The Integron Integrase Efficiently Prevents the Melting Effect of *Escherichia coli* Single-Stranded DNA-Binding Protein on Folded *attC* Sites

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Integrans play a major role in the dissemination of antibiotic resistance genes among bacteria. Rearrangement of gene cassettes occurs by recombination between *attI* and *attC* sites, catalyzed by the integron integrase. Integron recombination uses an unconventional mechanism involving a folded single-stranded *attC* site. This site could be a target for several host factors and more precisely for proteins able to bind single-stranded DNA. One of these, *Escherichia coli* single-stranded DNA-binding protein (SSB), regulates many DNA processes. We studied the influence of this protein on integron recombination. Our results show the ability of SSB to strongly bind folded *attC* sites and to destabilize them. This effect was observed only in the absence of the integrase. Indeed, we provided evidence that the integrase is able to counterbalance the observed effect of SSB on *attC* site folding. We showed that IntI1 possesses an intrinsic property to capture *attC* sites at the moment of their extrusion, stabilizing them and recombining them efficiently. The stability of DNA secondary structures in the chromosome must be restrained to avoid genetic instability (mutations or deletions) and/or toxicity (replication arrest). SSB, which hampers *attC* site folding in the absence of the integrase, likely plays an important role in maintaining the integrity and thus the recombinogenic functionality of the integron *attC* sites. We also tested the RecA host factor and excluded any role of this protein in integron recombination.

Integrans have been isolated on mobile elements responsible for the capture and dissemination of antibiotic resistance genes among Gram-negative bacteria (1). More recently, their importance has been highlighted by the discovery of integrans in the chromosomes (chromosomal integrans) of environmental strains of bacteria (2). This has led to the extension of their role from acquisition of resistance genes to a wider role in the adaptation of bacteria to different environments (3). The discovery that integron recombination was controlled by the SOS response has further connected these elements to bacterial adaptive responses (4). Integrans are composed of three key elements: an integrase gene (*intI*), which encodes a tyrosine (Y) recombinase performing the site-specific recombination (SSR) reaction; a primary recombination site (*attI*), where the incorporation of gene cassettes occurs; and a strong resident promoter (Pc) (5). Gene cassettes are devoid of promoters and correspond mostly to genes associated with an *attC* recombination site. The *attC* × *attI* recombination event catalyzed by IntI leads to cassette integration at the *attI* site, downstream of the Pc promoter, allowing gene cassette expression (6). IntI can also catalyze recombination between two consecutive *attC* sites, leading to cassette excision.

The structures of *attI* and *attC* sites differ significantly from that of the canonical Y-recombinase core sites (for a review, see reference 7), and integron site-specific recombination is described as an atypical mechanism, in comparison to the classical SSR largely used in bacteria for movement of mobile genetic elements such as prophages or genomic islands (8).

The major distinctive characteristic of integron SSR comes from the properties of the *attC* sites. Indeed, it was previously shown that the *attC* site recombines as a single-stranded DNA (ssDNA) folded structure, in contrast to the *attI* site, which is recombined in a double-stranded form (9). The length of natural *attC* sites varies from 57 to 141 bp. These sites include two regions

of inverted homology, R'-L' and L'-R', separated by a central region that is highly variable in length and sequence (Fig. 1A) (10). In contrast to the heterogeneity of their sequences, *attC* sites display a strikingly conserved palindromic organization (11) that can form secondary structures through DNA strand self-pairing (Fig. 1B). Upon folding, single-stranded *attC* sites show a structure resembling a canonical core site consisting of R and L boxes separated by an unpaired central segment (UCS), two or three extrahelical bases (EHBs), and a variable terminal structure (VTS), (12). The VTS varies in length among various *attC* sites, from 3 predicted unpaired nucleotides, such as for *attC_{aadA7}*, to a complex branched secondary structure in larger sites such as VCR (*Vibrio cholerae* repeat) sites (Fig. 1B).

It has been shown using a DNA-binding assay that IntI1 binds strongly and specifically to the bottom strand of *attC* ssDNA (13). *In vivo*, it has also been shown that only the bottom strand of the *attC* site is used as a substrate during gene cassette integration (9), thereby creating an atypical Holliday junction (aHJ) between the single-stranded *attC* and the double-stranded *attI* sites. We recently proved the implication of replication in the resolution of the aHJ and definitively ruled out the involvement of a second strand exchange of any kind in the *attC* × *attI* reaction (14).

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This work is dedicated to the memory of Guy Duval-Valentin.

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FIG 1 *attC* recombination sites and a model for *attC* folding. (A) Schematic representation of a double-stranded (ds) *attC* site. Inverted repeats R'', L'', L', and R' are indicated by gray boxes. The dotted line represents the variable central part. Conserved nucleotides are indicated. The asterisk shows the conserved G nucleotide, which corresponds to the extrahelical base (EHB) in the folded *attC* site bottom strand. The black arrow shows the cleavage point. (B) Secondary structures of VCR_{2/1} and *attCaadA7* site bottom strands (bs). Structures were determined by the UNAFOLD online interface at the Pasteur Institute. The four structural features of *attC* sites, namely, the UCS (unpaired central segment), the EHB, the stem, and the VTS (variable terminal structure), are indicated. The black arrow shows the cleavage points, and the asterisk shows the extrahelical G bases. Primary sequences of the *attC* sites are shown (except for the VTS of the VCR_{2/1} site). (C) Representation of both ssDNA and dsDNA pathways for *attC* site folding. In the ssDNA pathway (1) (red), horizontal gene transfer (conjugation, transformation, and phage infection) and replication (template of lagging strand) lead to the production of ssDNA favoring *attC* hairpin formation. In the dsDNA pathway (2) (green), an increase of supercoiling ensures cruciform extrusion. Origins of replication are shown as blue ovals, and replication complexes are shown as yellow ovals.

