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Dynamic stepwise opening of integron attC DNA hairpins by SSB prevents toxicity and ensures functionality

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ABSTRACT

Biologically functional DNA hairpins are found in archaea, prokaryotes and eukaryotes, playing essential roles in various DNA transactions. However, during DNA replication, hairpin formation can stall the polymerase and is therefore prevented by the single-stranded DNA binding protein (SSB). Here, we address the question how hairpins maintain their functional secondary structure despite SSB’s presence. As a model hairpin, we used the recombinogenic form of the attC site, essential for capturing antibiotic-resistance genes in the integrons of bacteria. We found that attC hairpins have a conserved high GC-content near their apical loop that creates a dynamic equilibrium between attC fully opened by SSB and a partially structured attC-6–SSB complex. This complex is recognized by the recombinase IntI, which extrudes the hairpin upon binding while displacing SSB. We anticipate that this intriguing regulation mechanism using a base pair distribution to balance hairpin structure formation and genetic stability is key to the dissemination of antibiotic resistance genes among bacteria and might be conserved among other functional hairpins.

INTRODUCTION

Bacteria have an impressive capability of acquiring antibiotic resistances using the integron recombination platform. The integron is the predominant tool for adaptation in gram-negative bacteria (1,2). It is a genetic platform that assembles, and rearranges promoterless gene cassettes through a unique recombination process in which the integron integrase IntI recognizes and recombines a folded single-stranded DNA hairpin called attC (3,4). attC sites can form 57 to 141 nucleotide (nt)-long hairpins (3), which display conserved structural features that are recognized by the integrase IntI (5,6). IntI’s specificity for folded attC hairpins is advantageous for bacteria, as it allows the coupling of recombination with the presence of single-stranded DNA (ssDNA) during replication or DNA exchange among bacteria (2). However, DNA hairpins can also have toxic or deleterious effects, for instance, replication arrest due to polymerase stalling (7). These effects are prevented by the single-stranded DNA binding protein (SSB) which binds to ssDNA with high affinity and low sequence specificity (8,9). Indeed, SSB has been shown to open short double-stranded DNA (dsDNA) stretches of up to 12 bp when migrating along ssDNA (10–12) and to hamper attC hairpin folding in vivo in the absence of IntI (13). However, it is unknown how SSB removes secondary structure of hairpins up to 141 nt in length and how hairpins maintain their functional folded structure in presence of SSB. Moreover, the recognition dynamics of folded attC by the integrase in presence of several thousands of SSB monomers per cell (14,15) is completely unknown. Here, we use an in vitro approach based on single-molecule Förster Resonance Energy Transfer (sm-FRET) (16) to describe how the structure of an attC hairpin balances both efficient opening by SSB to avoid toxicity, and reliable recognition by IntI to ensure functionality (Figure 1A). We found that attC hairpins have a conserved high GC-content near their apical loop that creates a dynamic equilibrium between attC fully opened by SSB and a partially structured attC-6–SSB complex. This complex is recognized by the integrase IntI, which extrudes the full hairpin upon binding while displacing SSB. In vivo recombination assays...
Figure 1. SSB requires a single-stranded DNA overhang to open the attC hairpin. (A) Schematic illustration of smFRET assay to study the SSB-IntI competition. The average distance between the donor (green) and acceptor (red) serves as a readout of the molecular conformation. (B) The secondary structure of the attCaadA7 hairpin predicted by RNAfold (43). (C) Binding of SSB to poly(dT)70. SSB binds in the SSB-65 binding mode at low protein-to-DNA ratios and in the SSB-35 binding mode at higher SSB concentrations (cSSB). (D) Inefficient SSB opening of the attC hairpin by SSB-65 and SSB-35 binding modes. (E) Increased hairpin opening efficiency due to the presence of a ssDNA overhang of 2, 3 or 9 dT nucleotides on the attC hairpin. (F) Hairpin opening efficiency quantified by SSB-35 proportion with ssDNA overhangs from 1 nt to 65 nt at cSSB = 1 μM. DNA breathing was reduced in the attC_Gcclamp hairpin (grey), which hence requires a longer overhang for opening. (G) Hydrodynamic radius of SSB-ssDNA complexes at cSSB = 1 μM obtained by FCS. SSB binds to ssDNA as short as 7 nt and the binding affinity increases with the length of ssDNA until 17 nt. The green line presents the control without SSB. All error bars depict the 95% confidence interval.

of a mutant abolishing the attC-6–SSB complex showed a reduced recombination frequency.

