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Long-range transcriptional interference in *E. coli* used to construct a dual positive selection system for genetic switches

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ABSTRACT

We have investigated transcriptional interference between convergent genes in *E. coli* and demonstrate substantial interference for inter-promoter distances of as far as 3 kb. Interference can be elicited by both strong σ^{70} dependent and T7 promoters. In the presented design, a strong promoter driving gene expression of a 'forward' gene interferes with the expression of a 'reverse' gene by a weak promoter. This arrangement allows inversely correlated gene expression without requiring further regulatory components. Thus, modulation of the activity of the strong promoter alters expression of both the forward and the reverse gene. We used this design to develop a dual selection system for conditional operator site binding, allowing positive selection both for binding and for non-binding to DNA. This study demonstrates the utility of this novel system using the Lac repressor as a model protein for conditional DNA binding, and spectinomycin and chloramphenicol resistance genes as positive selection markers in liquid culture. Randomized LacI libraries were created and subjected to subsequent dual selection, but mispairing IPTG and selection cues in respect to the wild-type LacI response, allowing the isolation of a LacI variant with a reversed IPTG response within three rounds of library generation and dual selection.

INTRODUCTION

Transcriptional interference (TI) has been defined as the *cis* active suppressive influence of one transcriptional process on a second one (1,2). This mechanism has been exploited in a genetic selection system for DNA binding in a number of studies (3–6). In this system, a weak promoter is driving the expression of the antibiotic resistance gene *aadA*. An opposing strong promoter interferes with transcription of the *aadA* gene. Upon occupation of an operator site at the strong promoter, the inhibition of transcription from the weak promoter is alleviated, thus leading to expression of the selectable resistance marker. Within this approach, the interfering promoter is directly adjacent to the suppressed gene.

We investigated whether TI can also be employed in engineered systems with two full-length genes facing each other, which would make it a versatile set-up for artificial genetic circuitry requiring inversely correlated gene expression. Using two converging fluorescent protein reporter genes, we demonstrate that TI can be very efficient even in this two gene layout, creating an inter-promoter distance of 1.5 kb. The introduction of spacers between the genes to further increase the inter-promoter distance to up to 3 kb decreased, but not completely abolished TI. Further, we show that long-range TI can also be elicited by a T7 RNA polymerase dependent promoter in the presence of functional T7 RNA polymerase.

Based on long-range TI, we have developed a transcriptional interference assay (TIA) that allows positive selection for both the DNA binding (ON) and for the non-binding (OFF) state. In synthetic biology the development and engineering of gene switches is crucial for constructing genetic circuits that allow sensing of environmental or internal cues. Predictive structural design of gene switches like conditionally active transcription factors is an exceptionally complex problem and instead, selections or high-throughput screenings from libraries are commonly used. Employed selection schemes must include selection for an ON and an OFF state, with the state being switched by an external cue. Ideally, the genetic selection system should enable selection for both states and avoid laborious and diversity decreasing subcloning between an ON and an OFF selection system. In recent years, several dual selection systems have been developed that rely on a combination of positive and negative selection (7–13). In contrast to positive selection markers, negative selection markers are detrimental when expressed at respective selective conditions and thus select for absence of the marker. Negative selections can be very effective, but

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harbor an inherent risk of enrichment for loss-of-function mutants.

To construct a genetic selection system, which allows positive dual selection, instead of fluorescent protein genes, positive selection markers were cloned into the TIA system. The chloramphenicol resistance marker *cat* driven by a strong, conditionally active promoter was cloned opposite to an *aadA* gene. This design enables positive selection for both the occupied and the unoccupied state of the operator region of the interfering promoter, as shown with the Lac repressor (LacI) as a model protein for conditional DNA binding. The feasibility of selections from large libraries in liquid culture with this system was demonstrated by functionally inverting the Lac repressor in a directed evolution approach.

The selection system described in this work makes use of a novel two gene TI approach that can be generalized for selection and screening of conditional DNA binders or genetic circuits whose output is a DNA binding protein or an orthogonal polymerase activity. Because of its nature as a dual positive selector for antibiotic resistances, the presented transcriptional interference assay (TIA) places very few restrictions on medium composition and is genetically and functionally robust.

MATERIALS AND METHODS

Plasmids

A construct of opposing genes coding for BFP (blue fluorescent protein) and mCherry was cloned into pSB4S5 (14) using EcoRI and PstI. BFP was obtained from pTagBFP-C (Evrogen) and mCherry (15) was obtained from a BioBrick. The mCherry gene had the weak promoter *bla P3* (16). To counteract low transcriptional efficiency a strong ribosomal binding site was used, which was designed with the Salis lab RBS calculator (17,18). The promotor of the BFP gene was exchangeable with EcoRI and XbaI. For inserted promoter sequences see Supplementary Table S2.

Variable spacer lengths between the BFP and the mCherry gene were created in 500 bp increments. First, a 'landing site' with an AgeI and a HindIII site was inserted between the genes. Spacer fragments were amplified from the *cat* gene from a pAK100 derived vector (19), with the forward primer adding an XmaI site (5′-ATGCC CGGGG AGAAA AAAAT CACTG GATAT ACCAC-3′) and the reverse primer an AgeI and a HindIII site (5′-TAGAA GCTTC TGACC GGTAA GTTGT CCATA TTGGC CAC-3′). The backbone with the landing site was digested with AgeI and HindIII, the amplified spacer segment with XmaI and HindIII, and the resulting fragments ligated. AgeI and XmaI are isocaudamers, producing compatible overhangs. The corresponding ligation results in a cloning scar not recognized by either of the two enzymes. Thus, the obtained plasmid again had unique AgeI and HindIII sites used for further rounds of spacer segment insertion.

T7 RNA polymerase (T7 RNAP) was amplified from the BioBrick BBa_K145001 (14), using the forward primer T7RNAP-fw-XbaI (5′-GATTC TAGAA TGAAC ACGAT TAACA TCGCT AAG-3′). The reverse primer was either T7RNAP-S-rv-PstI (5′-TCTCT GCAGT TATTA CGCGA ACGCG AAGTC-3′), introducing two stop codons directly behind the coding sequence and yielding an active T7 RNAP, or T7RNAP-rv-PstI (5′-TCTCT GCAGC GCGAA CGCGA AGTC-3′), yielding a T7 RNAP with a C-terminal, plasmid encoded 6xHis-tag disrupting its function. Each amplicon was cloned into pBAD-Kan with XbaI and PstI.

pSAH0016 was constructed by cloning the LacI selection cassette into pSB4K5 (14) using EcoRI and PstI. The backbone resistance *npt*II was then removed by PCR of the remaining plasmid using the primers pSB4X5-fw (5′-GTTAC ATTGT CGATC TGTTC ATGGT GAACA-3′) and pSB4X5-rv (5′-ACTAG CAGAA ATCAT CCTTA GCGAA AGCTA AGG-3′) and subsequent blunt end self-ligation. The *aadA* gene was cloned from a pSB4S5 (14) vector, the *cat* gene was obtained from a derivative of pAK100 (19). As *lacO* site, a symmetric, high affinity operator (20) was used.

For construction of pBAD-LacI the *lacI* coding region was amplified from a pAK100 derived vector with the primers LacI-fw-XbaI (5′-ATCTC TAGAA TGAAA CCAGT AACGT TATAC GATG-3′) and LacI-rv-PstI (5′-GATCT GCAGC TGCCC GCTTT CCAG-3′). Using XbaI and PstI, it was cloned into pBAD-Kan, a derivative of pBAD/His (Life Technologies) with a kanamycin resistance cassette instead of an ampicillin resistance.