We have previously shown that cellular bacterial processes delivering ssDNA, such as conjugation and replication, favor the proper folding of *attC* sites ("ssDNA pathway") (Fig. 1C) (14, 15). By developing a very sensitive *in vivo* assay, we also provided evidence that *attC* sites can recombine as cruciform structures by extrusion from double-stranded DNA (dsDNA) ("dsDNA pathway") (Fig. 1C) (14). Moreover, we showed an influence of DNA superhelicity on *attC* site extrusion and recombination *in vitro* and *in vivo* (14). We demonstrated that the proper folding of *attC* sites depends on both the propensity to form nonrecombinogenic structures and the length of their VTS.

Since integron recombination uses *attC* sites in a single-stranded form, the host cell factors that have affinity toward single-stranded DNA can potentially regulate integron recombina-

tion by acting on *attC* sites, impairing their folding and/or impeding the integrase activity.

In this paper, we examine the effects of several of these specific host factors on *attC* site folding. Among them, we chose to investigate the effect of single-stranded DNA-binding proteins (SSBs) and RecA.

Escherichia coli SSB is essential for cell viability (16). During replication, SSB binds tightly and cooperatively to ssDNA to prevent premature annealing. Indeed, single-stranded DNA has a natural tendency to revert to the double-stranded form, but SSB binds to the single strands, keeping them separate and allowing the DNA replication machinery to proceed (for a review, see reference 17). In addition, SSB is implicated in several other cellular processes, such as homologous recombination, mismatch repair, and excision repair (for a review, see reference 16). Moreover, recent studies found that SSB is not static when bound to DNA but could migrate randomly on single-stranded DNA. Indeed, SSB diffusion can melt short DNA hairpins and, as a consequence, remove these secondary structures spontaneously formed by DNA (18). This function is biologically important because RecA, which is necessary for recombinational DNA repair, does not bind well to secondary structures formed in ssDNA. SSB, through its ability to destabilize the intramolecular DNA base pairing within ssDNA, allows RecA to form a contiguous filament on the DNA (19).

RecA, a multifunctional DNA-binding protein with a ubiquitous distribution in nature, plays a central role in both homologous recombination and postreplicative DNA repair mechanisms (see above). In bacteria, it also plays a role in the SOS response as a coprotease for the autocatalytic cleavage of the LexA repressor, leading to derepression of the SOS regulon genes (20). The RecA protein binds strongly, and in long clusters, to ssDNA to form a nucleoprotein filament.

Since integron recombination uses a folded single-stranded *attC* site, we hypothesized that SSB could potentially act on the folded *attC* site and ultimately on integron recombination. SSB could bind parts of the folded *attC* site that remain as single-stranded DNA (i.e., the UCS and/or VTS) (Fig. 1B), thus destabilizing/unpairing recombinogenic *attC* structures and impeding integrase activity. We also studied the role of RecA in integron recombination.

By using a gel retardation assay, we showed that SSB can bind folded *attC* sites. Moreover, a replication slippage assay allowed us to conclude that this binding induces the destabilization of *attC* site folding *in vivo*. Interestingly, this effect of SSB was not observed in the presence of the integron integrase (Int11). We also provide evidence that, contrary to SSB, Int11 possesses an intrinsic property to capture *attC* sites at the moment of their extrusion, stabilizing and recombining them efficiently.

The stability of secondary structures in the cell must be limited to avoid genetic instability (mutations or deletions) and/or toxicity (replication arrest). SSB is able to restrain the formation of *attC* secondary structures in the absence of the integrase, ensuring the integrity of integron *attC* sites when recombination is not happening. We also demonstrated that, when expressed, the integrase is able to counterbalance this effect of SSB even when SSB is overexpressed.

By using a set of similar experiments, we demonstrated that RecA does not regulate integron recombination. We conclude

that the interplay between SSB and the *attC* sites represents another example of host regulation of *attC* site folding.

MATERIALS AND METHODS

Bacterial strains, plasmids, oligonucleotides, and media. Bacterial strains, plasmids, and oligonucleotide primer sequences are described in Tables S1, S2, and S3 in the supplemental material, respectively. *Escherichia coli* strains were grown in Luria-Bertani broth (LB) at 37°C. The following antibiotics were used: ampicillin (Ap) at 100 µg/ml, chloramphenicol (Cm) at 25 µg/ml, kanamycin (Km) at 25 µg/ml, spectinomycin (Sp) at 50 µg/ml, and tetracycline (Tc) at 15 µg/ml. 2,6-Diaminopimelic acid (DAP) was supplemented when necessary to a final concentration of 0.3 mM. Glucose and L-arabinose were added at final concentrations of 10 and 2 mg/ml, respectively. Chemicals were obtained from Sigma-Aldrich (France).

Integron cassette excision assay. (i) SSB overexpression assay. The two plasmids pBAD::intI1 (p7383) (Km resistant [Km^r]) and pHYD620 (p7305) (Sp^r) (which carries the *ssb* gene on a pSC101 vector; approximately 5 copies/cell) were introduced by transformation into an MG1655 Δ dapA derivative strain, which contains an insertion (in the *attB* lambda site) of a plasmid carrying a *dapA* gene interrupted by the two specific *attI1* and *attC_{aadA7}* sites (pB117 [strains ω B768 and ω C021]) (see Tables S1 and S2 in the supplemental material), *attC_{aadA7}* and *attC_{ereA2}* sites (pB647 [ω B661 and ω B726]), and *attI1* and *attII* sites (pB119 [ω B662 and ω B735])). These strains are unable to synthesize DAP, and as a result, they are not viable without DAP supplemented in the medium. Recombination between *att* sites causes excision of the synthetic cassette, restoring a functional *dapA* gene and allowing the strain to grow on DAP-free medium. As controls, we also transformed this MG1655 Δ dapA strain with the previously used pBAD::intI1 plasmid and with the control vector pCL1920 devoid of the *ssb* gene (p8008 [Sp^r] [ω C020, ω B725, and ω B734])). We also transformed MG1655 Δ dapA strains by pCL1920 or pHYD620 but without pBAD::intI1 (ω C018, ω C019, ω B723, ω B724, ω B732, and ω B733).

