bacteria is based on the T7 RNA polymerase/promoter combination. Several studies in the 1980's have led to the establishment of this very successful system [11,12], which is still used our days (mostly with variations from the original protocols). The gene encoding the T7 DNA-directed RNA polymerase (EC 2.7.7.6) (from here on referred to as T7 RNA polymerase), phage T7 *gene 1*, was first cloned by Davanloo *et al.* in 1984 [13]. This opened up new possibilities as the T7

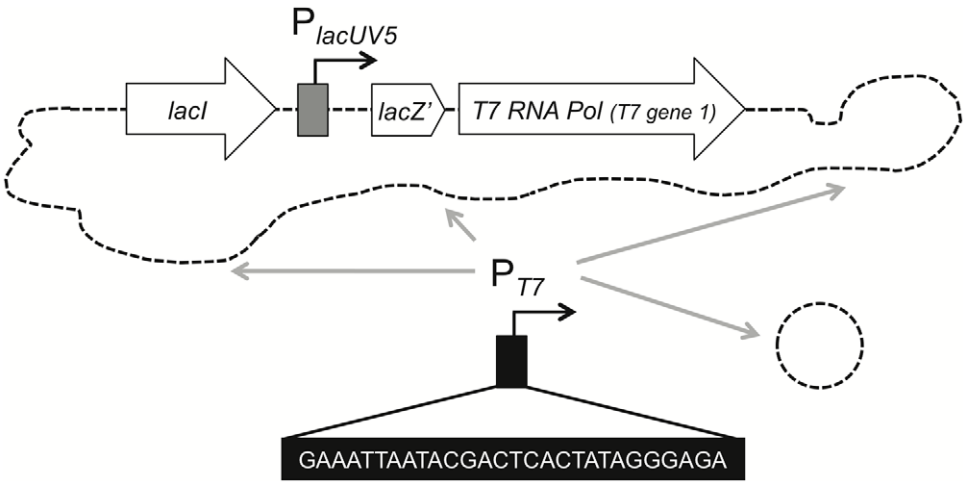

Figure 1. Schematic representation of the experimental setup. The rational behind this study was to engineer *V. cholerae* strains so that large gene clusters can be artificially expressed independent of growth condition restraints. The idea was to integrate the lacUV5-promoter controlled T7 RNA polymerase-encoded gene together with its repressor gene (*lacI*; both derived from *E. coli* BL21(DE3) [12]) into the *V. cholerae* chromosome. Using this strain, specific genes could be put under control of the T7 RNA polymerase solely by integrating the T7 RNA polymerase-dependent promoter sequence (indicated in black box; P_{T7}) at the respective locus on the chromosome using natural transformation. Alternatively, the P_{T7} sequence was integrated on a plasmid as is commonly done in *E. coli* overexpression systems.
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RNA polymerase "has a stringent specificity for its own promoters and will selectively transcribe DNA that has been linked to such a promoter" [13]. Such specificity was considered useful for directing the expression of only a subset of genes within a cell. The T7 RNA polymerase-dependent promoter is a "highly conserved sequence of 23 continuous base pairs including the start site for the RNA" [13]. Few if any of these conserved sequences are found in bacterial hosts causing this stringent specificity of the enzyme. The 22–23 base pairs consensus sequence of the T7 RNA polymerase promoter has been previously studied [14–16]. However, Ikeda *et al.* identified the essential bases of this specific promoter sequence by screening a randomly mutagenized library of T7 promoter sequences and correlating the *in vivo* activity to the sequences [17]. In our study the T7 Φ10 promoter was used, which encompasses 28 bp: GAAATTAATACGACTCACTA-TAGGGAGA [17,18]. We choose this strong expression system to have the best possible outcome as our study is aimed at proof of principle. Below we explain the cloning strategy and the experimental execution in detail. Furthermore, we provide evidence that the target gene cluster was indeed highly expressed in the genetically engineered strain, which resulted in the desired phenotypes. Obstacles and future needs for improvement are also discussed.

The target of our first trial was the toxin-coregulated pilus (*tcp*) [19] gene cluster of *V. cholerae*. The *tcp* gene cluster is located on the *Vibrio* pathogenicity island 1 (VPI-1) [20] previously termed TCP-ACF element [21]. The reason for this choice was that this cluster has been extensively studied *in vivo* and also under virulence inducing conditions *in vitro* (e.g. through the use of AKI growth conditions [22,23]). The TCP pilus is essential for intestinal colonization as it allows the establishment of microcolonies in the host [19], which was shown both in humans and in the infant mouse animal model of cholera [24–27]. The bundle-forming TCP pilus is a member of Type IVb pili, which "are found almost exclusively on enteric bacteria" [28]. The TCP pilus (classical and El Tor type) has been characterized based on mutant analysis, electron microscopy (EM; such as TEM, SEM, STEM, and three-dimensional high-resolution field emission scanning electron microscopy, FESEM), crystallization of the major pilin TcpA, and CryoEM reconstruction of full length TCP [28–32]. Based on *in vivo* and *in vitro* data it was concluded that a major function of this pilus is to mediate interactions between bacteria through microcolony formation that is required for intestinal colonization [30]. Indeed, Kirn *et al.* provided evidence that autoagglutination correlates well with intestinal colonization in the infant mouse model [30]. Thus, we hypothesized that induced expression of the *tcp* cluster would provide us with phenotypes such as *in vitro* autoagglutination/microcolony formation due to TCP production. It should be noted that artifical induction of the *tcp* cluster was already performed earlier by expressing *toxT*, which encodes the major transcriptional activator of virulence genes in *V. cholerae* [33], from an inducible promoter [29]. This method led to the production of the major subunit of TCP, TcpA, both in the classical and El Tor biotypes of *V. cholerae*. Furthermore, TCP fibers of *V. cholerae* O1 biotype El Tor could be visualized by EM upon *toxT* overexpression [29]. However, whereas autoagglutination of the classical biotype strain was readily observable under such artificial induction conditions, autoagglutination of a *V. cholerae* O1 El Tor strain (C6706) did not occur [31]. The author of this study speculated that the reduced level of TcpA protein and TCP fibers (~50%) for the O1 El Tor biotype compared to the classical strain O395 could partially explain this lack of autoagglutination [31]. To circumvent this issue, we aimed at high-level expression of the whole *tcp* cluster by directly inducing this cluster using the T7 RNA polymerase dependent system described above. The promoter sequence was thereby introduced upstream of the *tcpA* gene in a site-directed manner using the natural transformation-based TransFlp method as a tool [3,4]. Proof-of-principle was provided by successful TCP-mediated agglutination, microcolony formation, and the visualization of TCP fibers using scanning electron microscopy (SEM).