MATERIALS AND METHODS

Proteins

We used the catalytically inactive integrase mutant IntI1-Y312F, which is unable to cleave its recombination site but retains full binding ability (17). It was purified using a maltose binding protein (MBP) fusion. The IntI1-MBP fusion protein has been shown to retain full IntI1 functionality in vitro and in vivo (18). Purification was done in a two-step protocol using first an amylose column (MBPTrap HP, 5 × 1 ml, GE healthcare Life Sciences), followed by an ion exchange column (HiTrap SP HP, 5 × 1 ml, GE Healthcare Life Sciences) to remove contaminants. The Escherichia coli...
Single-Stranded DNA Binding Protein was obtained from Promega (Madison, USA).

DNA constructs

All oligonucleotides used in this study are listed in Supplementary Table S1. They were obtained from biomers.net (Ulm, Germany), PURIMEX (Göttingen, Germany), eurofins genomics (Ebersberg, Germany), or IBA lifesciences (Göttingen, Germany). Biotin or dye modifications at the ends of the oligonucleotides were attached as NHS esters to an amino-C6-dT or an amino-C6-dA base. Labeled DNA was purified using a reverse phase C18 column (Phenomenex) by High Performance Liquid Chromatography (HPLC).

smFRET

For smFRET measurements the partial duplex DNA constructs carrying one donor (Cy3) and one acceptor (Cy5) were immobilized at the duplex ends on a polyethylene glycol (PEG)-passivated quartz surface via biotin–neutravidin interaction at sample concentrations of 25 pM. The FRET imaging buffer contained 20 mM NaCl, 50 mM Tris–HCl in a saturated, aged Trolox-solution (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) at pH 8 with an oxygen scavenging system composed of 1 mg/ml glucose oxidase and 0.8 % (w/v) D-glucose, 1 kU/ml catalase. Single molecules were observed at 23°C in a custom-built prism-type total internal reflection microscope (19,20) at a frame rate of 33 s⁻¹. Proteins were added at the concentrations specified and incubated for 10 min prior to data acquisition. The FRET histograms were constructed using FRET time traces of 70 to 150 molecules per condition.

smFRET state finding algorithm

The custom state-finding pipeline consists of three main steps. First, a generic Hidden Markov Model (HMM) was used to find candidate states in the smFRET traces. Second, the found ‘HMM states’ are assigned to ‘molecular states’ in a user-assisted post-processing step. Third, the rates and state proportions are calculated from the resulting assignment. HMM was performed using the Matlab statistics and machine learning toolbox utilizing the Baum-Welch algorithm (21). First, the FRET efficiency values were quantized into 50 bins between 0 and 1. All traces from one experiment are fit globally by m+n HMM models, differing in their number of hidden states ranging from 1 to m+n, where m is the number of expected states and n the number of over-fitting states. First, the m+n states HMM is run and its states are initialized evenly between 0 and 1. The state number is then reduced by one, taking the previous model’s fitted states for initialization and removing the least probable state. This is repeated until all models from 1 to m+n are fit. Here, the maximum number of possible states m was estimated from previous knowledge and preliminary analysis using ‘Fast Step Transition and State Identification’ algorithm (22) and set to 4. The number of used over-fitting states n was set to 3. The over-fitting of states allows for the correction of misassigned states during the user-assisted post-processing and was found to greatly increase the robustness of the analysis (see Supplementary Figure S4). The user-assisted post-processing is required to map the ‘HMM states’, i.e. the results from the fitting, to the m ‘molecular states’, i.e. the actual FRET states characterizing a certain molecular conformation. The user is first presented with m+n FRET histograms summarizing the fits of the different HMM models, chooses the best fit and merges HMM states when necessary. To assist the user in assessing whether the state assignment is correct, the empirical cumulative distribution function (CDF) of the dwell times of each transition in the reassigned data is calculated. The empirical CDF is obtained by the Kaplan–Meier estimator (23) with right censoring provided by the cCDF function of MATLAB. Once the state assignment is performed, the newly ‘assigned state model’ is used for all further analysis. For the calculation of the transition rates and the state proportions from the ‘assigned state models’, the following parameters and filtering was used. A minimum dwell time of 10 frames was applied to account for the resolution limit and to avoid fitting of noise or blinking. Transition rates \( \hat{r} \) between pairs of states \( A \) and \( B \) were estimated using the maximum likelihood estimator for an exponential distribution, subject to right censoring and calculated by