After overnight growth in the presence of appropriate antibiotics (Sp, Cm, and Km), DAP, and 1% glucose, strains were cultivated for 6 h in the presence of 0.2% arabinose to allow *intI1* expression and plated onto agar containing either LB or LB plus DAP. Recombination activity was calculated as the ratio of the number of cells growing in the absence of DAP over the total number of cells.

(ii) RecA overexpression assay. In order to test RecA overexpression in the integron cassette excision assay, we proceeded exactly as described above for the SSB overexpression assay, but we transformed pGB2-ara-RecA_{Eco} (pB981 [Sp^r], which carries the *recA* gene on a pSC101 vector) in place of pHYD620 (ω C071, ω C069, ω B720, ω B722, ω B729, and ω B731) and the control vector pGB2 (pB495) in place of pCL1920 (ω C070, ω C068, ω B719, ω B721, ω B728, and ω B730).

(iii) *ssb-200* and *recA269::Tn10* assays. In order to test the effects of SSB and RecA inactivation in the integron cassette excision assay, we proceeded exactly described above for the SSB and RecA overexpression assays. pBAD::intI1 (p7383) (Km^r) was introduced by transformation into MG1655 Δ dapA, MG1655 Δ dapA *ssb-200*, and *recA269::Tn10* derivative strains, which contain an insertion (in the *attB* lambda site) of a plasmid carrying a *dapA* gene interrupted by the two specific *attI1* and *attC_{aadA7}* sites (pB117 [ω B768, ω B769, ω B149, ω B848, ω B849, and ω B645])) (see Tables S1 and S2 in the supplemental material), *attC_{aadA7}* and *attC_{ereA2}* sites (pB647 [ω B661, ω B771, ω B660, ω B718, ω B851, and ω B717])), and *attI1* and *attII* sites (pB119 [ω B662, ω B770, ω B150, ω B727, ω B850, and ω B646])).

Replication slippage assay. To determine reversion frequencies, 100 ml of *E. coli* strains GJ1885 and GJ1890 (GJ1885 *ssb-200*) (ω 6737 and ω 6738), transformed by the pBR derivative plasmids (p5087, p5088, p6671, p6672, p8558, and p8559), was grown to an optical density at 600 nm (OD₆₀₀) of 0.8 in LB medium containing ampicillin from 4 ml of a culture grown overnight. Reversion frequencies were calculated as the

number of Cm^r cells in the population, which was determined by plating the equivalent of 90 ml of cells onto medium containing ampicillin and chloramphenicol. Total viable cells were determined by plating onto medium containing ampicillin. The frequency represents the mean of 4 independent experiments. The partial or complete deletion of the *attC* site was confirmed for several Cm^r reversion events by sequencing. To study the effect of the integrase on reversion frequency, we performed the same experiment by employing the previously used GJ1885 and GJ1890 strains. We transformed them with the pBAD::intI1Y312F plasmid (p8741) (Sp^r). To determine reversion frequencies, 100 ml of these strains (ω 8749, ω 8752, and ω 8755) was grown to an OD₆₀₀ of 0.8 in LB medium containing ampicillin and kanamycin with 1% glucose (integrase gene repression) or 0.2% arabinose (integrase gene expression) from 4 ml of a culture grown overnight.

Protein purification. Bacterial strain BL21(DE3)/pLysS (21) was transformed with pET-derived plasmids expressing the His-IntI integrase (p4634 [ω C004]). Protein purification was carried out as described previously by Johansson and collaborators (22) and with the minor modifications described previously by Demarre and collaborators (23). *E. coli* SSB was provided by Sigma (product code S3917).

Electrophoretic mobility shift assay. SSB was incubated with 0.6 pmol of ³²P-labeled DNA oligonucleotides corresponding to the bottom strands of the wild-type (wt) (ω 32) or the synthetic paired (ω 42) or the synthetic unpaired (ω 36) VCR_{2/1} site derivative (see Tables S2 and S3 in the supplemental material). The electrophoretic mobility shift assay (EMSA) was carried out as described previously by Frumerie and collaborators (24). Quantifications were made by using Image Gauge version 4.0.0 (Fuji Photo Film Co. Ltd.), manually defining the lanes and using the automatic peak search function. All other functions were at default values.

***attC* site folding assay.** The 100-bp double-stranded *attI1*-containing fragment was generated by annealing oligonucleotide attI1-F with the complementary oligonucleotide attI1-R. The 120-bp double-stranded *aadA7* fragment was obtained by PCR amplification using the p4136 vector as the template and primers aadA7-F and aadA7-R. The 131-bp double-stranded VCR fragment was obtained by PCR amplification using the p1880 vector and primers VCR-F and VCR-R. The 119-bp double-stranded *aadA7* mutant fragment was obtained by PCR amplification using the p4250 vector as the template and primers aadA7-F and aadA7-R.

Under standard conditions, the melting temperature of a 50-µg/ml solution of each fragment was measured as the OD₂₆₀ in a thermostatic spectrophotometer at 37°C. Fragments were prechilled to 4°C and preheated to 95°C. To analyze the effect of IntI1, proteins were added to the fragment solution 30 min after the beginning of the measurement. The OD₂₆₀ measurement of the proteins alone was previously performed and subtracted from the measurement obtained with the mixture in order to determine accurate values of the fragment solution.

