ADDITIONAL MATERIALS

Bacterial strains and media

Bacterial strains used in this study are DH5α (Laboratory collection), Π1, β2163 (Demarre et al, 2005), UB5201 (Martinez and de la Cruz, 1990) and UB5201-Pi (Bouvier et al, 2005). JTT1 (gal-25, λ⁻, pyrF287, fnr-1, rpsL195 (StrR), iclR7, trpR72) and SD7 (JTT1 topA10 gyrB226) have been described by Pruss et al, (1982).

Escherichia coli strains were grown in Luria Bertani (LB) at 37°C or 30°C (for the thermosensible origin of replication). Antibiotics were used at the following concentrations: ampicillin (Ap), 100 µg/ml, chloramphenicol (Cm), 25 µg/ml, kanamycin (Km), 25 µg/ml. Thymidine (Thy) and diaminopimelic acid (DAP) were supplemented when necessary to a final concentration of 0.3mM. Glucose and L-arabinose were added at respectively 10 and 2mg/ml final concentration.

DNA procedures

Standard techniques were used for DNA manipulation and cloning (Sambrook et al, 1989). Restriction and DNA-modifying enzymes were purchased from New England Biolabs and Roche. DNA was isolated from agarose gels using the QIAquick gel extraction kit (Qiagen). Plasmid DNA was extracted using the miniprep or midiprep kits (Macherey-Nagel, Qiagen).

PCR were performed with the Taq DNA polymerase (Promega) according to the manufacturer's instructions. PCR products were purified using the QIAquick PCR purification kit (Qiagen). 1% agarose electrophoresis gels were used. The sequence of each constructed *attC* site was verified using an ABI BigDye Terminator v.3.1 sequencing kit and an ABI Prism 3100 Capillary GeneticAnalyzer (Applied Biosystem).

Plasmid constructions

pSW::VCRs construction procedure

VCR mutant sites were constructed by the annealing of complementary partially overlapping primers. After annealing, the primers' end reconstitutes the *EcoRI* and *BglI* enzyme restriction sites. These products are ligated into the pSW23T plasmid linearized by *Eco*RI/*Bgl*I. Π1, a [Pir+] DH5α derivative that requires Thy to grow in MH medium, was used as a cloning strain.

pTSC::*attC*s construction procedure

$attC_{aadA7}$ and $VCR_{2/1}$ were reconstituted by the annealing of the two complementary partially overlapping primers. After annealing, the products are ligated into the pTSC29 plasmid linearized by SmaI. DH5α was used as a cloning strain.

Nicked pSW23T::*aadA7* construction procedure

The pSW23T::*aadA7* nicked plasmids were constructed by the annealing of two complementary primers reconstituting the Nt-BtsI cleavage site. We used two sets of primers which reconstitutes after annealing either MfeI or NaeI enzyme restriction sites. These products are ligated into the pSW23T::aadA7 plasmid linearized by MfeI or NaeI. The two orientations for each cloning site are selected; Π1, a [Pir+] DH5α derivative that requires Thy to grow in MH medium, was used as a cloning strain.

The nicking of each of the 4 constructions is performed by digestion of the Nt-BtsI restriction enzyme (Biolabs). The efficiency of the reaction was controlled thanks to the differential migration of the supercoiled and nicked molecules on electrophoresis gel.

Topoisomers production

We treated the pSW::*attC* substrates with the topoisomerase I protein which catalyze the relaxation of negatively supercoiled DNA. 1µg of supercoiled DNA plasmids (pSW::$attC_{aadA7}$ and pSW::$VCR_{2/1}$) was incubated at 37°C in a volume of 100µL with 10 units of Topoisomerase I (Biolabs) for 2h using the suggested buffer. The reactions were stopped with EDTA and proteinase K treatment and DNA was purified using the QIAquick PCR purification kit (Qiagen).