\[
\hat{r} = \frac{N_{A\rightarrow B}}{N_A \cdot \hat{\tau}_A},
\]

where \( N_{A\rightarrow B} \) is the number of transitions from \( A \) to \( B \) observed in all traces, \( N_A \), the total number of transitions out of state \( A \), including those occurring due to the trace ending, and \( \hat{\tau}_A \) the average dwell time of state \( A \). The 95% confidence intervals were estimated using the inverse Fisher information, assuming a normal distribution, as

\[
\hat{\sigma} = \frac{\hat{r}}{\sqrt{N_{A\rightarrow B}}}.\]

State proportions were also calculated from the ‘assigned state model’. The proportion of time spent in each state was estimated from the Viterbi path. Bootstrapping was then performed to obtain an error for the state proportions introduced through state assignment. For all states, FRET traces were re-sampled 10 000 times with replacement. The standard deviation was then estimated from the empirical distribution and used to estimate a 95% confidence interval assuming a normal distribution.

FCS

FCS samples used a partial duplex DNA to attach the ATTO-655 fluorophore. Measurements were performed in 20 mM NaCl, 50 mM Tris–HCl, pH 7.4. The DNA concentration was 1 nM and protein was added at concentrations as indicated. Fluorescence intensity data was acquired using a home-built confocal microscope combined with time-correlated single photon counting (24). Excitation of ATTO-655 was achieved by a pulsed laser source with a wavelength of 640 nm. FCS data analysis was performed using a software correlator and an FCS fitting tool box from PicoQuant (Berlin, Germany).
Free energy profiles

Free energy profile barrier heights were calculated from transition rates using an Arrhenius equation and a 1 ms\(^{-1}\) attempt frequency. The closed hairpin structure was set as a zero reference state. The reaction coordinate axis is arbitrarily scaled.

Electrophoretic mobility shift assay

Oligonucleotides were obtained from Sigma-Aldrich, labeled at the 5′ end with radioactive phosphate transferred from \(\gamma\text{-}^{32}\text{P}\text{ATP}\) (PerkinElmer) by T4 polynucleotide kinase (ThermoFisher Scientific) and purified with MicroSpin G-25 columns (GE Healthcare). Each reaction contained 500 ng Poly[d(I-C)], 12 mM HEPES NaOH pH 7.7, 12 % glycerol, 4 mM Tris–HCl pH 8.0, 60 mM KCl, 1 mM EDTA, 0.06 mg/ml BSA, 1 mM DTT, 10 % Tween-20, 0.01 nmol of the corresponding \(32\text{P}\)-labeled DNA oligonucleotide and the amount of protein indicated, in a final volume of 20 μl. The samples were incubated at 30 °C for 10 min without the probe followed by 20 min with the probe, then loaded to a 5% native polyacrylamide gel (acylamide/ bisacylamide 37:5:1), with 0.5× TBE as buffer. The gel was run for 2 h at 40 mA with 0.5× TBE as running buffer and visualized using chemo-luminescence film (Amersham Hyperfilm™).

Calculation of base pair compositions of \(attC\) sites

The list of \(attC\) sites was obtained from the authors of the INTEGRALL database (25) containing 263 \(attC\) sites with less than 95 % sequence identity, all originating from mobile integrons. For each site, the GC-content was calculated for the core region (defined as the R-box, the unpaired central spacer, and the L-box) and for the apical region (defined as the rest of the stem and the variable terminal structure).