RESULTS

SSB binds folded *attC* sites. It was shown previously that single-stranded DNA-binding protein (SSB) destabilizes secondary structures in ssDNA during major cellular processes such as DNA replication, repair, and homologous recombination (19, 25, 26). We therefore hypothesized that SSB can bind folded *attC* sites and destabilize them. To determine if this was the case, we investigated the binding capacity of SSB on folded *attC* sites using an electrophoretic mobility shift assay (EMSA). In order to mimic the *attC* site's bottom strand, we used a VCR derivative bottom-strand (VCR₄bs) substrate, a single-stranded oligonucleotide of ~90 bases containing a shorter version of the VCR_{2/1}bs site (23). In this substrate, the central part of the site (VTS) was removed and replaced with GAA (Fig. 2A), a modification previously described and shown to be fully recombinogenic (23).

As controls, we constructed two other synthetic *attC* sites, namely, the unpaired and paired VCR₄bs site derivatives. To ob-

FIG 2 Comparison of SSB binding to several VCR₂₇₁ site derivatives. (A) Nucleotide sequences and secondary structures of the bottom strands (bs) of VCR₂₇₁ site derivatives. Structures were determined by the UNAFOLD online interface at the Pasteur Institute. VCR_dbs is a VCR derivative used in EMSA in which the variable terminal structure (VTS) was replaced by GAA. The black arrow shows the cleavage point in the R box, and the asterisk shows the extra-helical G nucleotide. We introduced mutations (boldface type) into this VCR_dbs site in order to generate, after folding, paired (pd) and unpaired (unpd) VCR_dbs site derivatives. The ΔG (free energy) values of VCR_dbs, paired VCR_dbs, and unpaired VCR_dbs folding are -21.7 , -37.2 , and $-5.3 \text{ kcal mol}^{-1}$, respectively. (B) EMSA of the binding of SSB to VCR₂₇₁ site derivatives. Equal quantities of radiolabeled VCR site derivative DNA fragments (0.6 pmol) were incubated with increasing amounts (0 , 13 , 26 , 52 , and 104 nM) of SSB. Complexes I and II probably represent several degrees of SSB multimerization. (C) Densitometry analysis of EMSA. Binding of SSB was measured by monitoring the fraction ($\times 100$) of the labeled DNA substrate bound by SSB in complexes I and II to labeled (bound and free) DNA, detected by an automatic peak search of Image Gauge.

tain a fully paired VCR_dbs site derivative, we introduced specific mutations in the unpaired-strand parts of the folded VCR_dbs site in order to introduce complementarity and pair them. Finally, to obtain the unpaired VCR_dbs derivative, we introduced specific mutations in the double-stranded parts of the folded VCR_dbs site in order to unpair them (Fig. 2A).

The results from EMSA are presented in Fig. 2B. In the presence of SSB, distinct shifted bands (bands I and II), corresponding to SSB-DNA complexes, can be seen for both VCR_dbs and un-

paired VCR_dbs site derivatives. Bands I and II probably correspond to different SSB-binding modes and/or different types of intertetramer cooperativity (see Discussion). As expected, for the paired VCR_dbs derivative, we failed to observe distinct and significantly shifted bands. The band intensities on the gels were quantified, confirming the ability of SSB to bind single-stranded parts of *attC* sites very efficiently (Fig. 2C). We observed, among free DNA, higher electrophoretic migration bands in the VCR_dbs and paired VCR_dbs derivative lanes. Note that these supplementary bands probably correspond to DNA dimer formation favored by the presence of inverted repeats. The presence of these DNA dimers does not alter the interpretation of the results.

SSB destabilizes folded *attC* sites. We hypothesized that the consequence of SSB binding on the folded *attC* sites could be a destabilization effect. Indeed, Reddy and collaborators have previously shown the ability of SSB to efficiently and rapidly bind stem-loops, melting these secondary structures and facilitating the binding of RecA (26). To study this, we developed a replication slippage assay. Indeed, a direct correlation between the stability of secondary structures and the frequency of replication slippage was demonstrated previously (27). The model proposed is that the formation of a stem-loop structure between a pair of flanking direct sites during replication permits a slippage event. This results in precise or nearly precise loss of the structured region. As SSB is essential in *E. coli*, the slippage assays were performed in the wild-type SSB context or with an *ssb-200* mutant. The *ssb-200* mutation corresponds to a G→A substitution at nucleotide 41, resulting in an amino acid substitution at position 4 (G4D). This mutation induces a *tex* (transposon excision) phenotype, leading to an increase in the precise excision frequency of a Tn10 derivative (Tn10dKan) (28). Interestingly, Reddy and collaborators showed that *tex* of the *ssb-200* mutant was not affected in any other functions mediated by SSB, such as replication, recombination, or repair. This suggests that the observed *tex* phenotype of this mutant is probably due only to an incapability of the SSB-200 protein to destabilize stem-loop structures formed by the inverted repeats of Tn10dKan. Thus, if the previously observed binding of SSB on the *attC* folded sites induces their melting, it is very likely that such an effect would not be observed with SSB-200.