To discriminate the genotypes used in the mock selection experiments, an mCherry based reporter for *lacO* binding was introduced into the pBAD derived expression plasmid by Gibson assembly, replacing the *rrnB* terminator region. The resulting pBAD derivative was named pLO2. The reporter is flanked by BioBrick terminators BBa_B1006 and BBa_B1002 for transcriptional isolation.

The sequences of all reporter cassettes are given in the supplementary material.

Strains

Cloning and experiments to demonstrate long-range TI with fluorescent proteins were done in *E. coli* strain XL1 blue (Stratagene), unless noted otherwise. LacI selection experiments and protein expressions were done in the *lacI* deficient strain RV308 (*lacI*$^{q-}$, su-, *ΔlacX74*, *gal*, IS II::OP308, *strA*) (21).

Fluorescent TIA

XL1 blue cells transformed with the respective operatorless fluorescent reporter plasmid were picked into a U-bottom 96-well plate with lid with 200 μl DYT (16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl) with 100 μg/ml spectinomycin per well and incubated for 16 h at 37°C under orbital shaking.

Following the growth period of 16 hours, the cells were pelleted by centrifugation of the plate at 3200g for 5 min. The supernatants were removed and the pellets were resuspended in 200 μl PBS each. Both BFP and mCherry fluorescence were directly measured in the cell suspensions using the multimode plate reader Infinite M1000 PRO (Tecan). For BFP detection an excitation wavelength of 402 nm and an emission wavelength of 457 nm was used, for detection of

mCherry fluorescence 587 and 610 nm, respectively. Values were blank subtracted and divided by the measured optical density at 600 nm for normalization.

Fluorescent T7 TIAs were done with RV308 *E. coli* co-transformed with the T7 reporter pSAH0112 and a T7 RNA polymerase gene in pBAD-Kan coding for either an active (T7 RNAP+) or an inactive (T7 RNAP-) polymerase. Here, cells were picked into DYT with 50 μg/ml kanamycin and 0.00002% arabinose per well and incubated for 16 h at 32°C under shaking. The further protocol was the same for all fluorescent reporter assays.

TIA with antibiotic resistance genes

For LacI based assays, RV308 cells co-transformed with expression and reporter plasmid were grown overnight at 32°C in DYT containing 25 μg/ml chloramphenicol and 50 μg/ml kanamycin. Cultures were diluted 1000-fold in DYT with 50 μg/ml kanamycin, 0.02% arabinose and either no or 1 mM IPTG.

Diluted cell suspensions were transferred to a 96-well F-bottom microplate (200 μl per well) with lid and grown for 3 h at 32°C in an Infinite M1000 PRO (Tecan) under orbital shaking. Afterwards selective antibiotic was added (spectinomycin or chloramphenicol) from 100× stock solutions. Cultures were grown for another 3 h in the microplate reader while recording the optical density at 600 nm every 5 min. Examples for recorded growth curves are given in Supplementary Figure S3. For calculation of doubling rates, the recorded growth curves were blank subtracted, $\log_2$ transformed, and the slopes of the simple linear regressions in the exponential growth phase (30–90 min) were determined.

From the doubling rates for different antibiotic concentrations x, EC50 values were calculated by fitting the doubling rates to $Y(x) = Y0 + a/\left(1 + e^{-\frac{x-EC50}{b}}\right)$ constraining a to a maximum of 1.7.

Expression of lac repressor

RV308 cells were transformed with pBAD-Kan containing the desired *lacI* coding sequence. An overnight culture in DYT with 50 μg/ml kanamycin was used to inoculate 750 ml DYT containing 50 μg/ml kanamycin. This culture was incubated for 2 h at 32°C and 200 rpm, cooled down to 16°C and then induced by the addition of arabinose to a final concentration of 0.2%. After an expression time of 16 h at 16°C, cells were pelleted at 6000g and the pellet frozen at −80°C. The pellet was resuspended in 20 ml of 50 mM sodium phosphate buffer at pH 8.0 containing 500 mM NaCl, 20 mM imidazole, 2.5% glycerol, 1 mM DTT, 10 mM $MgCl_2$, 0.1% tween 20, 20 mg lysozyme and a tablet of cOmplete protease inhibitor cocktail, EDTA-free (Roche). Cells were lysed by sonication, then 1000 U DNAse I were added. After 30 min of incubation on ice the suspension was cleared by centrifugation at 48 000g for 1 h at 4°C. The supernatant was passed through a 0.22 μm PVDF filter and loaded onto a 1 ml Ni-NTA column pre-equilibrated with 50 mM sodium phosphate buffer pH 8.0 containing 500 mM NaCl, 20 mM imidazole, 2.5% glycerol and 1 mM DTT. The column was washed with equilibration buffer and the bound protein eluted with 20 mM sodium phosphate buffer pH 7.4 containing 300 mM NaCl and 200 mM imidazole. The protein containing fractions were dialyzed overnight into 200 mM potassium phosphate buffer pH 7.6 containing 5% glucose and 1 mM DTT.

Fluorescence polarization assay

For an *in vitro* characterization of the wild-type LacI and 5B3His, fluorescence polarization assays were performed with purified and dialyzed Lac repressor. Assay buffer was dialysis buffer supplemented with 5 ng/μl poly(dI-dC) (Affymetrix) and 10 nM double-stranded symmetrical Lac operator (5′-ATTGT GAGCG CTCAC AAT-3′) labeled with fluorescein on both 5′ ends (Sigma Aldrich). Measurements were performed in black half-area 96-well microtiter plates (50 μl per well) using the multimode plate reader Infinite M1000 PRO (Tecan) at 470 nm excitation and 550 nm emission. IPTG response curves were measured at the lowest protein concentration at which the lower affine state (with IPTG for wild-type LacI, without IPTG for LacI mutant 5B3His) still caused a detectable polarization increase, which was 0.1 μM for wild-type LacI and 1 μM for 5B3His. IPTG was added in concentrations spanning 10 mM to 1.22 μM in a $\log_2$ dilution series.

Mock selections

Plasmid pLO2-wt, -a2 or -a3 were each co-transformed with the reporter plasmid into RV308 *E. coli*. Each of the three expression plasmids contains an additional mCherry based *lacO* reporter for easy determination of genotype frequencies. All were grown separately over night at 32°C in DYT containing 25 μg/ml chloramphenicol and 50 μg/ml kanamycin and mixed in a 1:1:1 ratio for the first selection step. Mixtures of transformants were used to inoculate cultures of 5 ml DYT containing 50 μg/ml kanamycin, 0.02% arabinose, 5 μg/ml chloramphenicol and either no or 1 mM IPTG in congruence with the selection conditions used afterwards. After 3 h of incubation at 32°C, 500 μl of these cultures were transferred to 100 ml of fresh pre-warmed medium with the same composition but additional selection antibiotic, which was 250 μg/ml spectinomycin in the absence of IPTG (ON-selection) or 500 μg/ml chloramphenicol in the presence of IPTG (OFF-selection). Those selection cultures were then incubated for further 8 h at 32°C, monitoring the optical density and transferring cultures to fresh selection medium to maintain cultures below an OD_{600} of 0.4. After selection, glycerol stocks were prepared from the cultures. The second selection step, switching from ON- to OFF-selection and vice versa, was performed accordingly, with the difference that the 3 h pre-selection cultures were inoculated from glycerol stocks of the first selection step instead of from overnight cultures.