Table 1. Bacterial strains and plasmids.

Strains or plasmids	Genotype*	References
***V. cholerae* strains**		
A1552	Wild type, O1 El Tor Inaba, Rif^R	[42]
A1552-GFP	A1552 with mTn7-gfp, Rif^R, Gm^R	[36]
AT7RNAP	A1552 containing mTn7-T7RNAP, Rif^R Gm^R	this study
AΔctxAB	A1552ΔctxAB::FRT, Rif^R	this study
AΔctxAB-T7RNAP	A1552ΔctxAB::FRT containing mTn7-T7RNAP, Rif^R Gm^R	this study
AΔctxAB-[P_{T7}]-tcp	A1552ΔctxAB::FRT, [P_{T7}]-tcp::FRT, Rif^R	this study
AΔctxAB-[P_{T7}]-tcp-T7RNAP	A1552ΔctxAB::FRT, [P_{T7}]-tcp::FRT, containing mTn7-T7RNAP, Rif^R Gm^R	this study
Plasmids		
pBR-Tet_MCSI	pBR322 derivative deleted for Tet promoter and part of tet^R gene; Amp^R	[7]
pBR-[P_{T7}]-GFP	*gfp* gene preceded by T7 RNA polymerase-dependent promoter sequence; Amp^R	this study
pUX-BF13	oriR6K, helper plasmid with Tn7 transposition function; Amp^R	[37]
pGP704::Tn7	pGP704 with mini-Tn7	[41]
pGP704-mTn7-T7RNAP	pGP704 with mini-Tn7 carrying *lacI* and P_{lacUV5}-driven T7 DNA-directed RNA polymerase gene; Amp^R	this study

*VC numbers according to [47].

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Materials and Methods

Bacterial strains and plasmids

V. cholerae strains and plasmids used in this study are indicated in Table 1. *E. coli* strains DH5α [34] and BL21(DE3) [12] were used as hosts for cloning purposes and to test T7 RNA polymerase-dependent expression constructs. Bacterial mating between *V. cholerae* and *E. coli* was done using *E. coli* strain S17-1λpir [35] as the donor strain.

Media and growth conditions

Overnight cultures of *V. cholerae* and *E. coli* strains were grown in LB medium with shaking at 30°C. Bacterial strains used for gene expression profiling, phenotypic characterization and SEM were also cultured in LB either in the absence or in the presence of

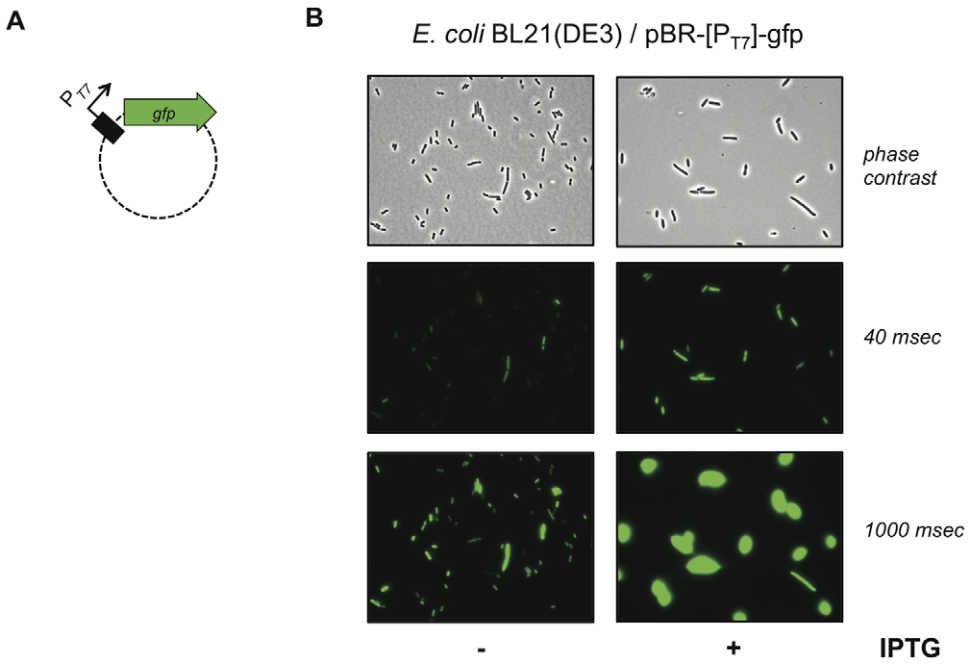

Figure 2. Functionality of T7 RNA polymerase dependent reporter plasmid. Plasmid pBR-[P_{T7}]-GFP (panel A) was transferred into chemically competent *E. coli* BL21(DE3) cells. Transformed bacteria were grown in the absence or presence of 1 mM IPTG as indicated and tested for GFP expression using epifluorescence microscopy (panel B). Panel B upper row: phase contrast images showing all cells; middle row: green fluorescence channel with short exposure time (40 msec); lower row: green fluorescence channel with longer exposure time (1000 msec).