We constructed a pBR325 derivative plasmid carrying an *attC*_{aadA7} site inserted into the *cat* resistance gene in such way that the loss of this *attC* site by “replication slippage” would lead to the reconstitution of a functional *cat* gene (p5087) (see Materials and Methods) (Fig. 3A). This plasmid was transformed into an *ssb-200* mutant strain ($\omega 6738$ [28]) and into its *ssb* wt parental strain, GJ1885 ($\omega 6737$ [28]). The Cm^r reversion frequency obtained for the parental strain was 5.5×10^{-11} . Interestingly, the frequency of reversion for the *ssb-200* strain was approximately 14-fold higher (7.6×10^{-10}) (Fig. 3B). Note that several of the Cm^r clones were sequenced in order to confirm deletion of the *attC* sites (Fig. 3B). These results suggest that SSB reduces the *attC* site loss mediated by replication slippage events, probably by binding and destabilizing the single-stranded folded *attC* site. To confirm that the obtained frequency of replication slippage is not influenced by the local context (transcription, replication, and nucleotide sequences), we performed a set of experimental controls. *attC* sites were cloned into further pBR derivatives in both orientations within the *cat* resistance gene. Moreover, the *cat* gene was cloned in both orientations on the plasmid (see Materials and Methods). In all cases, we obtained the same order of Cm^r reversion frequencies for the four tested construc-

FIG 3 *In vivo* effects of SSB and IntI1 on *attC* site folding. (A) Experimental setup of the replication slippage assay. The model proposed is that folding of the *attC* site between the pair of flanking direct repeats (EcoRI restriction sites) (black rectangles) permits a replication slippage event. This results in precise or nearly precise *attC* deletion, thus reconstituting a functional *cat* resistance gene (*Cm^r* reversion frequencies). The precise *attC* deletion was measured by sequencing (*attC* deletion frequencies). (B) Effects of SSB and IntI1 on *Cm^r* reversion and *attC* deletion frequencies (see Materials and Methods). The replication slippage assay was used to study the effects of IntI1Y312F and SSB on *attC_{aadA7}* site folding. The *Cm^r* reversion frequencies and *attC* deletion frequencies are represented in the absence (–) and in the presence (+) of IntI1 for the GJ1885 wild-type strain and the GJ1885 *ssb-200* strain (GJ1890). The results represent means of four independent experiments (see Materials and Methods). Error bars show the standard deviations.

tions in the wt context and in the *ssb-200* context (data not shown, and see Materials and Methods; see also Tables S1 and S2 in the supplemental material). As an additional control, we constructed a synthetic derivative of the *attC_{aadA7}* site presenting a lower free energy of single-stranded *attC* site folding ($\Delta G = -5.1$ kcal mol⁻¹) than the natural *attC_{aadA7}* site ($\Delta G = -19.1$ kcal mol⁻¹). For this, we introduced specific mutations into the double-stranded parts of the natural *attC_{aadA7}* site in order to unpair them. We called this synthetic *attC* site an unpaired *attC_{aadA7}* site derivative (Fig. 4A). As with the *attC_{aadA7}* site, we also observed a decrease in *attC* deletion frequencies in the *ssb-200* context (~13-fold) (ω8609) compared to the wt context (ω8605) (Fig. 4B). This result shows that the probable destabilization effect of SSB is not only on the *attC* sites but also more generally on single-stranded folded secondary structures. We also cloned the unpaired *attC_{aadA7}* site derivative in the opposite orientation within the *cat* resistance gene, and we obtained the same order of *Cm^r* reversion and *attC* deletion frequencies for this construct in both the wt context (ω8604) and the *ssb-200* context (ω8608) (data not shown).

Altogether, these assays confirmed that SSB can destabilize folded *attC* sites in a nonspecific manner.

FIG 4 Specificity of the *in vivo* effects of SSB and IntI1 on *attC* site folding. (A) Secondary structures of the *attC_{aadA7}* site bottom strand (bs) and the unpaired *attC_{aadA7}* site derivative. Structures were determined by the UNAFOLD online interface at the Pasteur Institute. The black arrow shows the cleavage point. Primary sequences of the *attC* sites are shown. The ΔG (free energy) values of *attC_{aadA7}* bs and unpaired *attC_{aadA7}* bs folding are -19.1 and -5.1 kcal mol⁻¹, respectively. (B) The replication slippage assay was used to study the effect of SSB on folding of the *attC_{aadA7}* site (black bar) and the unpaired *attC_{aadA7}* site derivative (gray bar). The relative deletion rates obtained for the *ssb-200* strain versus GJ1885 (*ssb*-WT) are indicated. The results represent means of four independent experiments. (C) The replication slippage assay was used to study the effect of IntI1Y312F on folding of the *attC_{aadA7}* site (black bar) and the unpaired *attC_{aadA7}* site derivative (gray bar). The relative deletion rates obtained in the presence of integrase versus in its absence (*intI1*+/+/*intI1*-) are indicated. The results represent means of four independent experiments.

The integrase stabilizes *attC* site folding. We showed a destabilizing effect of SSB on folded *attC* sites, in the absence of integrase. As the integrase likely stabilizes the folded *attC* structure, we could expect this effect to be counterbalanced in the presence of this protein. In order to test this hypothesis, we assessed the effect of IntI1 expression on the slippage frequency in the assay described above. In strain GJ1885 (ω6791), we introduced plasmid p8741 carrying the *intI1* gene under the control of the inducible *P_{bad}* promoter (ω8749). We chose to use an *intI1* gene mutated for the catalytic tyrosine (Y312F) in order to observe the effect of IntI1 binding without an influence of potential recombination events that could occur between copies of the plasmid. It was previously shown that when replacing the reactive nucleophilic tyrosine with the inert phenylalanine (IntI1Y312F), the ability of the integrase

FIG 5 *In vitro* effect of IntI1 on *attC_{aadA7}* (A), *VCR_{2/1}* (B), *attI1* (C), and *attC_{aadA7m}* (D) site folding. The OD₂₆₀ of each fragment (50 μ g/ml) was measured at 37°C either after prechilling (prechilled *attC_{aadA7}*, *VCR_{2/1}*, *attI1*, and *attC_{aadA7m}*) or after preheating for 3 min at 95°C (preheated *attC_{aadA7}*, *VCR_{2/1}*, *attI1*, and *attC_{aadA7m}*) in the absence of IntI1 as well as after the addition of IntI1 (2 pmol) 30 min after the beginning of the measurement with prechilling (prechilled *attC_{aadA7}* + IntI1, *VCR_{2/1}* + IntI1, *attI1* + IntI1, and *attC_{aadA7m}* + IntI1) or after preheating at 95°C (preheated *attC_{aadA7}* + IntI1, *VCR_{2/1}* + IntI1, *attI1* + IntI1, and *attC_{aadA7m}* + IntI1). The OD₂₆₀ measurement of the proteins alone was previously performed and subtracted from the measurements obtained with the mixture in order to determine accurate values for the fragment solution. Results represent means of three independent experiments. Error bars show the standard deviations.