In vivo recombination assay

Wild-type and mutant \(attC\) sites were constructed by annealing two overlapping, phosphorylated oligonucleotides (Supplementary Table S1), fully complementary except for the overhangs corresponding to EcoRI and BamHI restriction sites. The sites were then ligated into the pSW23T vector carrying a chloramphenicol (Cm) resistance marker (27). Successful \(attC\) vector carrying a chloramphenicol (Cm) resistance marker was verified by sequencing. The recombination experiment was performed using a previously developed suicide conjugation assay (27). Briefly, pSW23T vectors were transferred through conjugation from the \(p\)2163 strain into a recipient \(E.\ coli\) DH5α strain, so that the transferred strand carried the bottom strand of \(attC\) sites. The recipient strain harbored the pBAD plasmid allowing the expression of IntI1 integrase (28) and a pSU plasmid with an \(attI\) site (27). Successful \(attI\) × \(attC\) recombination in the recipient strain conferred its resistance to Cm, allowing us to measure the rate of recombinant cells (Cm\(^{2}\)) among the total population of recipient cells (Cm\(^{2}\)) by plating. Recombination frequencies correspond to the average of three independent experiments.

RESULTS

SSB requires a single-stranded DNA overhang to open DNA hairpins

In a first step, we characterized the interaction of SSB with the \(attC_{\text{aadA}}\) hairpin of 70 nt (3) (Figure 1B). We used the tetrameric SSB from \(E.\ coli\) (9), as \(E.\ coli\) is a common host of antibiotic resistance integrons. SSB has multiple binding modes, depending on temperature, salt concentration and protein-to-DNA ratio, which are named after the number of nt occluded by SSB: SSB-17, SSB-35, SSB-56 and SSB-65 (29–33). In our low-salt condition (20 mM NaCl, 50 mM Tris–HCl, pH 7.5), SSB complexed unstructured ssDNA of 70 nt (poly(dT)\(_{70}\)) with its major binding modes SSB-35 and SSB-65 (Figure 1C). The low FRET efficiency population (\(E_{\text{FRET}}\approx 0.1\)) of poly(dT)\(_{70}\) vanished upon addition of SSB in SSB-65 (\(E_{\text{FRET}}\approx 0.7\)) at 100 pM SSB concentration (\(c_{\text{SSB}}\)), and gradually transitioned into SSB-35 (\(E_{\text{FRET}}\approx 0.3\)) above 1 nM \(c_{\text{SSB}}\), in accordance with previous reports (32). Next, we performed the SSB titration on the \(attC\) hairpin, where the presence of the SSB-characteristic FRET populations reflects hairpin opening. Indeed, SSB addition decreased the closed hairpin population (\(E_{\text{FRET}}\approx 0.9\)), and SSB-35 and SSB-65-like \(E_{\text{FRET}}\) populations occurred (Figure 1D). However, the \(c_{\text{SSB}}\) required for these populations were 1000-fold higher than for poly(dT)\(_{70}\), and even at 2 μM \(c_{\text{SSB}}\), 75 % of the hairpins remained closed.

The opening efficiency of the \(attC\) hairpin by SSB was largely increased by the presence of a single-stranded DNA overhang (ssDNA overhang). This effect is demonstrated by the increase of the SSB-35 population with increasing ssDNA overhang (Figure 1E). We quantified this effect by calculating the proportion of SSB-35 at \(c_{\text{SSB}} = 1 \text{ μM for ssDNA overhang lengths of 1–65 nt (Figure 1F, cyan curve). The observed increase in opening efficiency with the ssDNA overhang suggests that SSB requires binding to the ssDNA overhang prior to hairpin opening and that its minimal interaction site is 3 nt, as this is the apparent minimal ssDNA overhang required. However, this result disagrees with SSB’s reported minimal binding site of 17 nt (33,34).