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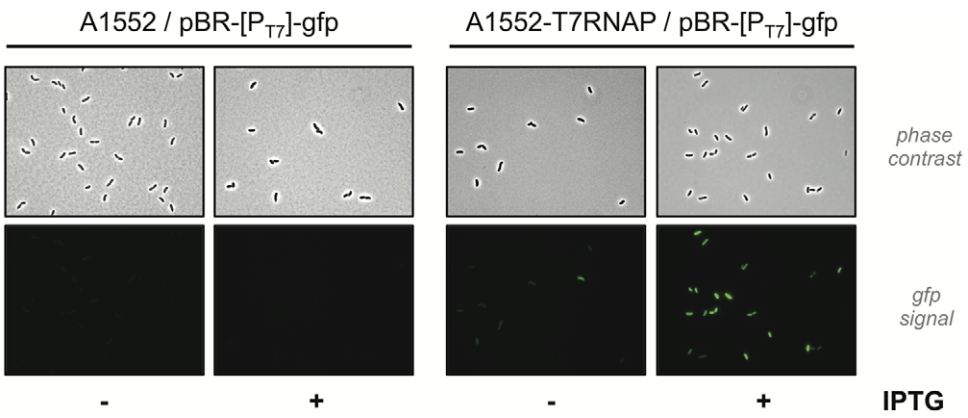

Figure 3. Testing for T7 RNA polymerase-dependent expression of *gfp* reporter construct in *V. cholerae*. Plasmid pBR-[P_{T7}]-GFP was transferred into *V. cholerae* strain A1552 (WT; on the left) or its T7 RNA polymerase derivative AT7RNAP (on the right). Plasmid-containing bacteria were grown in rich medium either in the absence (−) or in the presence (+) of the inducer IPTG. Expression of *gfp* driven by the T7 RNA polymerase-dependent promoter was visualized by epifluorescence microscopy (green channel; lower row; same exposure time was applied to all samples). The corresponding phase contrast images are shown above.
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1 mM IPTG. Ampicillin was added for plasmid maintenance at a concentration of 100 μg/ml whenever required. Fifty μg/ml gentamicin was used to select mTn7-T7RNAP-containing *V. cholerae* cells after bacterial mating with *E. coli* cells. *E. coli* cells were counter-selected by using *Vibrio* selective medium (TCBS; Fluka).

Construction of mini-Tn7 transposon carrying the gene encoding for T7 RNA polymerase

Plasmid pGP704-mTn7-T7RNAP was generated by ligating the *Sma*I-digested vector pGP704::Tn7 (Table 1) with the *lacI*-P_{lacUV}-T7RNAP cluster-containing and *Sca*I-digested PCR fragment. PCR was performed using primer pair LacI-before and T7 RNA pol after (Table S1) and genomic DNA of *E. coli* strain BL21(DE3) as template [12]. The construct was verified by sequencing.

Construction of T7 RNA polymerase-dependent transcriptional reporter plasmid

Plasmid pBR-[P_{T7}]-GFP was constructed as follows. The *gfp* coding region (including the Shine-Dalgarno sequence) was PCR-amplified using oligonucleotides P[T7]-GFP-up-P and P[T7]-GFP-down-*Bam*HI (Table S1) and genomic DNA of strain A1552-GFP [36] as template. The upstream primer had a 5′ phosphorylated and non-priming overhang containing the T7 RNA polymerase dependent promoter sequence (as indicated in Fig. 1). The fragment was digested with *Bam*HI and cloned into *Eco*RV/*Bam*HI double-digested vector pBR-Tet_MCSI [7]. Correct ligation was validated by colony PCR and sequencing.

Construction of *Vibrio cholerae* strains

V. cholerae strains carrying the gene encoding T7 RNA polymerase on the chromosome were created by triparental mating between the respective *V. cholerae* strain (Table 1), *E. coli* strain S17λpir/pUX-BF13 [37], and *E. coli* strain S17λpir/pGP704-mTn7-T7RNAP. The latter suicide plasmid consists of vector pGP704 as backbone and the mini-Tn7 transposon [38] containing the gene cluster *lacI*-P_{lacUV}-T7RNAP as cargo. Mini-Tn7 transposons (mTn7) insert site-specifically and at a neutral site in Gram-negative bacteria including *V. cholerae* [38,39].

The T7 RNA polymerase-dependent promoter sequence was site-specifically integrated into the chromosome using natural transformation and FLP recombination (TransFLP method; [3,4]). Oligonucleotides for the design of the transforming PCR fragment are indicated in Table S1. The *ctx* operon was likewise deleted (using PCR fragment ΔctxAB-FRT-Kan-FRT; primer indicated in Table S1).

Quantitative reverse transcription PCR (qRT-PCR)

V. cholerae strains were grown for several hours in LB containing 1mM IPTG until they reached an optical density at 600 nm of ~2. IPTG-induced strain AΔctxAB-[P_{T7}]-tcp-T7RNAP was harvested at the same time though the OD_{600} values varied due to extensive clumping of the cells (autoagglutination as described in the result section). Harvesting of cells, RNA preparation, reverse transcription and qPCR were performed as previously described [7]. Expression levels were normalized to the housekeeping gene *gyrA*. Primers used for qRT-PCR are listed in Table S1.

Epifluorescence microscopy

Visualization of bacterial cells at the microscopic level was done using a Zeiss Axio Imager M2 epifluorescence microscope. Image acquisition was performed using the Zeiss AxioVision software steering a high-resolution camera (AxioCam MRm). Images were rotated, cropped and magnified using Zeiss AxioVision and ImageJ.

Scanning electron microscopy (SEM)

Bacterial cells were grown as described. At the time of harvest, cells were spotted onto a silicon wafer. Attachment was allowed to occur for several minutes before the cells were fixed for 1 hour with 1.25% glutaraldehyde and 1% tannic acid in 0.1 M phosphate buffer, pH 7.4. Cells were washed in phosphate buffered saline and then further fixed for 30 minutes in 1.0% osmium tetroxide in the same buffer. The bacteria, attached to the wafers, were dehydrated in a graded alcohol series and then dried by passing through the supercritical point of carbon dioxide (Leica Microsystems CPD300). The samples were coated with a 2 nm layer of osmium using an osmium plasma coater (Filgen OPC60). Images of the bacteria were taken with a field emission scanning electron microscope (Merlin, Zeiss NTS) using an acceleration voltage of 2 kV and the in-lens secondary electron detector.