to attack the DNA was lost, but the *attC* site binding capability was preserved (22). The results showed that in the IntI1Y312F expression context (arabinose induction), the Cm^r reversion and *attC* deletion frequencies were 25-fold higher than those in the absence of the integrase (glucose repression) (Fig. 3B and 4C). This suggests that IntI1, contrary to SSB, increases the *attC* site loss mediated by replication slippage events, probably by stabilizing folded *attC* sites. To confirm that this observed stabilizing effect of IntI1 on *attC* folding is specific to *attC* sites, we performed the same experiment by using the previously used unpaired *attC_{aadA7}* derivative (Fig. 4A), which theoretically lost its IntI1-binding boxes (ω 8752). As expected in this case, we obtained a very low and probably nonsignificant stabilizing effect of IntI1 (6-fold higher) compared to that of the wild-type *attC_{aadA7}* site (25-fold higher) (Fig. 4C). We demonstrated here that the integron integrase stabilizes the folding of *attC* sites in a specific manner.

In vitro study of effects of IntI1 and SSB on *attC* site folding.

In order to specify the effect of the integrase on *attC* site folding, we performed additional *in vitro* experiments. Indeed, it has been shown that IntI1 possesses the intrinsic property to efficiently catalyze strand transfer *in vitro* between *attC* fragments in the absence of other bacterial proteins, suggesting the ability of the integrase to favor *attC* site folding or to stabilize it (29, 30). To

confirm this observation, we set up an *in vitro* folding assay to allow us to easily monitor the single-stranded or double-stranded folding of short DNA fragments containing *attC* sites. The variations in optical density measured at 260 nm (OD₂₆₀) directly reflect the DNA hybridization modifications. Indeed, the OD₂₆₀ was comparatively lower when the DNA fragment was in the double-stranded form rather than in the single-stranded form. The preheated *attC_{aadA7}* fragment led to an OD₂₆₀ close to 1.2, corresponding to the fully ssDNA form of the fragment (Fig. 5A, red), and the prechilled *attC_{aadA7}* fragment led to an OD₂₆₀ close to 0.6, corresponding to the fully dsDNA form (Fig. 5A, blue). By stabilizing the temperature to 37°C, the OD₂₆₀ of the preheated fragment decreased to 0.9, corresponding to the partially dsDNA form of the fragment. We also obtained the same partially dsDNA structure from the prechilled fragment. These results were also reproduced by using *VCR_{2/1}*-containing fragments (Fig. 5B) but not for *attI1* sites (sites not forming a hairpin structure) (Fig. 5C). This confirms that *attC* sites share a partially dsDNA form, in contrast to *attI1*, which remains fully double stranded. Addition of IntI1 to both prechilled and preheated *attC_{aadA7}* (Fig. 5A) and *VCR_{2/1}* (Fig. 5B) fragments led to an OD₂₆₀ close to 1.2, suggesting that the enzyme favors the formation of the fully single-stranded folded *attC* site and stabilizes this structure. In contrast, no such effect

FIG 6 *In vitro* effect of SSB on *attC_{aadA7}* (A) and *VCR_{2/1}* (B) site folding. The OD₂₆₀ of each fragment (50 μ g/ml) was measured at 37°C either after prechilling (prechilled *attC_{aadA7}* and *VCR_{2/1}*) or after preheating for 3 min at 95°C (preheated *attC_{aadA7}* and *VCR_{2/1}*) in the absence of SSB as well as after addition of SSB (2 pmol) 30 min after the beginning of the measurement with prechilling (prechilled *attC_{aadA7}* + SSB and *VCR_{2/1}* + SSB) or after preheating at 95°C (preheated *attC_{aadA7}* + SSB and *VCR_{2/1}* + SSB). The OD₂₆₀ measurement of the proteins alone was previously performed and subtracted from the measurements obtained with the mixture in order to determine accurate values for the fragment solution. Results represent means of three independent experiments. Error bars show the standard deviations.

was found by using the *attI1* site, suggesting that this activity of IntI1 is highly specific to *attC* sites. As a supplementary control, we tested a mutated *attC_{aadA7}* site (*attC_{aadA7m}*) (Fig. 5D) where the extrahelical G base has been deleted in order to prevent the specific binding of IntI1 on the L box (Fig. 1B) (22). As expected, by using this mutated site, we failed to observe any influence of IntI1 on *attC* site folding, highlighting the specificity of the IntI1 properties for the *attC* sites.

Interestingly, when SSB was used instead of IntI1, perturbation of the fragments' folding was observed only for preheated DNA. This suggests that the protein binds the single-stranded fragments, keeps them in a single-stranded form (Fig. 6A and B), and does not intervene in the *attC* folding property, as observed with IntI1. This also highlights the specificity of the effect observed with IntI1, confirming that the folding effect was due to an intrinsic property of the enzyme to favor and stabilize the folding of DNA strands and not to an unspecific DNA avidity.

In conclusion, while both SSB and IntI1 bind the folded *attC* site, the consequences of their binding would be antagonistic, since SSB seems to destabilize the folded *attC* site, while IntI1 assists with *attC* site folding.