***In vitro* detection of cruciform**

Potential cruciform loops were detected by S1 nuclease sensitivity. For these, we prepared plasmids isolated from exponentially growing cells. Indeed, it has previously been shown that the level of supercoiling of plasmids is lower in starved cells (stationary phase) than during the exponential bacterial grown (Balke and Gralla, 1987). 1 µg of supercoiled plasmid DNA (pSW::$attC_{aadA7}$ and pSW::$VCR_{2/1}$) were incubated at 37°C in a volume of 100µL with 50 units of S1 nuclease (Fermentas) for 45 min using the suggested buffer. The reactions were stopped with EDTA and proteinase K treatment. DNA was purified using the QIAquick PCR purification kit (Qiagen) and digested with NcoI or BglII for pSW::$attC_{aadA7}$ and NcoI or ScaI for pSW::$VCR_{2/1}$.

Analysis of recombination events and point localization

Note for all the recombination assays, recombination frequencies correspond to the average of at least three independent trials. Recombination events were checked by Polymerase chain reaction (PCR) using the GoTaq Flexi DNA (Promega) on eight randomly chosen clones per experiment. MFD/SW23begin were used for analysis of *attI* x *attC* co-integrates formation. The recombination point was precisely determined by sequencing with SW23beg (*attI* x *attC* co-integrates), MRV (*attI* x *attC* co-integrates obtained with the pTSC plasmids). Sequences were

verified using an ABI BigDye Terminator v.3.1 sequencing kit and an ABI Prism 3100 Capillary GeneticAnalyzer (Applied Biosystem). Primers were obtained from Sigma-Aldrich (France) and are listed in Table S2.

In vivo recombination assay

Suicide conjugation assay

This conjugation assay was based on that of Biskri et *al.* 2005 and was previously implemented in Bouvier et *al.* 2005 (Biskri et al, 2005; Bouvier et al, 2005). Briefly, the *attC* sites provided by conjugation are carried on a suicide vector from the R6K- based pSW family that is known to use the Pir protein to initiate its own replication. This plasmid also contains an RP4 origin of transfer (*oriT*$_{RP4}$). The orientation of the oriT sequence determines which of the two strands is transferred. The donor strain β2163 carries an RP4 integrated in its chromosome which requires DAP to grow in rich medium and sustains pSW replication through the expression of a chromosomally integrated *pir* gene. The recipient strain UB5201, which contains the pBAD::*intI1* [Ap^R] (expressing the IntI1 integrase) and the pSU38Δ::*attI1* [Km^R] (carrying the *attI1* site), is devoid of a *pir* gene and therefore cannot sustain replication of the suicide vector. The only way for the pSW vector to be maintained in the recipient cell is to form a co-integrate by *attC* x *attI* recombination. The recombination activity is calculated as the ratio of transconjugants expressing the pSW marker [Cm^R] to the total number of recipient clones [Ap^R, Km^R].

We also perform the same assay but using a pir+ recipient cell (UB5201-Pi) insuring the pSW::*attC* replication once transferred. In this case, after the overnight conjugation, cells are resuspended into 2 ml of LB followed by total DNA plasmid extraction. The obtained DNA is used to transform the DH5α pir- cells. The recombination activity is calculated as the ratio of cells expressing the pSW::*attC* marker [Cm^R] to the total number of Km^R clones.

Recombination assay with a replicative double-stranded substrate

This assay allows supplying the *attC* site on double strand replicative plasmid. Three plasmids, pBAD::*IntI1*, pSU38::*attI1* and pSW::*attC*, harboring the different *attC* site derivatives were transformed into a pir+ cell (UB5201-Pi). This strain allows the pSW::*attC* replication (see the description of the pSW plasmid family above). After overnight growth in the presence of appropriate antibiotics and 0.2% arabinose to allow the intI1 expression, cells were harvested and total plasmid DNA extracted. This was then introduced by transformation into the DH5α pir- cell. Transformants were selected for Cm^R (the pSW::*attC* marker). As pSW::*attC* cannot replicate in the pir- DH5α strain, Cm^R clones correspond to *attC* x *attI* recombination events. Recombination activity is calculated as the ratio of Cm^R to Km^R transformants. In order to control the implication of the *attC* site as a single-stranded form in this replicative test, we constructed an *attC* site lacking the double-stranded R'' box (see Table S1 and S2). In this *attC* site, the R'/R'' box of the folded *attC* site is affected. As expected, we obtained a very low frequency of recombination ($1.25x10^{-6}$+/- $5.06x10^{-7}$).