Before and after each selection step a sample of cells were plated for assessment of genotype frequencies. The assay for genotype determination was done in 96-well microtiter plates with lids. For each population a sample of 21 colonies was assayed by picking each colony into two separate wells, both filled with 200 μl of M9 medium containing 0.2% casamino acids, 1 mM thiamine hydrochloride, 50 μg/ml

kanamycin, and 100 μg/ml spectinomycin. Medium in one of the two wells was additionally supplemented with 1 mM IPTG. The plate was incubated for 16 h at 37°C under orbital shaking. Measurement of mCherry fluorescence was done with 587 nm excitation and 610 nm emission in an Infinite M1000 PRO (Tecan).

To determine single-round enrichment efficiencies for each separate selection step over 24 h, similar mock selections with LacI mutants a2 and a3 were performed. Here, 5 μl of overnight cultures were used to inoculate 5 ml DYT with 50 μg/ml kanamycin, 0.02% arabinose, and 5 μg/ml chloramphenicol. These inoculated cultures were grown for 3 h at 32°C. Normalizing with the OD_{600}, the two genotypes were mixed at a ratio of 1:100 000 with that genotype in excess, which was selected against in the following selection. A sample of the mixture was diluted 1:100 and plated. 1 ml of the mixture was then used to inoculate a 20 ml culture with DYT containing 50 μg/ml kanamycin, 0.02% arabinose, and either 125 μg/ml spectinomycin when selecting for the constitutive *lacO* binder a3, or 500 μg/ml chloramphenicol when selecting for the non-binder a2. Cultures were incubated for 24 h at 32°C, keeping the cultures' OD_{600} below 0.4. Then a sample was taken, diluted 1:100 and plated. Genotypes were determined by red fluorescence (a2) or the absence thereof (a3) directly on the plate in a FUSION-SL imaging system (Vilber Lourmat) with Epi-Red LED lighting and the filter F-695 Y5.

Library generation

LacI libraries were generated by random mutagenesis with the Genemorph II Random Mutagenesis Kit (Stratagene) according to the manufacturer's instructions with the primers used for *lacI* cloning (LacI-fw-XbaI and LacI-rv-PstI). In the first round of library generation from wild-type *lacI*, 100 ng of target template were used to achieve a medium to high mutation rate, in subsequent rounds 500 ng were used for a low to medium mutation rate. The error-prone PCR product was digested and ligated into pBAD-Kan, dialyzed against water and electroporated into electrocompetent RV308 *E. coli* already bearing the reporter plasmid. Library sizes were 9×10^6 for the initial, highly randomized library and about 5×10^5 for the two subsequent, moderately randomized libraries.

Library selection

Glycerol stocks of libraries or previous selection rounds were used to inoculate 100 ml DYT, taking care to achieve at least 20-fold coverage of the library, containing 50 μg/ml kanamycin, 0.02% arabinose, 5 μg/ml chloramphenicol, and, to prime the system for the following selection, either no or 1 mM IPTG. After 3 h of incubation at 32°C, this culture was used to inoculate the selection culture of 100 ml DYT to an OD_{600} of 0.005. The selection culture contained 50 μg/ml kanamycin, 0.02% arabinose, either no or 1 mM IPTG, and as respective selection antibiotic either chloramphenicol (OFF selection) or spectinomycin (ON selection). The selection culture was incubated for 6–8 h at 32°C, then pelleted and the supernatant removed. Part of the pellet was used for plasmid preparation, the other was stored as a glycerol stock. For selection for inverted LacI behavior the initial library was subjected to an 8-h selection for *lacO* binding by 250 μg/ml spectinomycin in the presence of 1 mM IPTG followed by a 6-h selection with 250 μg/ml chloramphenicol without IPTG. From this selection cycle, the pool was again diversified by error-prone PCR and the new library subjected to a similar selection, only the chloramphenicol counter selection was more stringent with 8 h of selection at 500 μg/ml chloramphenicol. A third round of library generation and selection and a subsequent fourth round of selection without prior randomization were performed with selection conditions that were identical to round two. In addition, the selection from the initial library was done in parallel in congruence with the wild-type LacI operation logic, omitting IPTG in the ON priming and selection step and adding 1 mM IPTG during the OFF step.

RESULTS

Long-range TI assessment by fluorescent proteins

In order to test for long-range TI of converging promoters, a BFP (blue fluorescent protein) gene was cloned opposite to an mCherry gene, resulting in an inter-promoter distance of 1.5 kb. mCherry expression was driven by the weak *bla P3* promoter, whereas the promoter driving BFP expression was varied in its strength ranging from no promoter activity (scrPX) to high promoter activity (conP01), and both BFP and mCherry fluorescence was measured (Figure 1). As expected, BFP signals increased with increasing promoter strength. mCherry fluorescence was maximal without an opposing promoter (scrPX) and reduced with increasing interfering promoter strength. The weakest tested interfering promoter conP05 already markedly reduced mCherry expression. The strongest of the used interfering promoters, conP01, led to a 170-fold reduction of mCherry fluorescence.

With the strongest interfering promoter, conP01, the possibility to exert transcriptional interference on mCherry expression over even longer inter-promoter distances was investigated. Spacers between the BFP and the mCherry gene were inserted in 500 bp increments up to a spacer size of 1.5 kb, resulting in a total inter-promoter distance of about 3 kb. Again, the constructs were tested by measuring mCherry and BFP expression (Figure 2). With increasing spacer length TI became less efficient, leading to an increase of mCherry expression. However, even with the maximal spacer length tested, substantial TI was still evident. Further, BFP expression was reduced with spacer sizes longer than 500 bp.

T7 RNA polymerase TIA reporters

To demonstrate modularity and versatility of the system, we further constructed a TIA reporter, in which the interfering promoter is T7 bacteriophage RNA polymerase dependent. The T7 promoter is thus driving BFP expression when an active T7 RNA polymerase (RNAP) is present. As expected, only T7 RNAP+ led to BFP expression and interfered with transcription of the mCherry gene (Figure 3).