SSB does not modulate integron recombination. We tested the recombination of *attC* sites under conditions of SSB overexpression and in an *ssb-200* mutant context. We used an excision assay described previously (31). This assay allowed us to directly measure *attC* recombination through restoration of the *dapA* reading frame. In an MG1655 Δ *dapA* strain, we inserted a plasmid containing a *dapA* gene interrupted by two specific recombination sites, *attI1* and *attC_{aadA7}* (ω B768) (Fig. 7A), *attC_{aadA7}* and *attC_{ereA2}* (ω B661), or *attI1* and *attI1* (ω B662) (see Table S1 in the supplemental material). Integrase expression causes site-specific recombination and excision of the synthetic cassette, restoring a functional *dapA* gene and allowing these strains to grow on DAP-free medium (Fig. 7A). We transformed these strains with the pBAD::intI1 multicopy plasmid carrying the *intI1* gene under the control of the P_{bad} promoter (p7383) and a second plasmid, p7305 (pHYD620 from reference 28), containing the *ssb* gene, ensuring the overexpression of the SSB protein. pCL1920, the pHYD620 progenitor plasmid (p8008) (devoid of the *ssb* gene), was used as a control. Integrase-driven recombination frequencies were calculated as the ratio of the number of cells growing in the absence of DAP over the total number of cells. Results are presented in Fig. 7B.

The frequencies of excision in the absence (vector) and presence (*ssb*⁺) of SSB overexpression were significantly similar for each tested *att* combination. These results show that SSB overexpression does not affect integron recombination.

We also tested the recombination of these three *att* combinations in the above-described *ssb-200* context (ω B769, ω B771, and ω B770). For this, we constructed an MG1655 Δ *dapA* *ssb-200* strain (ω B704) (see Table S1 in the supplemental material). Results are presented in Fig. 7B. Once more, excision frequencies in the wt and *ssb-200* strains were similar for each tested *att* combination. These results show that SSB did not affect integron recombination.

As a control, we also performed both assays described above in the absence of integrase expression (Fig. 7B). We observed a few recombination events only for the *attI1* $\times$ *attI1* reaction, probably due to slippage events and/or homologous recombination between both identical 58-bp-long *attI1* sites.

Effect of RecA on integron recombination. We tested the recombination of *att* sites under conditions of RecA overexpression (*recA*⁺) and in a *recA*-deleted mutant. Tests were performed by using the above-described *in vivo* excision assay. We also tested the recombination of the three previously used *att* combinations (see Materials and Methods; see also Tables S1 and S2 in the supplemental material). The frequencies of excision in the RecA overexpression conditions and *recA*-deleted strains are significantly similar for each tested *att* combination (Fig. 8). These results show that RecA does not affect integron recombination.

We also performed these assays in the absence of integrase expression (Fig. 8). As in the SSB studies, we observed recombination events only for the *attI1* $\times$ *attI1* combination, probably due to slippage events and/or homologous recombination (only in the *recA*⁺ strains) between both *attI1* sites.

FIG 7 *In vivo* effect of SSB on cassette excision. (A) Experimental setup of the cassette excision assay. The *dapA* gene is interrupted by a synthetic cassette (black line) encountered by the *attI1* (black triangle) and *attC*_{aadA7} (white triangle) sites. Recombination leads to the excision of the integron cassette and restores a functional *dapA* gene. (B) Excision frequencies of the *attI1* × *attI1*, *attC*_{aadA7} × *attC*_{ereA2}, and *attI1* × *attC*_{aadA7} cassettes in the WT or *ssb-200* strain (bottom) and in the presence of the pCL1920 control vector (vector) or the pHYD620 plasmid (*ssb*⁺), which overexpresses the SSB protein (bottom). Black bars represent the results obtained in the presence of IntI1, and gray bars represent those obtained in the absence of IntI1. “nd” indicates “nondetected” recombination events. Results represent means of three independent experiments. Error bars show the standard deviations.

FIG 8 *In vivo* effect of RecA on cassette excision. Shown are excision frequencies of the *attI1* × *attI1*, *attC*_{aadA7} × *attC*_{ereA2}, and *attI1* × *attC*_{aadA7} cassettes in the WT or *recA*-deleted strain (bottom) and in the presence of the pGB2 control vector (vector) or the pGB2-*recA* plasmid (*recA*⁺), which overexpresses the RecA protein (top). Black bars represent the results obtained in the presence of IntI1, and gray bars represent the results obtained in the absence of IntI1. “nd” indicates “nondetected” recombination events. Results represent means of three independent experiments. Error bars show the standard deviations.

DISCUSSION

Biological functions of secondary structures. It is now obvious that DNA is not always present in its canonical double-stranded form but can also form secondary structures through intrastrand base pairing. These structures are strongly favored in single-stranded DNA and can also be extruded from dsDNA at a lower rate. These structures have been found to be involved in biological functions (15). Indeed, hairpins play an essential role in initiation of replication and transcription and are present in a majority of origins of transfer (*oriT*), ensuring conjugation (15). Moreover, to date, there are three examples of recombination systems using secondary structures as the substrates: *attC* integron recombination (9), CTX-phage integration (32), and IS608 transposition (33). In all of the examples described above, secondary structures could represent a supplementary way to expand information storage in DNA, in addition to the primary base sequence.

It is also known that palindromes that are too long and too stable pose a threat to genome stability. Indeed, they cannot be maintained *in vivo* (for a review, see reference 15), either because they are inviable, i.e., intrinsically toxic to the cell, or because they are genetically unstable, i.e., partially mutated or deleted (34). It is assumed that inviability is caused by an arrest of the replication fork, as it is unable to process these secondary structures, and that instability is caused by the presence of proteins, such as SbcCD, destroying these structures. This leads to constraints on the size and perfection of the inverted repeats that can be maintained *in vivo*.