Calculation of the probability to fold a recombinogenic *attC* site from single-stranded DNA

UNAFOLD software was used to compute the probability to form active *attC* sites from single-stranded DNA. We consider that to be folded properly, *attC* sites need to form the R and L boxes (Figure 1). A proper L box is characterized by the presence of the extrahelical G16. If we constrain the proper pairing of A17, we can observe that the most energetically favorable fold is by far the proper fold (data not shown). Based on the assumption that we are in an equilibrium state, this means that the majority of the molecules that pair the A17 properly should have this proper fold. We computed the probability to pair the A17 properly, using the hybrid-ss function of UNAFold.

Calculation of the free energy of cruciform formation

The free energy of cruciform formation (ΔGc) was computed as the sum of four terms: the energy to melt the double stranded DNA (ΔGdb), the folding energy of the bottom and top strands (ΔGbot and ΔGtop), and the energy contributed by the change in superhelicity (ΔGs). ΔGdb, ΔGbot and ΔGtop were computed using UNAFOLD. ΔGs was computed according to JF Marko and ED Siggia (Marko and Siggia, 1995) for a superhelix density of -0.06 and a plasmid size of 2kb.

	Plasmids name and description
p929	pSU38Δ::attI1, ori_{p15A} [Km^R] Biskri et *al.*, 2005
p3938	pBAD::intI1, ori_{ColE1} [Ap^R], Demarre et al, 2007
p7523	pTSCaadA7 (ori-), fwd/rev aadA7 fragment in pTSC29 digested, *oripSC101ts* [Cm^R] (this study)
p7546	pTSCaadA7 (ori+), fwd/rev aadA7 fragment in pTSC29 digested,), *oripSC101ts* [Cm^R] (this study)
p7545	pTSCVCR (ori-), fwd1/rev1 and fwd2/rev2 VCR fragment in pTSC29 digested, *oripSC101ts* [Cm^R] (this study)
p7544	pTSCVCR (ori+), fwd1/rev1 and fwd2/rev2 VCR fragment in pTSC29 digested, *oripSC101ts* [Cm^R] (this study)
p4136	pSW23T::aadA7 (B), $oriT_{RP4}$, $oriV_{R6K}$ [Cm^R] (Bouvier et al., 2005)
p7945	pSW23T::aadA7 (T), $oriT_{RP4\ INV}$, $oriV_{R6K}$ [Cm^R] (this study)
p4192	pSW23T::aadA7 Mut3 (B), $oriT_{RP4}$, $oriV_{R6K}$ [Cm^R] (Bouvier et al., 2005)
p1880	pSW23T::$VCR_{2/1}$ (B), $oriT_{RP4}$, $oriV_{R6K}$ [Cm^R] (Biskri et al, 2005)
p2656	pSW23T::$VCR_{2/1}$ (T), $oriT_{RP4\ INV}$, $oriV_{R6K}$ [Cm^R] (Bouvier et al, 2005)
p3615	pSW23T::ereA2 (B), $oriT_{RP4}$, $oriV_{R6K}$ [Cm^R] (Bouvier et al., 2009)
p4392	pSW23T::ereA2 (T), $oriT_{RP4\ INV}$, $oriV_{R6K}$ [Cm^R] (Bouvier et al, 2009)
p3616	pSW23T ::oxa2 (B), $oriT_{RP4}$, $oriV_{R6K}$ [Cm^R] (Bouvier et al, 2009)
p4390	pSW23T :: oxa2 (T), $oriT_{RP4\ INV}$, $oriV_{R6K}$ [Cm^R] (Bouvier et al, 2009)