To learn more about the binding of SSB to ssDNA we used Fluorescence Correlation Spectroscopy (FCS) (35). FCS rendered the hydrodynamic radius (\(R_{h}\)) of fluorescently labelled ssDNA of 1 to 35 nt in the absence (green) and the presence (blue) of 1 μM SSB (Figure 1G). SSB binding to the ssDNA increased the \(R_{h}\) and is characterized by the divergence of the two curves. SSB showed binding to ssDNA as short as 7 nt and its binding affinity increased exponentially with increasing ssDNA until the plateau at 17 nt (Figure 1G, Supplementary Figure S1, Supplementary text). We infer that binding of SSB to ssDNA between 7 and 17 nt is governed by a subset of the amino acids involved in the SSB-17 binding mode, composed of Arg-56, Phe-60 and Trp-40, 54 and 88 (31,33,36,37). This hotspot binds to a total of 7 nt of ssDNA according to the crystal structure (Supplementary Figure S2). The binding of 7 nt by SSB does not represent a stable binding mode, but rather a loose association, characterized by a high dissociation constant \(K_{D}\) (SSB-7) ≈ 10 μM (Supplementary Equation S2). It further rationalizes why a binding of SSB to 8 nt was re-
ported once, but could have never been confirmed (38). In summary, FCS revealed SSB’s minimal association site of 7 nt, but did not explain how an ssDNA overhang of 3 nt on the attC hairpin is sufficient for the attC hairpin opening by SSB.

The discrepancy between the 3 nt ssDNA overhang required for hairpin opening and the 7 nt required for SSB binding can be explained by DNA breathing, describing the spontaneous opening of double-stranded DNA (dsDNA) at ssDNA–dsDNA junctions (39,40). In the attC hairpin, breathing is facilitated by the two AT bp at the end, which expose 4 nt of ssDNA adding to the 3 nt ssDNA overhang to reach 7 nt. We tested this hypothesis by a GC-clamp for smFRET time traces that identifies a GC-clamp that reduced breathing by mutating the AT bp at the end to thermodynamically more stable GC bp (Figure 1F, gray line). Indeed, hairpin opening by SSB was shifted to longer ssDNA overhangs (Figure 1F, Supplementary Figure S3), confirming that dsDNA opening requires SSB to bind to ssDNA provided by an ssDNA overhang and by breathing DNA of both strands. We observed opening of attC hairpins without a ssDNA overhang with a small percentage (Figure 1D), which can be explained by a low, but non-zero probability that DNA breathing exposes 7 nt. We further found that hairpin opening by SSB is 5-fold more efficient when proceeding in a 3′ to 5′ direction (Supplementary Figure S3, Supplementary text). The directionality was observed only for hairpin opening, and not for binding to ssDNA. Thus, E. coli SSB is less efficient in capturing breathing DNA in the 5′ to 3′ direction due to the directional wrapping of ssDNA around the SSB tetramer, as was previously proposed for SSB from Deinococcus radiodurans and E. coli (10).

SSB–attC hairpin complex structures are highly dynamic

Next, we investigated the pathways and kinetics of SSB opening the attC hairpin. For this, a state-finding algorithm for smFRET time traces that identifies $E_{FRET}$ states, their lifetimes and transitions was used. However, available algorithms failed to identify states across varying SSB concentrations, as they are biased toward highly populated areas of intrinsically noisy FRET traces. To overcome this limitation, we developed a two-step state-finding pipeline (Supplementary Figure S4) that overfits the data with more states than expected to ensure sparsely populated states to be identified (Materials and Methods). The user-assisted post-processing then allows merging of states if necessary. We confirmed the algorithm by reproducing the results of the well-studied interaction of SSB with poly(dT)$_{70}$ (32) (Supplementary Figure S4).