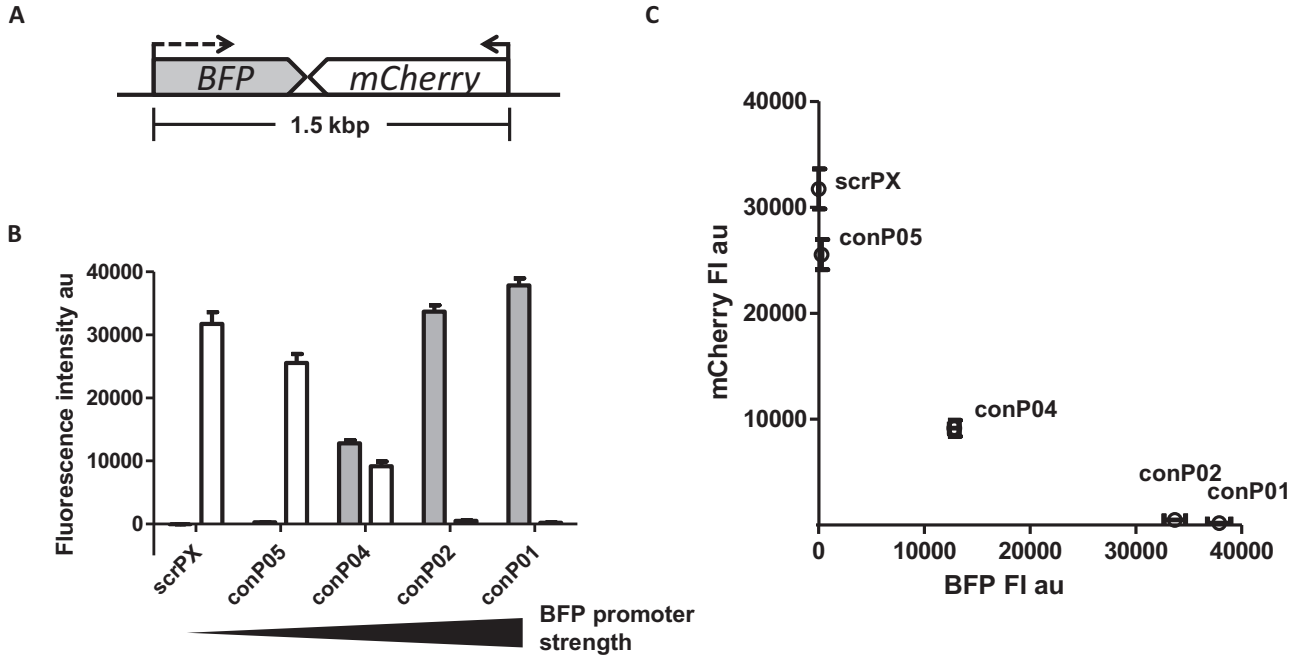

Figure 1. Transcriptional interference with variable interfering promoter strength assessed by expression of fluorescent proteins. (**A**) A BFP and an mCherry gene are directly opposing each other on a pSB4S5 vector. BFP expression is driven by promoters of variable strength, the mCherry gene by the weak *bla P3* promoter. (**B**) BFP (gray) and mCherry (white) fluorescence signals with increasing promoter strength of the BFP gene. (**C**) Plot of BFP fluorescence against mCherry fluorescence. Data points represent means with standard errors from eight biological replicates.

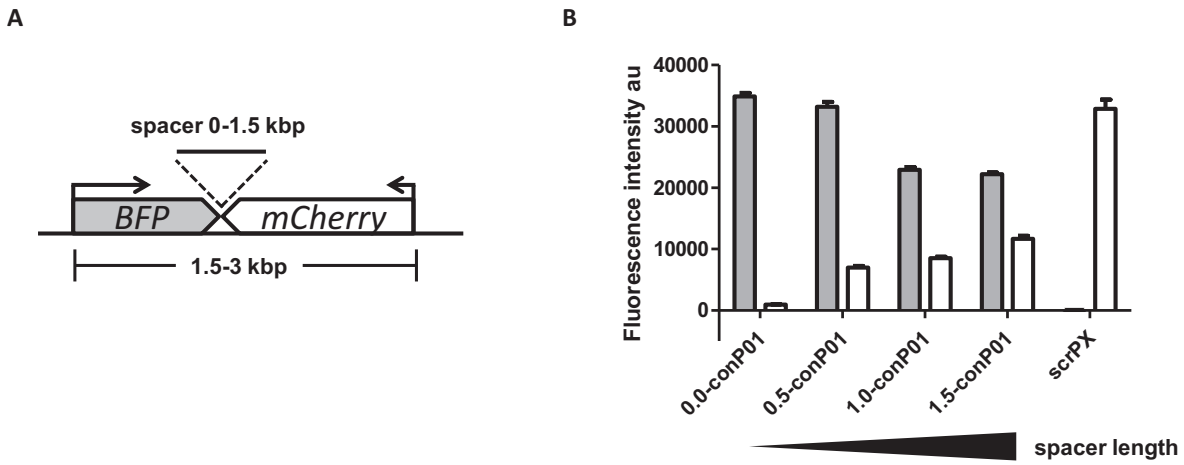

Figure 2. Transcriptional interference with variable inter-promoter distances assessed by expression of fluorescent proteins. (**A**) A BFP and an mCherry gene are directly opposing each other on a pSB4S5 vector. BFP expression is driven by the strong promoter conP01, the mCherry gene by the weak *bla P3* promoter. Between the two genes, spacers have been added in 500 bp increments. (**B**) BFP (gray) and mCherry (white) fluorescence signals with increasing inter-promoter distance and, as control, with a scrambled promoter in front of the BFP gene (scrPX). Data points represent means with standard errors derived from eight biological replicates.

LacI selection system

To employ long-range TI for construction of a dual genetic selection system for conditional DNA binders, antibiotic resistance genes instead of fluorescent proteins were used. A schematic of the LacI selection/reporter system consisting of two opposing transcriptional units is shown in Figure 4A. The rightward strong σ^{70} dependent promoter driving chloramphenicol acetyltransferase (CAT) expression is expected to interfere with expression of the spectinomycin resistance gene *aadA* from its native, weak promoter. Lac repressor being co-expressed from an additional plasmid should inhibit the strong promoter in the absence of IPTG, alleviating the transcriptional interference and thus allowing transcription from the weak promoter. Conversely, addition of IPTG should restore transcriptional interference and high expression of CAT (Figure 4B and C). Indeed, this genetic circuitry caused a conditional response for resistance against both chloramphenicol and spectinomycin: In

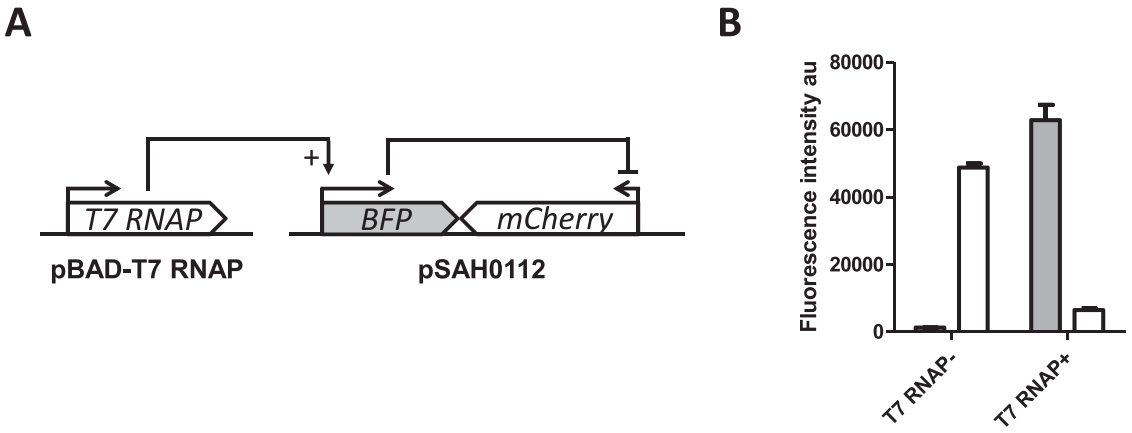

Figure 3. T7 RNAP activity sensitive TIAs with fluorescent protein or antibiotic resistance genes. (**A**) Schematic of the T7 RNAP system with fluorescent TIA reporter plasmid. Expression of T7 RNAP leads to transcription from the T7 promoter driving BFP expression and inhibiting transcription from the mCherry gene. (**B**) BFP (gray) and mCherry (white) fluorescence signals, when expressing an inactive (T7 RNAP-) or an active (T7 RNAP+) T7 RNA polymerase. Data points represent means with standard errors derived from four biological replicates.

the absence of IPTG, the cells displayed a high resistance against spectinomycin and a low resistance against chloramphenicol, whereas in the presence of IPTG it was the other way around. In liquid culture, differences in growth rates between the two states were apparent over a wide range of antibiotic concentrations with maximal differences of about 1 doubling/h at a chloramphenicol concentration of 500 μg/ml or at a spectinomycin concentration of 250 μg/ml, respectively (Figure 4D and E). When done on LB agar as a spot assay at identical antibiotic concentrations, wild-type LacI under chloramphenicol pressure grew only in the presence of IPTG, and under spectinomycin pressure only in the absence of IPTG (Supplementary Figure S2).