p6823	pSW23T::VCR56 (B), EcoRI/BglII fwd/rev VCR56 fragment in pSW23T digested (this study)
p6824	pSW23T::VCR58 (B), EcoRI/BglII fwd/rev VCR58 fragment in pSW23T digested (this study)
p4893	pSW23T::VCR-GAA (B), EcoRI/BglII fwd/rev VCR-GAA fragment in pSW23T digested (this study)
p4191	pSW23T::VCR-TTC (B), EcoRI/BglII fwd/rev VCR-TTC fragment in pSW23T digested (this study)
p7329	pSW23T::VCR-GC (B) EcoRI/BglII fwd/rev VCR-GC fragment in pSW23T digested (this study)
p7330	pSW23T::VCR-TA (B) EcoRI/BglII fwd/rev VCR-TA fragment in pSW23T digested (this study)
p7332	pSW23T::VCR97a (B), EcoRI/BglII fwd/rev VCR-97a fragment in pSW23T digested (this study)
p7527	pSW23T::VCR97b (B), EcoRI/BglII fwd/rev VCR-97b fragment in pSW23T digested (this study)
p7589	pSW23T::VCR100 (B), EcoRI/BglII fwd/rev VCR-100 fragment in pSW23T digested (this study)
p7333	pSW23T::VCR116a (B), EcoRI/BglII fwd1/rev1 and fwd2/rev2 VCR-116a fragment in pSW23T digested (this study)
p7528	pSW23T::VCR116b (B), EcoRI/BglII fwd1/rev1 and fwd2/rev2 VCR-116b fragment in pSW23T digested (this study)
p7114	pSW23T::VCRb (B), EcoRI/BglII fwd1/rev1 and fwd2/rev2 VCRb fragment in pSW23T digested (this study)
p6730	pSW23T::VCR139 (B), EcoRI/BglII fwd1/rev1 and fwd2/rev2 VCR-139 fragment in pSW23T digested (this study)
p6731	pSW23T::VCR147a (B), EcoRI/BglII fwd1/rev1 and fwd2/rev2 VCR-147a fragment in pSW23T digested (this study)
p6990	pSW23T::VCR147b (B), EcoRI/BglII fwd1/rev1 and fwd2/rev2 VCR-147b fragment in pSW23T digested (this study)
p7591	pSW23T::VCR147c (B), EcoRI/BglII fwd1/rev1 and fwd2/rev2 VCR-147c fragment in pSW23T digested (this study)
p6938	pSW23T::VCR147d (B), EcoRI/BglII fwd1/rev1 and fwd2/rev2 VCR-147d fragment in pSW23T digested (this study)
p6814	pSW23T::VCR164 (B), EcoRI/BglII fwd1/rev1 and fwd2/rev2 VCR-164 fragment in pSW23T digested (this study)
p7684	pSW23T::VCR180 (B), EcoRI/BglII fwd1/rev1, fwd2/rev2 and fwd3/rev3 VCR-180 fragment in pSW23T digested (this study)
p7781	pSW23T::VCRΔR'' (B), EcoRI/BglII fwd1/rev1 VCRΔR'' fragment in pSW23T digested (this study)
p8426	pSW23T::aadA7 (B), bottom strand cleaved, MfeI fwd/rev Nb-BtsI (MfeI) fragment in pSW23T digested (this study)
p8427	pSW23T::aadA7 (B), top strand cleaved, MfeI fwd/rev Nb-BtsI (MfeI) fragment in pSW23T digested (this study)
p8428	pSW23T::aadA7 (B), bottom strand cleaved, NaeI fwd/rev Nb-BtsI (NaeI) fragment in pSW23T digested (this study)
p8429	pSW23T::aadA7 (B), top strand cleaved, NaeI fwd/rev Nb-BtsI (NaeI) fragment in pSW23T digested (this study)

Table S1: Plasmids used and constructed in this study

Table S2: Primers used in this study

Sequences are given in 5' → 3' direction.