The analysis of SSB opening the attC hairpin shows four distinct FRET states (Figure 2A and B). In addition to the expected closed hairpin (red), SSB-35 (cyan) and SSB-65 (green), a state at $E_{FRET} \approx 0.85$ was found (dark blue). We hypothesized this state to be a partially folded hairpin bound by SSB, as we also observed for artificial hairpins (Supplementary Figure S5). We proposed this hairpin to be composed of six successive GC bp close to the apical loop of attC and hence termed the complex attC-6–SSB (inset of Figure 2B). To confirm its existence, we designed a negative control, attC$_{AT}$, in which the GC stretch was replaced by AT bp reducing the probability of attC-6–SSB formation. Indeed, the attC-6–SSB population was significantly reduced and the hairpin was more often completely opened by SSB, indicated by the increased SSB-35 population (Figure 2C). Additionally, a positive control containing only the GC-stretch, while all other bases were replaced by dT (attC$_{Gonly}$), showed a prominent attC-6–SSB population (Figure 2D). We conclude that SSB opening of the attC hairpin involves a partially closed hairpin–SSB complex.

The opening pathway of attC by SSB was reconstructed using the transition rates (Figure 2E, Supplementary Figure S6) and shows that SSB opens the attC hairpin via the attC-6–SSB conformation. The initial transition increases with $c_{SSB}$, because it depends on SSB binding to available ssDNA at the end of the hairpin. From attC-6–SSB, the complex transitions to the SSB-65 state through opening of the 6 bp with a $c_{SSB}$-independent rate. The transition to SSB-35, describing the addition of a SSB monomer, is expectedly dependent on $c_{SSB}$. The transitions back to the hairpin follow the reverse pathway with $c_{SSB}$ independent rates describing either dissociation events, or base pairing of attC-6–SSB. In conclusion, attC opening by SSB proceeds in a dynamic and stepwise fashion stabilizing an on-pathway intermediate of a partially closed hairpin termed attC-6–SSB.

**IntI recognizes the attC-6–SSB complex and displaces SSB**

We investigated the role of the attC-6–SSB state for the biological functionality of attC recombination sites, by performing experiments with both SSB and IntI under competition for attC (Figure 3A). To minimize IntI-fluorophore interactions, the donor fluorophore was repositioned to the apical loop, without affecting the interaction with SSB (Supplementary Figure S7). When IntI was added to the hairpin and bound it, the low $E_{FRET}$ population of the closed hairpin remained (Figure 3A). However, when SSB was added in the absence of IntI, the closed hairpin population disappeared and was replaced by the characteristic SSB populations, which are highlighted by a blue outline for simplicity (Figure 3A). Keeping the $c_{SSB}$ constant while adding increasing concentration of IntI (Figure 3A) allowed studying their binding competition, as the appearance of a peak at low $E_{FRET}$ (orange) indicated the displacement of SSB from the hairpin. The results show that even at a 100-fold molar excess of SSB, IntI efficiently displaces SSB from 50% of the hairpins. At equimolar concentration, >90% of the hairpins are closed. This effect is specific to IntI’s target site, the attC bottom strand, as it was significantly less pronounced on the non-recombinogenic attC top strand (Supplementary Figure S8). We concluded that IntI can outcompete SSB for its recombination site.

We hypothesized that IntI’s efficient binding of the hairpin despite SSB is due to the recognition of the attC-6–SSB state, which exposes part of the attC hairpin. To test this, we used the attC$_{AT}$ hairpin, which has a greatly reduced attC-6–SSB population at $c_{SSB} = 1 \mu$M (Figure 2C). After confirming that this mutation does not affect IntI binding using a gel-shift assay (Supplementary Figure S9), we performed the smFRET competition assay (Figure 3B). Remarkably, at a concentration ratio of 1:100, IntI could only displace SSB in 8% of the cases on attC$_{AT}$ compared to 44% on the
attC<sub>wt</sub>. Even at equimolar concentration, only 45% of the hairpins were bound by IntI. This supports the hypothesis that IntI recognizes the attC-6–SSB state and subsequently displaces SSB from the hairpin and that the lack of the attC-6–SSB state significantly reduces the recognition by IntI.

To test whether the reduced recognition of the attC<sub>AT</sub> hairpin by IntI due to the action of SSB is also visible in vivo, we compared the recombination frequencies of the attC<sub>wt</sub> and the attC<sub>AT</sub> hairpins using a well-established in vivo recombination assay (27). Indeed, the results show that IntI recombines the attC<sub>AT</sub> hairpin approximately 50% less efficient than the attC<sub>wt</sub> (Figure 3C). These results show that the attC-6–SSB state plays an important role for the recognition by IntI and therefore for the functionality of the integron both in vitro and in vivo.