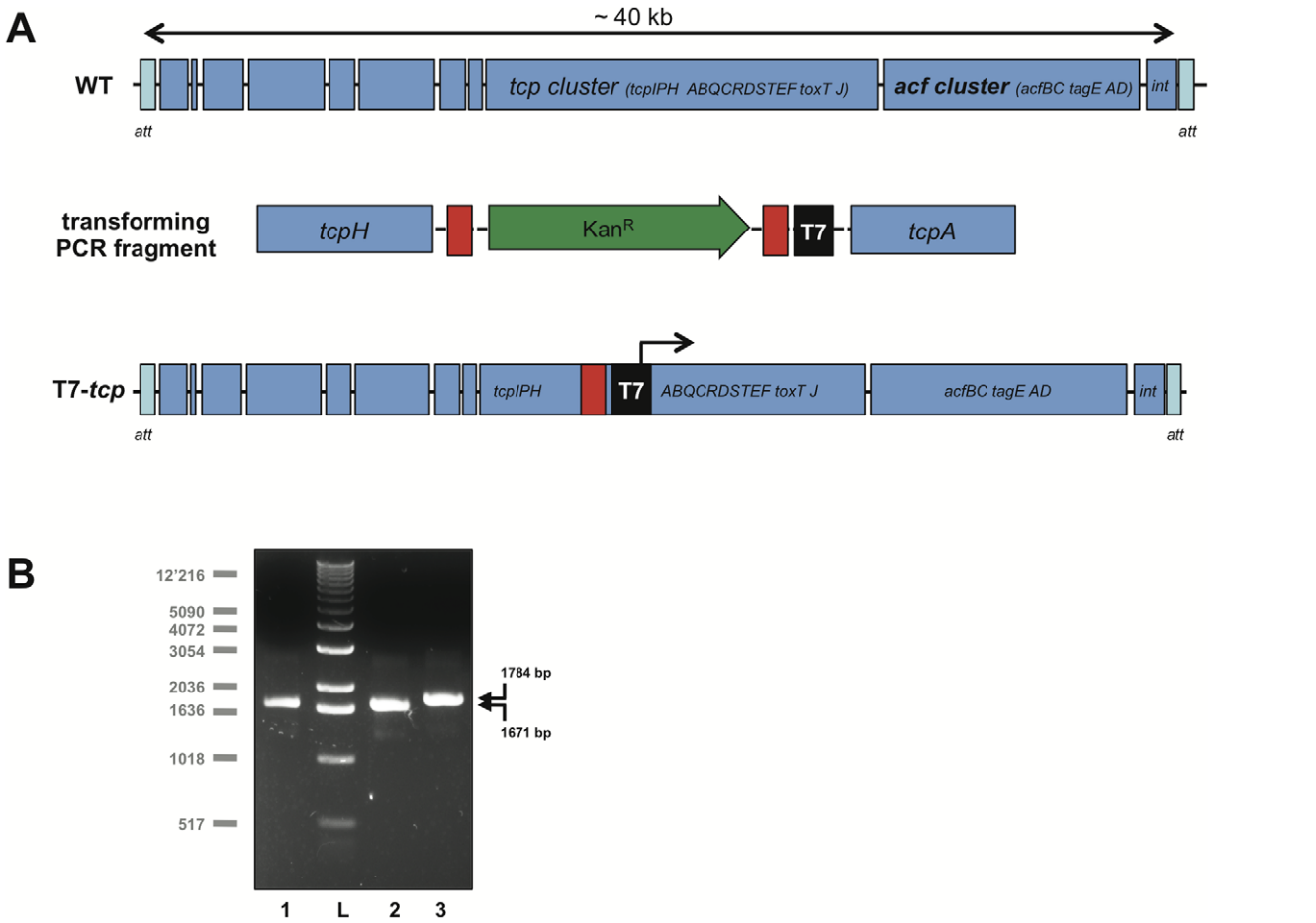

Figure 4. Insertion of the T7 RNA polymerase-dependent promoter sequence by TransFLP. A: Schematic representation depicting the strategy to integrate the T7 RNA polymerase-dependent promoter sequence into the *V. cholerae* chromosome. Upper row: the *Vibrio* pathogenicity island (VPI-1 or *tcp* island) is indicated for the WT strain of *V. cholerae* (freely adapted from [46] and based on [47]; not to scale). Middle row: the transforming PCR-derived DNA fragment included parts of the genes *tcpH* and *tcpA* as flanking regions (in blue) to allow homologous recombination with the chromosome. In addition the PCR fragment carried the FRT-site (red rectangles) flanked kanamycin resistant cassette (*aph*; green arrow) and the T7 RNA polymerase-dependent promoter sequence (black box; according to Fig. 1). Lower row: the structure of the VPI-1 island after natural transformation and FLP recombination of the WT strain using the PCR fragment indicated in the middle row as transforming DNA material. **B: PCR-based verification of site-directed insertion.** The T7 RNA polymerase-dependent promoter sequence was inserted into the *V. cholerae* genome by chitin-induced natural transformation followed by FLP-mediated excision of the antibiotics resistance cassette (TransFLP; [3,4]). The correctness of the resulting strain was tested by PCR using primer pair T7tcp-chk-up & T7tcp-chk-down and genomic DNA as template. The expected fragment sizes for the wild type (A1552; lane 1) and the *ctx* minus parental strain (AΔctxAB-T7RNAP; lane 2) (both 1′671 bp in length) as well as for the newly created T7 RNA polymerase-dependent promoter-containing strain AΔctxAB-[PT7]-tcp-T7RNAP (lane 3; 1′784 bp) are indicated by arrows. L, 1 kb ladder (Invitrogen; sizes indicated on the left).
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Results and Discussion

The aim of this study was to genetically engineer *V. cholerae* strains so that large gene clusters could be artificially expressed *in vitro*. Consequently, the experimental design was as follows (Fig. 1): First, we integrated the T7 RNA polymerase encoding gene (*T7 gene 1*) preceded by the lacUV5 promoter and the Lac repressor gene (*lacI*) into the *V. cholerae* chromosome. The T7 specific promoter sequence was delivered on a plasmid (control) as well as upstream the *tcp* operon. The latter was accomplished using chitin-induced natural transformation followed by FLP recombination. Expression of *tcp* and expected phenotypes were confirmed.