A) Primers used to generate the pSW::*attC* derivatives plasmids.

attC	sites	Sequences
VCR_{56}	**rev**	GGGCTGACAACAGTCCCTCTTGAGGCGTTTGTTATAACAGATCTG
	fwd	AATTCAGATCTGTTATAACAAACGCCTCAAGAGGGACTGTTGTCAGCCCCTT
VCR_{58}	**rev**	GGGCTGCGAAGCAGTCCCTCTTGAGGCGTTTGTTATAACAGATCTG
	fwd	AATTCAGATCTGTTATAACAAACGCCTCAAGAGGGACTGCTTCGCAGCCCCTT
VCR_{GAA}	**rev**	GGGCTGACAACGCCTTGCGTTGACAGTCCCTCTTGAGGCGTTTGTTATAACAGATCTG
	fwd	AATTCAGATCTGTTATAACAAACGCCTCAAGAGGGACTGTCAACGCAAGGCGTTGTCAGCCCCTT
VCR_{TTC}	**rev**	GGGCTGACAACGCGAAGCGTTGACAGTCCCTCTTGAGGCGTTTGTTATAACAGATCTG
	fwd	AATTCAGATCTGTTATAACAAACGCCTCAAGAGGGACTGTCAACGCTTCGCGTTGTCAGCCCCTT
VCR_{TA}	**rev**	GGGCTGACAACGCTAAAAAATGCGTTGACAGTCCCTCTTGAGGCGTTTGTTATAACAGATCTG
	fwd	AATTCAGATCTGTTATAACAAACGCCTCAAGAGGGACTGTCAACGCATTTTTTAGCGTTGTCAGCCCCTT
VCR_{GC}	**rev**	GGGCTGACAACGCGCCCCCCGGCGTTGACAGTCCCTCTTGAGGCGTTTGTTATAACAGATCTG
	fwd	AATTCAGATCTGTTATAACAAACGCCTCAAGAGGGACTGTCAACGCCGGGGGGCGCGTTGTCAGCCCCTT
VCR_{97a}	**rev**	GGGCTGACAACGCACTACCGCTCAATGGGACTGGAAACGCCACGCGTTGACAGTCCCTCTTGAGGCGTTTGTTATAACAGATCTG
	fwd	AATTCAGATCTGTTATAACAAACGCCTCAAGAGGGACTGTCAACGCGTGGCGTTTCCAGTCCCATTGAGCGGTAGTGCGTTGTCAGCCCCTT
VCR_{97b}	**rev**	GGGCTGACAACGCACTACCACTAAACTCAAACACAACAACAGCGCGTTGACAGTCCCTCTTGAGGCGTTTGTTATAACAGATCTG
	fwd	AATTCAGATCTGTTATAACAAACGCCTCAAGAGGGACTGTCAACGCGCTGTTGTTGTGTTTGAGTTTAGTGGTAGTGCGTTGTCAGCCCCTT
VCR_{100}	**rev**	GGGCTGACAACGCACTACCACTAAACTCAAACACAACAACAGCCCGGCGTTGACAGTCCCTCTTGAGGCGTTTGTTATAACAGATCTG
	fwd	AATTCAGATCTGTTATAACAAACGCCTCAAGAGGGACTGTCAACGCCGGGCTGTTGTTGTGTTTGAGTTTAGTGGTAGTGCGTTGTCAGCCCCTT
VCR_{116a}	**rev1** **rev2**	GACAGTCCCTCTTGAGGCGTTTGTTATAACAGATCTG GGGCTGACAACGCACTACCACTAAACTCAAACACAACAACTCAATGGGACTGGAAACGCCACGCGTT
	fwd1 **fwd2**	AATTCAGATCTGTTATAACAAACGCCTCAAGAGGGACTGTCAACGCGTGGCGTTTCCAGTCC CATTGAGTTGTTGTGTTTGAGTTTAGTGGTAGTGCGTTGTCAGCCCCTT
VCR_{116b}	**rev1** **rev2**	AATGGGCGTTGACAGTCCCTCTTGAGGCGTTTGTTATAACAGATCTG GGGCTGACAACGCACTACCACTAAACTCAAACACAACAACAGCAACCACCGCGGCTC
	fwd1	AATTCAGATCTGTTATAACAAACGCCTCAAGAGGGACTGTCAACGCCCATTGAGCCGCGGTGG