LacI mock selections

To demonstrate the feasibility of selections for conditional DNA binders, three distinct *lacI* genotypes were used in competition experiments under a selection scheme equivalent to that used for library selections. The wild-type LacI as a conditional *lacO* binder was selected from a background of two LacI mutants non-responsive to IPTG. The LacI mutant a2 (V15A, L62S, G65D, A82S, L205H, W220R, Q317K) displays no appreciable *lacO* binding irrespective of IPTG availability, whereas the mutant a3 (A72S, D88G, P127Q, E137G, Q335*) is a very strong, constitutive binder. For mock selection experiments, an expression plasmid with an additional mCherry based reporter (pLO2) was used, allowing unambiguous determination of the genotype by comparing the fluorescence ratio with and without IPTG and overall fluorescence (Supplementary Figure S1).

As expected, when selecting for low *lacO* occupancy by 500 μg/ml chloramphenicol with 1 mM IPTG, wild-type LacI had a fitness advantage over a3, but a slight disadvantage over a2 (Figure 5A). Conversely, during selection for *lacO* binding by spectinomycin (250 μg/ml) in the absence of IPTG wild-type LacI had a fitness advantage over a2, but a slight disadvantage over a3 (Figure 5B). Subsequently applying those two selection regimen in both possible orders results in an overall selective advantage of conditionally *lacO* binding wild-type LacI over the other two genotypes and thus allows its enrichment from a 1:1:1 mixture of the three LacI variants (Figure 5C and D).

To determine enrichment factors over 24 h for both the ON and the OFF selection in a single selection round, mock selections of the *lacO* non-binder a2 (forming red fluorescent colonies) against the strong, constitutive binder a3 (forming non-fluorescent colonies) were performed. For the selection for *lacO* binding by spectinomycin pressure starting at a ratio of 100,000:1 (a2:a3), after 24 h of selection 85.5% (609 out of 712 colonies) were non-fluorescent, corresponding to an enrichment factor of 8.6×10^4. Selecting for a2 from a 100 000-fold excess of a3 by 500 μg/ml chloramphenicol for 24 h yielded 91.1% fluorescent colonies (288 out of 316 colonies), which is equivalent to an enrichment factor of 9.1×10^4.

LacI library selections and characterization

To employ the TIA for a challenging genetic selection from large libraries we aimed for isolating a functionally inverted Lac repressor, which binds *lacO* only in the presence of IPTG. An initial random library from wild-type *lacI* with 9×10^6 individual clones was created by error-prone PCR. Twenty clones were randomly chosen for sequencing and phenotypic characterization. The majority of the sampled clones were apparent constitutive non-binders of *lacO*, whereas 2 of the 20 sampled clones displayed wild-type like behavior with reduced switchability. In addition to selection for inverted response to IPTG (with IPTG during ON selection and no IPTG during OFF selection), a selection in accordance with LacI wild-type operation logic (without IPTG during ON selection and with 1 mM IPTG during OFF selection) was performed from the initial library. After each selection round, 20 clones were randomly chosen, sequenced and characterized by antibiotic resistance

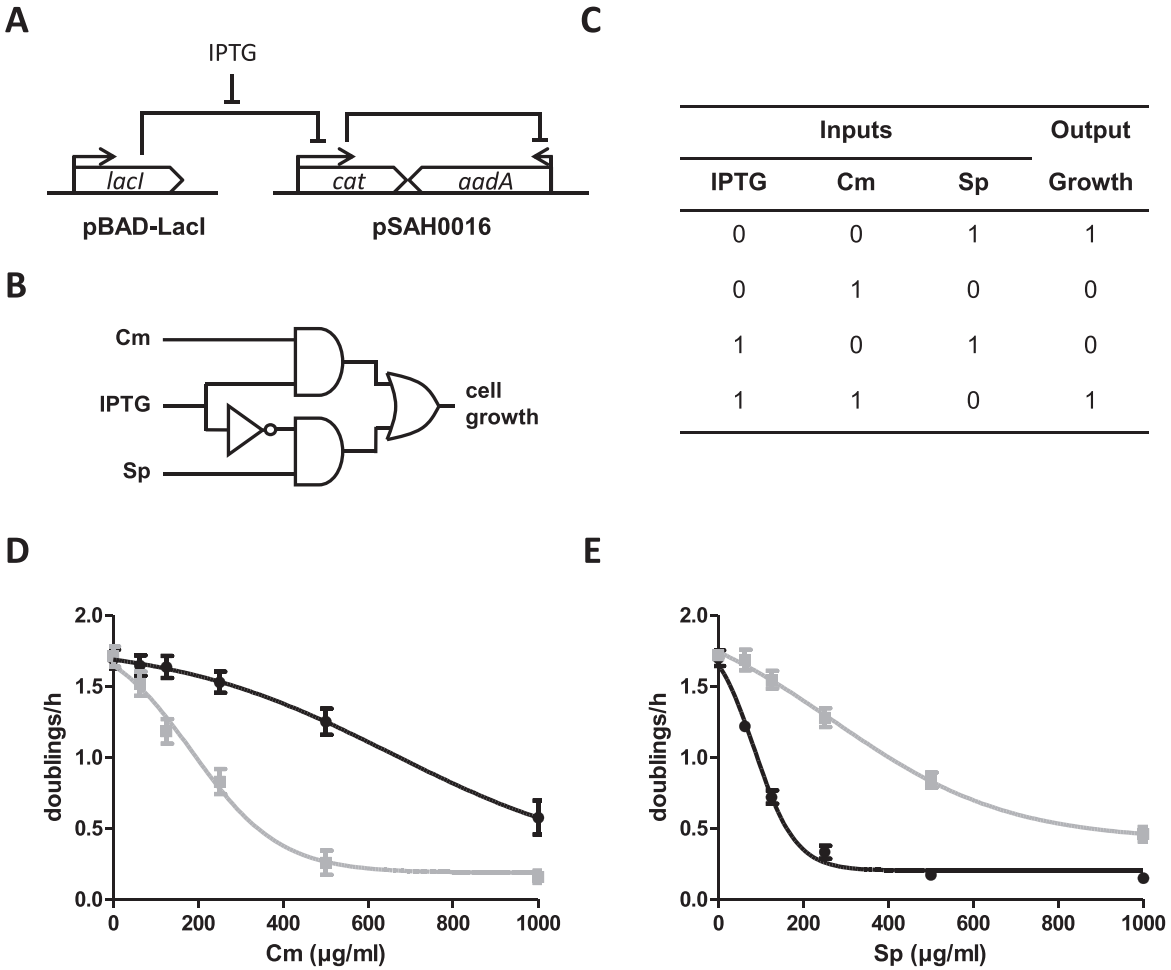

Figure 4. LacI selection system. (**A**) Schematic of the synthetic circuit with wild-type LacI operation logic. The TIA consists of two plasmids: The reporter plasmid pSAH0016 has two opposing antibiotic resistance genes, one of which (*cat*) has a strong, but conditional promoter, whereas the other gene (*aadA*) is driven by a weak promoter and is thus susceptible to transcriptional interference by the strong promoter. The expression plasmid pBAD-LacI expresses wild-type Lac repressor under control of an arabinose inducible promoter. In the absence of ITPG, wild-type LacI inhibits expression from the strong promoter. (**B**) Boolean logic circuit and (**C**) truth table for intended system behavior with use of either chloramphenicol or spectinomycin. (**D**) Growth rates under chloramphenicol pressure with the reporter plasmid and pBAD-LacI with (black) and without (gray) addition of 1 mM IPTG. (**E**) Growth rates under spectinomycin pressure. Data points represent means with standard errors from four separate experiments.