Integration of T7 RNA polymerase encoding gene into the *V. cholerae* chromosome

As the T7 RNA polymerase has a very stringent specificity towards the T7-specific promoter sequences [40] we decided to integrate the encoding gene (T7 *gene 1*; [13]) into the *V. cholerae* genome (Fig. 1). To do so we amplified the T7 *gene 1* preceded by the lacUV5 promoter and the lac repressor gene *lacI* from the λ(DE3) prophage contained in the lysogenic *E. coli* strain BL21(DE3) [12] using PCR and primers LacI-before and T7 RNA pol after (Table S1). After restriction enzyme digestion (see Material and Methods) the PCR fragment was ligated into the likewise digested miniTn7 transposon [37] harbored on plasmid pGP704::Tn7 [41]. After triparental mating between the *E. coli* strains S17-1λpir/pGP704-mTn7-T7RNAP (carrying the construct-containing transposon), S17-1λpir/pUX-BF13 (providing the Tn7 transposition *in trans* [37]), and *V. cholerae* wild type strain

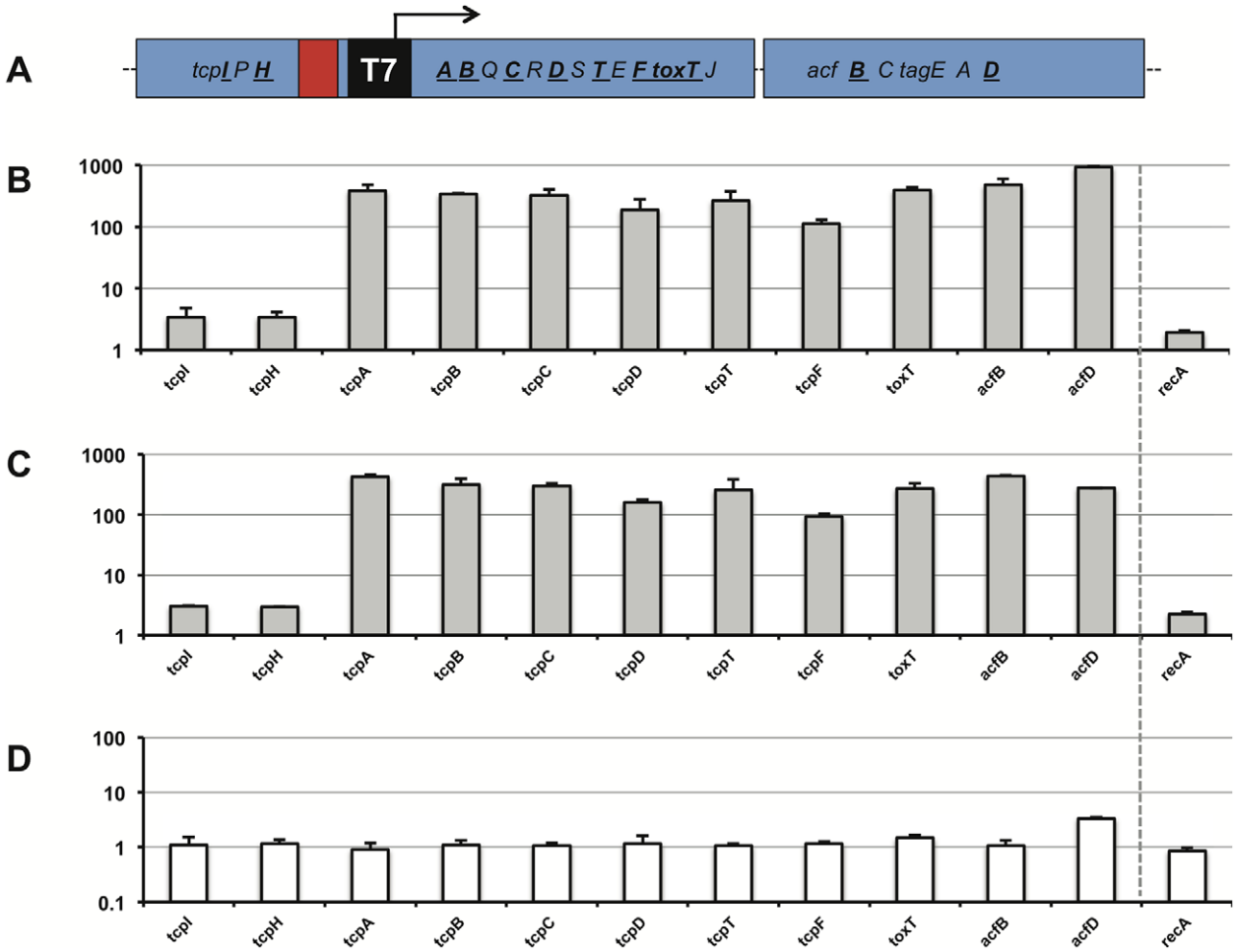

Figure 5. Expression of VPI-1 genes in a T7 RNA polymerase-dependent manner. Expression of genes upstream and downstream the T7 RNA polymerase dependent promoter sequence was tested using qRT-PCR. **A: Schematic representation of the genomic region of interest in the final strain AΔctxAB-[P_{T7}]-tcp-T7RNAP.** Genes whose expression was tested by qRT-PCR in panels B–C are indicated in bold and underlined. The P_{T7} promoter is indicated as a black rectangle with a black arrow on top. **B–D: Comparison of gene expression between the engineered *V. cholerae* strain and its parental strains.** Gene expression was measured in the engineered *V. cholerae* strain containing both the T7 RNA polymerase gene and the P_{T7} promoter sequence and compared to its two parental strains each containing only one of both elements. Bacteria were grown in rich medium in the presence of 1 mM IPTG. Genes indicated on the X-axis were tested for their expression level using qRT-PCR (normalized to the housekeeping gene *gyrA*). The values shown on the Y-axis depict the relative expression difference between strains AΔctxAB-[P_{T7}]-tcp-T7RNAP ([P_{T7}]$^+$, T7RNAP$^+$) and AΔctxAB-[P_{T7}]-tcp ([P_{T7}]$^+$, T7RNAP$^-$)(panel B), between strains AΔctxAB-[P_{T7}]-tcp-T7RNAP ([P_{T7}]$^+$, T7RNAP$^+$) and AΔctxAB-T7RNAP ([P_{T7}]$^-$, T7RNAP$^+$)(panel C), and between the two control strains AΔctxAB-T7RNAP ([P_{T7}]$^-$, T7RNAP$^+$) and AΔctxAB-[P_{T7}]-tcp ([P_{T7}]$^+$, T7RNAP$^-$) (panel C), respectively. Relative expression levels of *recA* are shown for comparison reason. Average of two independent biological replicates.
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A1552 [42] the resulting *V. cholerae* strain AT7RNAP was selected on gentamicin-containing TCBS agar plates. Single colonies were isolated, grown on LB agar plates, and verified by colony PCR for the correct and site-directed integration of the transposon (data not shown).