	fwd2	TTGCTGTTGTTGTGTTTGAGTTTAGTGGTAGTGCGTTGTCAGCCCCTT
VCR_b	**rev1**	GAAACGCCGCGCGTTGACAGTCCCTCTTGAGGCGTTTGTTATAACAGATCTG
	rev2	GGGCTGACAACGCGCTACCACTAAACTCAAACACAACAACAGCAACCACCGCGGCTCAATGGGACTG
	fwd1	AATTCAGATCTGTTATAACAAACGCCTCAAGAGGGACTGTCAACGCGGGGCGTTTCCAGTCCCATTGAGCCGCGGTGG
	fwd2	TTGCTGTTGTTGTGTTTGAGTTTAGTGGTAGCGCGTTGTCAGCCCCTT
VCR_{139}	**rev1**	ACCAGCGCGTTGACAGTCCCTCTTGAGGCGTTTGTTATAACAGATCTG
	rev2	GGGCTGACAACGCGCTGACTACCACTAAACTCAAACACAACAACAGCAACCACCGCGGCTCAATGGGACTGGAAACGCC
	fwd1	AATTCAGATCTGTTATAACAAACGCCTCAAGAGGGACTGTCAACGCGCTGGTGGCGTTTCCAGTCCCATTGAGC
	fwd2	CGCGGTGGTTGCTGTTGTTGTGTTTGAGTTTAGTGGTAGTCAGCGCGTTGTCAGCCCCTT
VCR_{147a}	**rev1**	ACCGCGCAGCGCGTTGACAGTCCCTCTTGAGGCGTTTGTTATAACAGATCTG
	rev2	GGGCTGACAACGCGCTGCGCGACTACCACTAAACTCAAACACAACAACAGCAACCACCGCGGCTCAATGGGACTGGAAACGCC
	fwd1	AATTCAGATCTGTTATAACAAACGCCTCAAGAGGGACTGTCAACGCGCTGCGCGGTGGCGTTTCCAGTCCCATTGAGC
	fwd2	CGCGGTGGTTGCTGTTGTTGTGTTTGAGTTTAGTGGTAGTCGCGCAGCGCGTTGTCAGCCCCTT
VCR_{147b}	**rev1**	GAAACGCCACGCGTTGACAGTCCCTCTTGAGGCGTTTGTTATAACAGATCTG
	rev2	GGGCTGACAACGCACTACCACTAAACTCAAACACAAGCGTTAAACTACCGAACAACAGCAACCACCGCGGCTCAATGGGACTG
	fwd1	AATTCAGATCTGTTATAACAAACGCCTCAAGAGGGACTGTCAACGCGTGGCGTTTCCAGTCCCATTGAGCCGCGGTGG
	fwd2	TTGCTGTTGTTCGGTAGTTTAACGCTTGTGTTTGAGTTTAGTGGTAGTGCGTTGTCAGCCCCTT
VCR_{147c}	**rev1**	GACTGGAAACGCCACGCGTTGACAGTCCCTCTTGAGGCGTTTGTTATAACAGATCTG
	rev2	GGGCTGACAATTTACTACCACTAAACTCAAACACAAGCGTTAAACTACCGAACAACAGCAACCACCGCGGCTCAATGG
	fwd1	AATTCAGATCTGTTATAACAAACGCCTCAAGAGGGACTGTCAACGCGTGGCGTTTCCAGTCCCATTGAGCC
	fwd2	GCGGTGGTTGCTGTTGTTCGGTAGTTTAACGCTTGTGTTTGAGTTTAGTGGTAGTAAATTGTCAGCCCCTT