assays (Figure 6, Supplementary Table S1). After a single cycle of ON/OFF selection for wild-type response to IPTG, all sampled clones showed pronounced conditional DNA binding according to LacI wild-type operation logic. In contrast, a single round of dual selection for inverted response to IPTG yielded only clones with no or marginal, non-inverted response to IPTG. After a second round of error-prone PCR based library generation and ON/OFF selection, first clones with slightly inverted behavior were sampled. A third round of randomization and dual selection was conducted, and one clone showed pronounced functional inversion of IPTG response (5B3), bearing the mutations A72T, H163, G297V and Q335*. Up to now, all sampled clones yielded unique sequences. After a fourth selection cycle, this time without prior randomization, another LacI variant with markedly inverted response to IPTG was dominant among the sampled clones (6B3: Q26R, M42I, V66L, H202Y, Q227D, P320S, V324G, L346*). For both isolated pronounced Lac inverters (5B3 and 6B3), addition of IPTG resulted in a noticeably lowered chloramphenicol resistance and a higher resistance against spectinomycin in the resistance assay (Figure 7). For 5B3, the difference between the lower and the higher affine state was considerably smaller than for wild-type LacI. 6B3 displayed a distinctively better switchability than 5B3.

To confirm inversed functional behavior of the first selected inverter 5B3 *in vitro*, we aimed to purify the protein. Since the C-terminal 6xHis-tag needed for protein purification was absent because of the premature stop codon, it was initially restored by mutating the amino acid position 335 back to a glutamine. This, however, disrupted conditional binding. Instead, a 6xHis-tag was cloned directly to the C-terminus of the selected protein (5B3His), which seems to be functionally well tolerated. The 5B3His protein was purified, and its IPTG response was assessed in a fluorescence polarization assay for *lacO* binding and compared to the

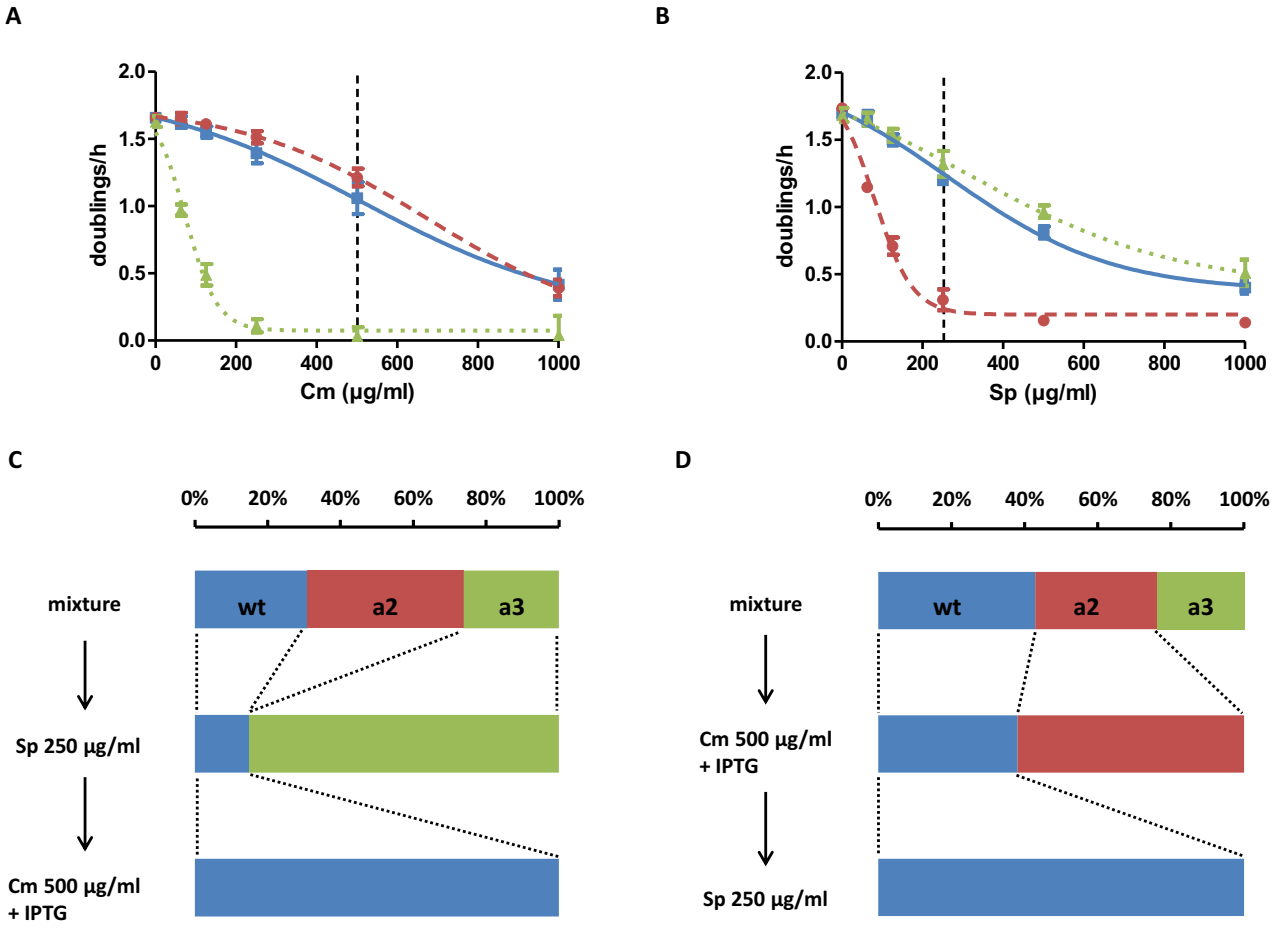

Figure 5. Fitness of used genotypes and mock selections from mixtures of equal cell numbers. (**A**) Growth rates of the three genotypes LacI wt (blue squares, solid line), a2 (red circles, dashed line) and a3 (green triangles, dotted line) in pLO2 (an expression plasmid with an additional reporter cassette for *lacO* binding) with the reporter plasmid pSAH0016 under chloramphenicol pressure in the presence of 1 mM IPTG. The vertical black dashed line represents the antibiotic pressure used for the mock selection experiments. (**B**) Growth rates under spectinomycin pressure in the absence of IPTG. (**C**) ON/OFF selection. A mixture of *E. coli* RV308 containing the reporter plasmid and either LacI wt, a2 or a3 was first subjected to 8 h of ON selection with 250 µg/ml spectinomycin and subsequent 8 hours of OFF selection with 500 µg/ml chloramphenicol in the presence of 1 mM IPTG. Genotype frequencies were determined before and after each selection step by assaying 21 clones each. (**D**) OFF/ON selection. The procedure was the same as for the ON/OFF selection, but with switched order of the two selection steps.

response of wild-type LacI (Figure 7F). In agreement with the *in vivo* assay, the response of 5B3His protein to IPTG was inverted in respect to the wild-type; 5B3His displayed increased DNA binding with increasing IPTG concentrations.