Functionality of T7 RNA polymerase in *V. cholerae*

In order to test whether the T7 RNA polymerase gene was functional in *V. cholerae* a transcriptional reporter gene was cloned onto a plasmid (Table 1). To accomplish this, the green florescent protein-encoding gene (*gfp*) was PCR-amplified from *V. cholerae* strain A1552-GFP [36]. Concomitantly with this amplification step we added the T7 promoter sequence "GAAATTAATAC-GACTCACTATAGGGAGA" (Fig. 1) upstream the *gfp* coding region by incorporating this sequence as 5′-overhang into the forward primer (Table S1). The respective P_{T7}-*gfp* fragment was ligated in the promoterless plasmid pBR-Tet_MCSI [7] yielding plasmid pBR-[P_{T7}]-gfp (Table 1). The functionality of this plasmid was first tested in the well-characterized *E. coli* strain BL21(DE3) [12](Fig. 2) as this strain has been extensively used over the years for T7 RNA polymerase dependent high-level expression of proteins. The respective *E. coli* transformant was grown in rich medium either in the absence or presence of the inducer IPTG and T7 RNA polymerase-dependent expression was visualized using epifluorescence microscopy (Fig. 2). Though low-level expression of *gfp* was also observed under uninduced growth conditions (as discussed below), the expression was significantly enhanced upon provision of the inducer. Thus, the functionality and usefulness of the reporter plasmid was established.

Next, the reporter plasmid was transferred in two different *V. cholerae* strains: the wild type strain A1552 and the newly constructed strains AT7RNAP (T7RNAP$^+$) (Fig. 3). Similar to the experiment described above for *E. coli*, we grew both transformants in rich medium without or with IPTG as the