**DISCUSSION**

Is this regulation mechanism by a high GC content close to the apical loop found only in the attC<sub>andA7</sub> hairpin or a common motif in Integron recombination sites? For this, we performed a bioinformatic analysis in which we compared the GC-content of the apical and the core region of 263 different attC sites occurring in vivo (Figure 4A). This analysis revealed that the apical part of the hairpins, close to the loop, has significantly more GC base pairs than the core region.

In conclusion, we propose that an attC-6–SSB like state is the key to maintaining attC functionality in the presence of SSB. This state is generated by a conserved, weighted base pair distribution along the hairpin.

Based on our results, we propose the following model of the interaction of SSB and IntI with attC hairpins both in vitro and in vivo (Figure 4B). SSB can open the attC hairpin, if it can bind to a single-stranded DNA overhang of at least 3 nt. However, the conserved base pair distribution of attC hairpins, featuring a high GC-content in the apical tip of the hairpin, does not allow SSB to open the hairpin irreversibly. Instead, the high GC-content creates a dynamic equilibrium transitioning on the seconds scale between the fully opened hairpin (SSB-65 and SSB-35) and a partially folded attC hairpin (attC-6–SSB). We show that IntI recognizes the short hairpin of the attC-6–SSB complex, binds to it as a monomer and then recruits a second monomer, while displacing SSB (Supplementary Figure S8). Assuming an SSB concentration of 1 μM, the attC<sub>andA7</sub> will transition on average every 4–5 s to the attC-6–SSB state and thus allowing IntI binding. This model can also explain the regulation of attC hairpins in living bacteria, where SSB is constitutively expressed, while IntI expression is suppressed.
Figure 3. IntI outcompetes SSB for the attC hairpin. (A) The attC hairpin is labeled at the apical loop to minimize IntI-fluorophore interactions. When IntI binds to the hairpin, the E<sub>FRET</sub> shows the closed hairpin. The SSB-characteristic populations form in the presence of 1 µM SSB, summarized by the blue outline. Upon addition of IntI to the SSB-attC complex, the low E<sub>FRET</sub> peak appears, representing the displacement of SSB by IntI. (B) The same competition experiment with attC<sub>AT</sub>, which does not stabilize attC-6–SSB, characterized by the difference in the histograms at 1 µM SSB compared to the wild type. Here, SSB displacement by IntI is much less efficient. (C) In vivo recombination frequencies show that attC<sub>AT</sub> recombination is approximately 50% less efficient than the wildtype attC site. Error bars show the standard deviation (P = 0.093).

in steady growth conditions and only activated by the SOS response, an indicator for the need of adaptation (41). In steady growth conditions, SSB assists replication by destabilizing attC hairpins. The frequent formation of the attC-6–SSB state should not present an obstacle for the replication machinery (42). However, as soon as IntI expression is induced, it recognizes the attC-6–SSB complex and displaces SSB even at great molar excess of SSB. As long as IntI is expressed, recombination is favoured over replication and the integron is active. This is crucial to bacteria, because the integron is their major tool for adapting to environmental stresses, for example, by acquiring antibiotic resistance genes.

In summary, we present here a regulation mechanism how attC DNA hairpins can be both genetically stable, which requires easy opening of the hairpins, and biologically functional, which requires stable folding of the hairpins. The key to this regulation is most likely an evolutionarily optimized GC-base pair distribution along the hairpin, which allows SSB to open the hairpin, while providing a recognition site for the recombining integrase IntI. In the case of attC hairpins, this regulation ensures antibiotic resistance acquisition and transmission in bacteria. There are many functional DNA hairpins in bacteria, which play critical roles during the recognition of the origin of replication, transcription, and recombination (4). We therefore anticipate that this elegant regulation mechanism is also used as a general approach by other hairpins.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.
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