Single-strand DNA-binding proteins. Hairpin formation in the cell is most likely to occur in the presence of ssDNA in the cell. The presence of ssDNA is not merely an intermediate state between its functional double-stranded forms. Indeed, ssDNA can be found in the cell during many of its physiological processes, such as lagging-strand synthesis during replication and DNA repair, but ssDNA is also tightly connected to horizontal gene exchange in bacteria, i.e., in bacterial conjugation, natural transformation, or viral infections. When produced, cellular ssDNA is not left naked; several proteins, such as RecA and SSB, bind it without sequence specificity. The best-characterized SSB is the one from the bacterium *E. coli*. SSB prevents premature annealing, stabilizes and protects the single-stranded DNA, and also removes the secondary structures (16, 17). It acts as a homotetramer that can migrate via random walk-along ssDNA, providing a mechanism by which it can be repositioned along ssDNA while remaining tightly bound (18). SSB can form different complexes and bind ssDNA with several binding modes, depending on the number of nucleotides bound per tetramer [(SSB)_n; $n = 35, 56, \text{ or } 65$]. ssDNA interacts with only two SSB subunits in the (SSB)₃₅ complex and with all four subunits in the (SSB)₆₅ complex, resulting in different extents of ssDNA compaction. The binding mode defines several intertetramer cooperativities: in the (SSB)₃₅-binding mode, cooperativity is “unlimited,” and tetramers can form long protein clusters, while in the (SSB)₆₅-binding mode, only dimers of tetramers (octamers) can be formed (“limited” cooperativity) (17). Moreover, SSB directs RecA binding to ssDNA and ensures the directionality of RecA polymerization across stem-loop structures in ssDNA (19), (26). Indeed, the RecA protein also binds ssDNA, forming a nucleoprotein filament. Recent single-molecule studies have shown how SSB can spontaneously migrate along ssDNA, melting unstable hairpins while stimulating RecA filament elongation (18).

In this paper, we were interested in examining the role of these ssDNA-binding proteins, SSB and RecA, in integron recombination and, more specifically, the role of SSB in *attC* site folding. Whereas SSB and RecA are not involved in integron recombination, we demonstrate here the involvement of SSB in the modulation of *attC* site folding in the absence of recombination.

The RecA protein does not regulate integron recombination.

In order to test the effect of RecA on integron recombination, *in vivo* tests were performed by using the integron cassette excision assay where the recombination sites are carried by the chromosome. Note that we also performed supplementary tests by using our suicide conjugation and replicative assays (data not shown). While the conjugation assay permits the delivery of *attC* sites as ssDNA, the test performed under replicative conditions supplies *attC* sites that have to be extruded as cruciforms from double-strand molecules. Our results show that whatever the delivery mode of *attC* sites, RecA does not influence integron recombina-

tion. Interestingly, even RecA overexpression did not affect integron recombination. This is relevant since we know that expression of integron integrases depends on the induction of the SOS response, which in turn produces a large excess of RecA. Note that this result does not exclude any regulatory role of RecA in *attC* site folding outside recombination. Nevertheless, it seems unlikely, since previous observations showed that the RecA protein does not, by itself, bind well to ssDNA secondary structures and needs SSB to remove such structures (19).

SSB modulates *attC* site folding. It was thought that SSB could have a role in integron recombination, as it plays extensive cellular roles in DNA replication, DNA repair, and DNA homologous recombination, where it destabilizes secondary structures in ssDNA (19, 25, 26). Since SSB is essential for cell viability, new experimental setups were developed to study its role in integron recombination and *attC* folding. Especially, we developed a replication slippage assay using a strain with a mutated form of SSB, SSB-200, which does not hamper cell viability but confers a transposon excision (*tex*) phenotype (28). This *tex* phenotype is probably directly linked to SSB-200 not destabilizing the stem-loop structures formed by the pair of inverted repeats of the transposon. In the *ssb-200* mutant strain, the frequency of reconstitution of a chloramphenicol resistance gene induced by an excision of the folded *attC* site by replication slippage was increased only in the absence of the integrase. This *in vivo* test confirmed that SSB can modify *attC*-mediated replication slippage, probably by destabilizing the folded *attC* sites. A DNA-binding assay allowed us to confirm the ability of SSB to strongly bind these sites. Note that even if the unpaired single-stranded parts contained in the folded *attC* site (and also the unpaired derivative site) are smaller than the SSB tetramer binding site ($n = 35$) (see above), SSB is able to bind to these sites. Indeed, the folded *attC* sites are probably not fixed structures and could “breathe,” inducing a transient, more unpaired state allowing SSB binding.

In conclusion, we demonstrated here that SSB, by “flattening” the folded *attC* sites, permits the maintenance of their integrity in the cell, avoiding their deletion or mutation and also ensuring cell viability.

Stabilization constraints and recombination of hairpins. Although ssDNA is present in many contexts within the cell, hairpin formation is strongly constrained by SSB binding. Proteins that function through hairpin binding are thus in competition with SSB for substrate availability. It could be the case for the integrase, since during recombination, folded *attC* sites need to be stable enough to resist SSB melting and coating. Therefore, we proposed that the integrase could capture *attC* sites at the moment of their extrusion, efficiently stabilizing and recombining them. Here, we confirmed this hypothesis and demonstrated that, when expressed, the integrase is able to counterbalance the effect of SSB even when SSB is overexpressed.

Finally, integron recombination efficiency is directed by a precise regulation of the stability of the folded *attC* sites. Bacteria have to find a subtle balance between the benefit provided by biological functions of secondary structures in DNA and the avoidance of such structures that could be detrimental. Here, this balance seems to have been reached through the production of SSB and integrase, even though other host factors may have a role in *attC* site folding. Both proteins are capable of recognizing and acting upon these secondary structures, mediating an antagonistic effect.

We previously demonstrated the influence of cellular processes

such as replication and/or horizontal gene transfer events (such as conjugation) on *attC* site folding and also the implication of superhelicity in cruciform extrusion. The multiple means of regulating *attC* site folding by the host show once again the network of cell processes that regulate integron recombination.

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