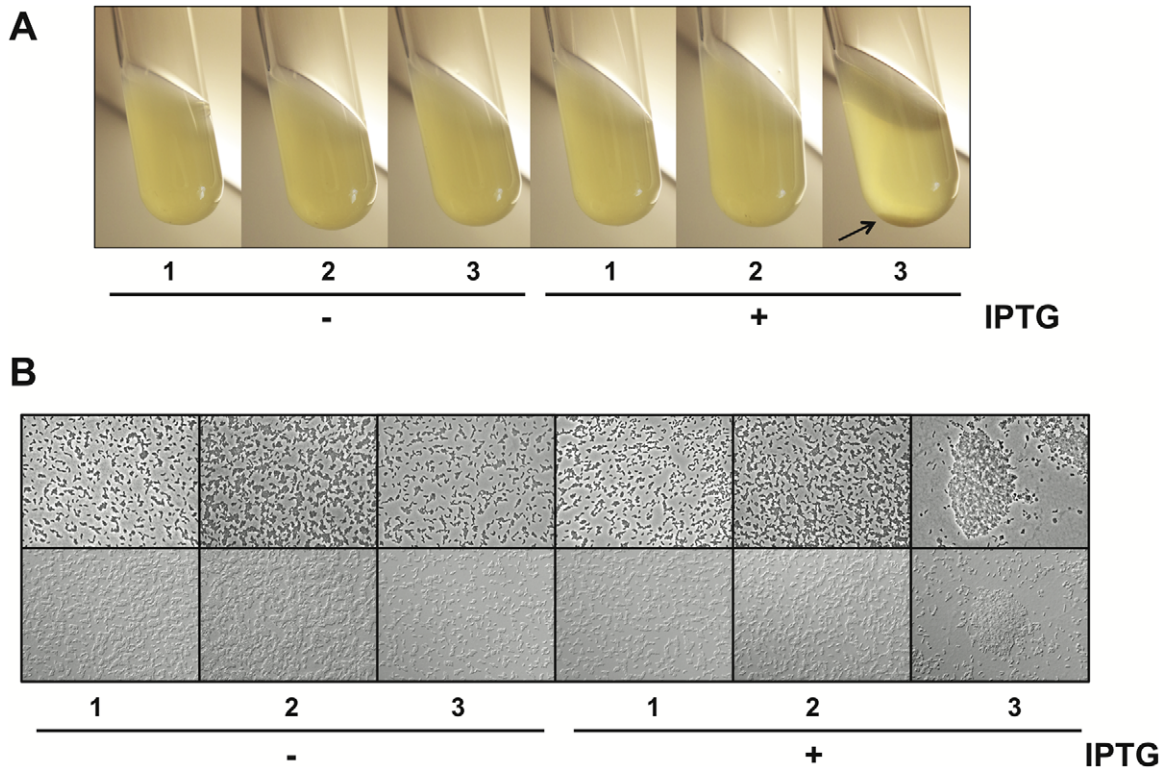

Figure 6. Phenotypes of T7 RNA polymerase-mediated expression of the *tcp* cluster. The *V. cholerae* strains AΔctxAB-[P_{T7}]-tcp (lane 1), AΔctxAB-T7RNAP (lane 2), and AΔctxAB-[P_{T7}]-tcp-T7RNAP (lane 3) were grown under shaking conditions in LB medium without or with supplementation of 1 mM IPTG. **A: Macroscopic observation of an autoagglutination phenotype.** Bacterial cultures were allowed to settle before pictures were taken. Agglutinated bacteria are indicated by a black arrow in the rightmost image. **B: Microscopic observation of a microcolony formation phenotype.** Microcolony formation of the cells was visualized using light microscope. Pictures were taken using phase contrast (upper panel) or DIC (lower panel).
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inducer of T7 RNA polymerase production. Whereas no green fluorescent signal was detectable under both conditions in the *V. cholerae* strain lacking T7 RNA polymerase (Fig. 3, left), *gfp* expression was unambiguously detectable in strain AT7RNAP and significantly enhanced in the presence of the inducer IPTG (Fig. 3, right). We conclude that the T7 RNA polymerase was functional in *V. cholerae* and that T7 RNA polymerase specific promoter sequences were not unspecifically transcribed (or at least not at high levels) in *V. cholerae* strains lacking T7 RNA polymerase. Thus, the system seemed feasible for induction of large gene clusters in a T7 RNA polymerase-dependent manner.

Integration of the T7 RNA polymerase specific promoter sequence upstream *tcpA* using the TransFLP method

As described above and as a proof-of-principle we were interested in artificially expressing the *tcp* cluster *in vitro* under non-virulence inducing conditions. As it has previously been shown that induced expression of *toxT* led to enhanced TcpA and cholera toxin (CT) production [29] and as *toxT* is embedded within the *tcp* cluster (Fig. 4) we decided to first attenuate the newly constructed *V. cholerae* strain (AT7RNAP) by deleting the *ctxAB* operon using chitin-induced TransFLP (data not shown) [2–4]. The resulting strain was named AΔctxAB-T7RNAP (Table 1). Next, to create an artificial induction system for the *tcp* gene cluster we needed to integrate the T7 RNA polymerase specific promoter sequence (Fig. 1) upstream the first gene whose gene product is directly involved in the toxin-coregulated pilus biosynthesis, namely *tcpA* (Fig. 4A). We did so by creating a PCR fragment consisting of the following features (Fig. 4A, middle row): 1) an FRT-site flanked antibiotic resistance cassette (here *aph* adding kanamycin resistance to the cells); 2) flanking regions at both ends spanning *tcpH* and *tcpA* to allow double-homologous recombination; 3) the specific T7 RNA polymerase promoter sequence (black box stating T7 in Fig. 4A). This PCR fragment was obtained in two rounds of PCR as described earlier [3] and used as transforming DNA in a chitin-induced transformation assay [2]. The acceptor strain in this assay was AΔctxAB-T7RNAP ($T7RNAP^+$). Transformants were selected on kanamycin-containing agar plates followed by the excision of the Kan^R cassette using FLP recombination as previously described [3]. The resulting *V. cholerae* strain, AΔctxAB-[P_{T7}]-tcp-T7RNAP, was verified for the integration of the T7 RNA polymerase specific promoter sequence by PCR using purified genomic DNA as template (Fig. 4B).

T7 RNA polymerase-dependent expression of *tcp* genes under non-virulence inducing conditions

The newly created strain AΔctxAB-[PT7]-tcp-T7RNAP ($[P_{T7}]^+$, $T7RNAP^+$) as well as the parental strains lacking either the T7 *gene 1* (AΔctxAB-[P_{T7}]-tcp) or lacking the T7 specific promoter sequence (AΔctxAB-T7RNAP) were grown under non-virulence inducing conditions. After harvesting the cells, RNA was extracted and qRT-PCR was performed (Fig. 5). The expression of diverse VPI-1 genes located either upstream (*tcpI* and *tcpH*) of the T7 specific promoter sequence or downstream of the latter (*tcpA,C,C,D,T,F, toxT, acfB,D*; Fig. 5) was determined. As depicted in Fig. 5 all genes located downstream of P_{T7} were at least 100-fold