DISCUSSION

This study demonstrates that long-range transcriptional interference of converging promoters can be observed and used in artificial genetic circuitry in *E. coli*. While interference of convergent transcriptional units over longer distances has been described in eukaryotes (22), comparable mechanistic studies on convergent TI in prokaryotes have been done only with the interfering promoter either directly bordering the coding sequence of the sensitive gene (2,23) or the promoters directly facing each other (24), and thus with substantially smaller inter-promoter distances. However, previously undertaken modeling of TI of convergent promoters in *E. coli* had suggested that interference by collisions of opposing RNAP complexes can be very efficient for long inter-promoter distances if one promoter is considerably stronger than the other (25). Assessment of TI with fluorescent proteins done in this study experimentally demonstrates high efficacy of TI with an inter-promoter distance of around 1.5 kb without additional spacers. Here, the strongest interfering promoter conP01 reduced mCherry expression driven by the weak promoter *bla P3* by more than two orders of magnitude.

Based on this construct, spacers were inserted between the BFP and mCherry gene in 500 bp increments. The spacers were derived from the coding sequence of the *cat* gene, assuring high RNAP processivity. Spacer introduction decreased achievable TI on mCherry expression, possibly because a certain proportion of RNAP collision events occurred in the spacer region instead of within the mCherry

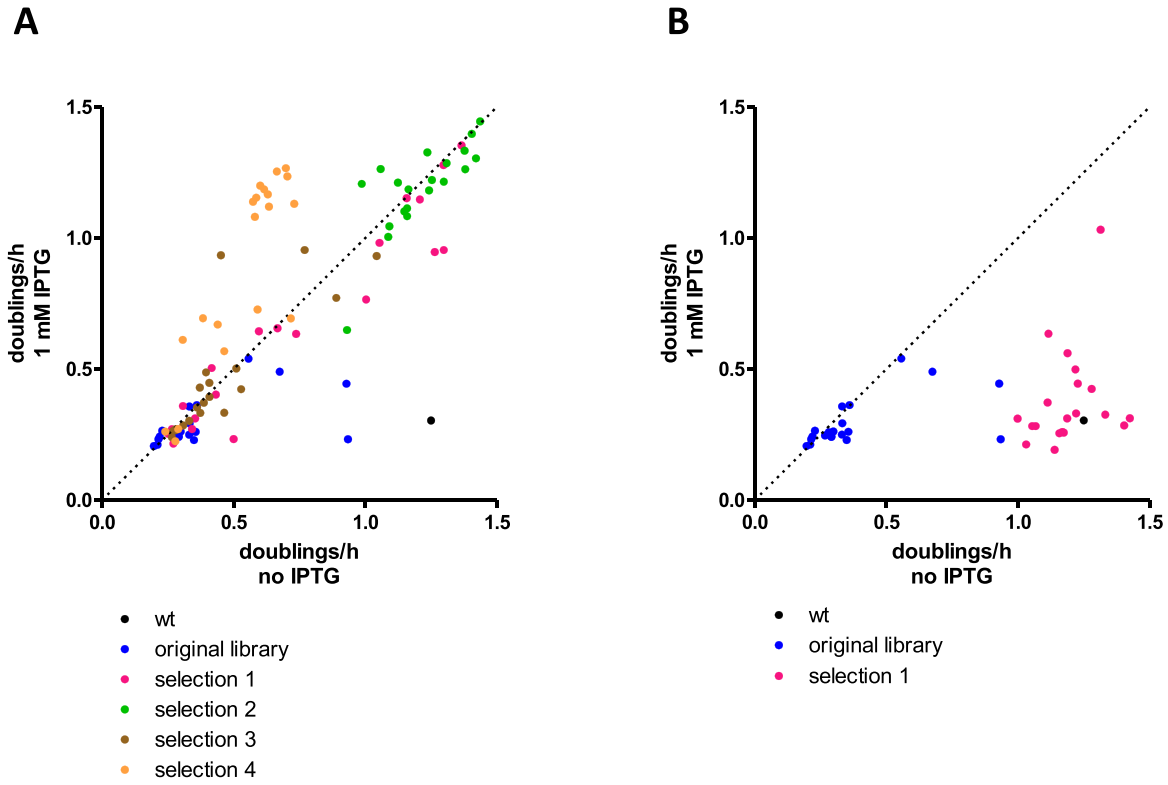

Figure 6. Phenotypic mapping of clones sampled from the initial library and after dual selection rounds for (**A**) inverted switching behavior (inverter selections) or (**B**) wild-type operation logic (repressor selection). For all clones, growth rates under spectinomycin pressure (250 μg/ml) were determined without and with 1 mM IPTG in antibiotic resistance assays with three biological replicates.

coding sequence, and thus creating more transcripts, which contain the complete mCherry coding sequence. However, substantial TI could still be observed for the largest inserted spacer of 1.5 kb. Additionally, with increasing spacer length a reduction of BFP expression was evident. This effect is most likely due to a decrease in transcript stability, e.g. because of the transcripts' total length increase or the repetitive nature of the spacers.

Further, we decided to investigate, whether long-range transcriptional interference can also be applied in systems with orthogonal RNA polymerase activity as output instead of DNA binding. The RNA polymerase from bacteriophage T7 is a single-subunit polymerase commonly used in synthetic biology in conjunction with promoters, which are strictly dependent on its activity. However, it was not at all clear, whether an active T7 promoter can exert TI on a transcriptional unit recruiting bacterial, multi-subunit RNAP. Heads-on collisions between elongating RNAP complexes are thought to induce stalling for both complexes (26). While stalled bacterial polymerases can apparently be re-activated by trailing RNAP complexes (27), stalled T7 RNAPs are instead displaced from the DNA strand by trailing T7 RNAPs (28), which might give the T7 RNAP a disadvantage in such collision events. Furthermore, it has been demonstrated, that single-subunit, processing T7 and T3 RNA polymerases can pass each other on opposite strands (29). If this applies to collisions of T7 RNAP with bacterial multi-subunit RNAP as well, it is expected to considerably reduce the degree of achievable TI. However, using a T7 promoter as interfering promoter in our systems shows substantial transcriptional interference with the expression of the opposite gene driven by a weak, bacterial promoter, if a functional T7 RNAP is present.

The demonstrated efficient long-range TI in the investigated two gene designs motivates its exploitation in more complex synthetic biological systems. Similar to the original TI-based selection system by Elledge and Davis for DNA binding proteins (3), we constructed a selection system (TIA) for conditional DNA binding by adding a *cat* gene driven by the interfering promoter. If the strong interfering promoter is under control of a DNA binding protein, this arrangement allows positive selection both for occupation of the operator by the DNA binding protein (ON state) and for absence of binding to the operator (OFF state). Recently published selection systems suitable for such dual selections employed a combination of a positive and a negative selection (7–13). The use of negative selection markers, however, creates an evolutionary pressure for loss-of-function mutations during selection, which can result in quick enrichment of false positives, especially in liquid culture. The selection systems using a single gene both as positive and negative selection marker, either a tetracycline/H^+ antiporter (8,9), the herpes simplex virus thymidine kinase (11), or a fusion of the thymidine kinase with a kanamycin resistance marker (13), reduce this problem. Here, most loss-of-function mutations acquired during negative selection will also incapacitate the marker in positive selection. In our system with dual positive selection there is no evolutionary pressure for loss-