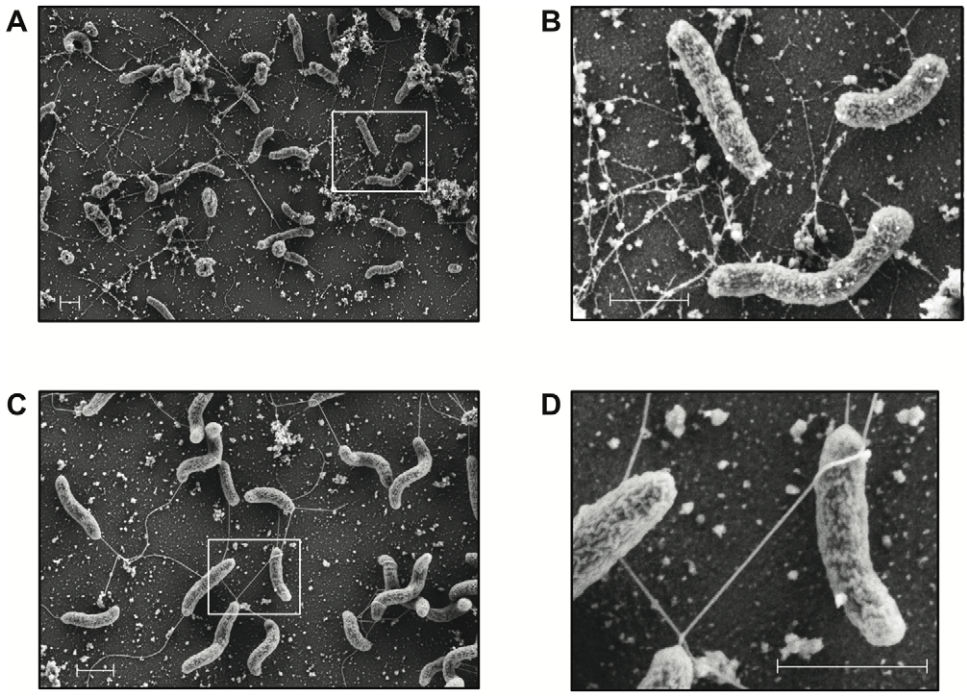

Figure 7. Visualization of TCP fibers by scanning electron microscopy. *V. cholerae* cells were grown in rich medium as described for Fig. 6. At that stage bacteria were transferred to silicon wafers and processed for SEM. A representative image of *V. cholerae* strain AΔctxAB-[P_{T7}]-tcp-T7RNAP containing both the T7 RNA polymerase gene and P_{T7} promoter preceding the *tcp* cluster is shown in panel A (EHT = 2.00 kV, WD = 3.7 mm, Mag = 13.48 k X). The control strain lacking the P_{T7} promoter sequence upstream the *tcp* cluster is shown in panel C (EHT = 2.00 kV, WD = 3.8 mm, Mag = 24.91 k X). The white rectangles in panel A and C indicate the regions that are magnified in panels B and D, respectively. Scale bar = 1 μm.
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induced compared to either parental strain (Fig. 5B and C). In contrast the expression of genes located upstream of P_{T7} were similar to the expression measured for the parental strains and in the range of the expression that was also observed for a house-keeping gene (*recA*). Importantly, neither the T7 RNA polymerase nor the P_{T7} promoter alone was sufficient to induce *tcp* expression (Fig. 5D) in contrast to the strain containing both elements (Fig. 5B, C). We conclude that the T7 RNA polymerase/promoter system is able to drive *tcp* expression in *V. cholerae*.

Phenotypes associated with T7 RNA polymerase-mediated *tcp* expression in *V. cholerae*

Taylor *et al.* provided evidence that *in vitro* induction of TCP (under virulence-inducing conditions of *V. cholerae* biotype classical) resulted in autoagglutination of the bacteria [19]. Such auto-agglutination led to the formation of macroscopic bacterial clumps and "large aggregates that collect rapidly at the bottom of the culture tube" [30]. Closer inspection of such clumps by SEM suggested that they are reminiscent of microcolonies formed upon intestinal colonization [30]. Furthermore, the authors of this study concluded that "the ability of TCP mutants to participate in microcolonies correlates with the ability of those strains to colonize the infant mouse" [30]. Consequently, we checked for auto-agglutination of *V. cholerae* as an *in vitro* phenotype after P_{T7}-driven expression of the *tcp* cluster (Fig. 6A). Indeed, autoagglutination was confirmed and fully dependent on the presence of the T7 RNA polymerase and the T7 RNA polymerase specific promoter sequence (Fig. 6A). In contrast, the parental strains harboring only one of those elements did not autoagglutinate. Furthermore, no cellular clumps were visible in the absence of autoinducer (-IPTG) even though we provided evidence that low expression also occured under those conditions (Fig. 3). This is consistent with the insufficient expression of *tcp* upon artificial *toxT* expression as discussed above [29].

As agglutinated bacteria are thought to resemble microcolonies formed *in vivo* [30] the bacterial cultures were observed using light microscopy (Fig. 6B). Whereas the bacteria were uniformly distributed in those cultures that also appeared homogenously at the macroscopic scale (Fig. 6A) the bacterial aggregates of strain AΔctxAB-[P_{T7}]-tcp-T7RNAP grown in the presence of inducer were reminiscent of microcolonies (Fig. 6B) and very similar in appearance to previously published studies [32].

For visualization of the TCP pili using SEM (Fig. 7), the bacterial cells were grown as described above and as illustrated in Fig. 6. Subsequently, the bacteria were allowed to attach to a silicon wafer before being processed for SEM. It should be noted that the macroscopic clumps observed in Fig. 6A were partly flushed from the surface upon immersion of the silicon wafer into the fixative. This is consistent with the TCP pilus being involved in cell-to-cell interactions more so than for attachment to the silicon wafer. Nevertheless, enough bacteria were retained on the surface to allow visualization the TCP fibers (Fig. 7). Indeed, a plethora of TCP fibers were visible in the T7RNAP$^+$ and P_{T7}-carrying strain (Fig. 7A+B), whereas no such fibers were visible in a strain lacking the P_{T7} promoter upstream *tcpA* (Fig. 7C+D). Interestingly, Jude and Taylor have recently demonstrated that *V. cholerae* O1 El Tor derived TCP pili are thinner in width than classical TCP pili [32], which is in accordance with the thin TCP fibers we observed in our SEM images (Fig. 7A+B).

Conclusions

In conclusion we successfully used the T7 RNA polymerase/promoter system in *V. cholerae*. To our knowledge this is the first time that this system was utilized in this organism though it was already suggested in earlier studies that "comparable T7 expression systems [to those in *E. coli*] can be developed in other types of cell" [12]. The advantage of this system is that the T7 RNA polymerase is highly selective for transcription from own promoters. A disadvantage that we encountered here and which was already reported earlier is that the lacUV5 promoter is not completely tight and thus leads to low expression of the T7 RNA polymerase even in the absence of inducer [12]. However, this problem has been addressed by others and several options to either tighten the promoter (e.g. using Lac repressor variants and/or production of increased levels of the repressor) or to reduce basal activity of undesired T7 RNA polymerase [43] have been suggested. As a next step we will exchange the lacUV5 promoter preceeding the T7 *gene 1* for the arabinose-inducible P_{BAD} promoter [44], which we have used with great success in earlier studies [7]. Judson and Mekalanos also used the P_{BAD} promoter in *V. cholerae* [45]. In their study P_{BAD} was outward-facing from a mariner-based transposon and as such used to transcriptionally fuse the promoter to different neighboring genes within the transposon library [45]. Here, we did not use random transposon insertion but instead we demonstrated target-specific gene expression as the T7-specific promoter was integrated by natural transformation and thus in a site-directed manner.

The data provided here demonstrate that the strategy depicted in Fig. 1 is functional. Thus, the combination of T7 RNA polymerase and the integration of the T7 RNA polymerase specific promoter sequence using natural transformation and FLP recombination (TransFLP) seems to be a promising tool to artificially induce gene clusters in *V. cholerae*.

Supporting Information

Table S1 Primers used in this study.
(DOCX)

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Author Contributions

Conceived and designed the experiments: MB. Performed the experiments: SB MB. Analyzed the data: MB. Contributed reagents/materials/analysis tools: MB. Wrote the paper: MB.

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