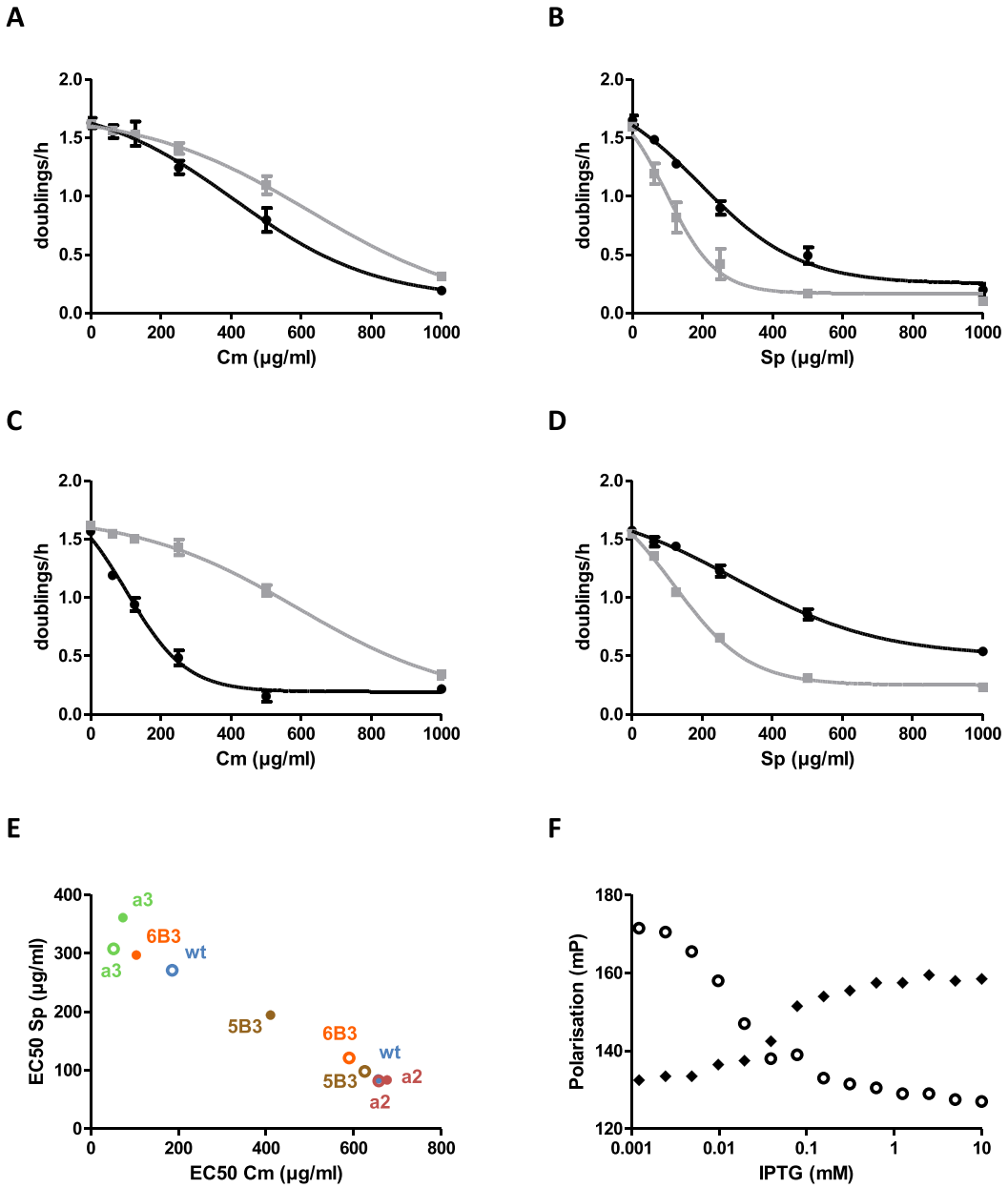

Figure 7. Characterization of selected Lac inverters 5B3 and 6B3. (**A**) Growth rates under chloramphenicol pressure with pBAD-5B3 with (black) and without (gray) addition of 1 mM IPTG and (**B**) growth rates under spectinomycin pressure. (**C**) Growth rates under chloramphenicol pressure with pBAD-6B3 with (black) and without (gray) addition of 1 mM IPTG and (**D**) growth rates under spectinomycin pressure. Data points represent means with standard errors from four separate experiments. (**E**) Plot of EC50 values for chloramphenicol against EC50 values for spectinomycin for used *lacI* genotypes in antibiotic resistance assay with 1 mM IPTG (filled circles) and without IPTG (open circles). (**F**) Binding of wild-type LacI (open circles) and 5B3His (filled diamonds) to symmetrical, fluorescein labeled 18 bp *lacO* as function of IPTG concentration assessed by fluorescence polarization.

of-function mutations, which is expected to make the selection system genetically robust. In liquid culture this is especially important, since false positive escape mutants with a considerable fitness advantage can quickly enrich due to the fact they are not restrained in growth as on solid media. However, genetic selection experiments in liquid culture have the distinct advantage of allowing the handling of large libraries, whereas on solid media library size is limited by plate surface area and the need to have spatially isolated colonies. Consequently, our selection assay has been developed to be used in liquid culture with chloramphenicol and spectinomycin as selection antibiotics: Both the chloramphenicol acetyltransferase and the aminoglycoside adenylyltransferase encoded by *aadA* are cytoplasmatic proteins, confining their protective activities to the respective bacterial cell producing them. Nonetheless, the system also works well on agar plates (Supplementary Figure S2). In addition, the integration of a further fluorescent reporter for convenient phenotypical assessment is well-tolerated.

The Lac repressor was chosen as model system for conditional DNA binding because of its high degree of switchability and its widespread use in molecular biology. In addition, phenotypes for a huge number of mutations have been characterized (30) and there is ongoing effort to improve its characteristics (31–33). Previously, the functional inversion of the Lac repressor by a directed evolution system from libraries created by error-prone PCR has been reported, highlighting the role of epistatic interactions between mutations required to achieve the inverted phenotype (10). This constitutes a considerable challenge for the selection of conditional DNA binders, as the occurrence of inverted phenotypes by random mutagenesis of wild-type *lacI* is expected to be an extremely rare event, since several mutations are necessary. If a selection system succeeds in selecting functionally inverted proteins, the selection for less drastic phenotypic changes (e.g. tighter occlusion, higher inducibility) should pose no problem, as they can be reached incrementally and evolutionary constraints are considerably smaller. This is illustrated in this study by selection in congruence with the wild-type LacI operation logic from a highly randomized library. Here, a single round of dual selection drastically reduced the frequency of mutations within the library and yielded only functional, highly switchable wild-type logic mutants, in contrast to the selection for an inverted response (Figure 6). Still, a number of different evolutionary trajectories to an inverted Lac repressor are apparently possible. Previously, other inverted Lac repressor variants have been reported, both of which have mutations mapping to the region between residues 91 and 97 (34,35). In the recent study evolving inverted LacI regulation, S97P was found to be a key substitution, which conferred an inverted phenotype in epistasis with sets of other mutations (10). The Lac inverters selected in our study had different sets of amino acid substitutions (5B3: A72T, H163, G297V, Q335* and 6B3: Q26R, M42I, V66L, H202Y, Q227D, P320S, V324G, L346*). For the Lac inverter 6B3 the substitutions Q26R, M42I and V66L seemed to be key substitutions on its evolutionary trajectory, as they were also found in other, slightly inverted phenotypes in selection rounds 2 (3B9) and 4 (6B10). The successful selection for a phenotypical inversion underscores the utility of this novel *in vivo* selection system enabling dual positive selection. The chemicals used for selection are commonly used antibiotics and the system places very few restrictions on usable media and strains; Strains need to be sensitive to chloramphenicol and spectinomycin and endogenous factors should not interfere with the used operator.

We expect that long-range TI as a construction principle for synthetic genetic circuitry has a number of further potential applications, in which inversely correlated gene expression is desired and thus constitutes a novel, valuable asset to the molecular toolbox.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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