VCR_{147d}	**rev1**	GAAACGCCACCGCGTTGACAGTCCCTCTTGAGGCGTTTGTTATAACAGATCTG
	rev2	GGGCTGACAACGCGACTACCACTAAACTCAAACACAACGTTAAACTACCGACAACAGCAACCACCGCGGCTCAATGGGACTG
	fwd1	AATTCAGATCTGTTATAACAAACGCCTCAAGAGGGACTGTCAACGCGGTGGCGTTTCCAGTCCCATTGAGCCGCGGTGG
	fwd2	TTGCTGTTGTCGGTAGTTTAACGTTGTGTTTGAGTTTAGTGGTAGTCGCGTTGTCAGCCCCTT
VCR_{147e}	**rev1**	GAAACGCCACGCGTTGACAGTCCCTCTTGAGGCGTTTGTTATAACAGATCTG
	rev2	GGGCTGACAACGTACTACCACTAAACTCAAACACAAGCGTTAAACTACCGAACAACAGCAACCACCGCGGCTCAATGGGACTG
	fwd1	AATTCAGATCTGTTATAACAAACGCCTCAAGAGGGACTGTCAACGCGTGGCGTTTCCAGTCCCATTGAGCCGCGGTGG
	fwd2	TTGCTGTTGTTCGGTAGTTTAACGCTTGTGTTTGAGTTTAGTGGTAGTACGTTGTCAGCCCCTT
VCR_{147f}	**rev1**	GACTGGAAACGCCACGCGTTGACAGTCCCTCTTGAGGCGTTTGTTATAACAGATCTG
	rev2	GGGCTGACAACTTACTACCACTAAACTCAAACACAAGCGTTAAACTACCGAACAACAGCAACCACCGCGGCTCAATGG
	fwd1	AATTCAGATCTGTTATAACAAACGCCTCAAGAGGGACTGTCAACGCGTGGCGTTTCCAGTCCCATTGAGCC
	fwd2	GCGGTGGTTGCTGTTGTTCGGTAGTTTAACGCTTGTGTTTGAGTTTAGTGGTAGTAAGTTGTCAGCCCCTT
VCR_{164}	**rev1**	ACGCGGCGTCGCGCGCAGCGCGTTGACAGTCCCTCTTGAGGCGTTTGTTATAACAGATCTG
	rev2	GGGCTGACAACGCGCTGCGCGCGCGCCGCACTACCACTAAACTCAAACACAACAACAGCAACCACCGCGGCTCAATGGGACTGGAAACGCC

	fwd1 **fwd2**	AATTCAGATCTGTTATAACAAACGCCTCAAGAGGGACTGTCAACGCGCTGCGCGCGACGCCGCGTGGCGTTTCCAGTCCCATTGAGC CGCGGTGGTTGCTGTTGTTGTGTTTGAGTTTAGTGGTAGTGCGGCGCGCGCGCAGCGCGTTGTCAGCCCCTT
VCR_{180}	**rev1** **rev2** **rev3**	CCACGCGGTGTCGGCGGTGTCGCGCGCAGCGCGTTGACAGTCCCTCTTGAGGCGTTTGTTATAACAGATCTG ACTACCACTAAACTCAAACACAACAACAGCAACCACCGCGGCTCAATGGGACTGGAAACG GGGCTGACAACGCGCTGCGCGCGCGCCGCCGCACCGC
	fwd1 **fwd2** **fwd3**	AATTCAGATCTGTTATAACAAACGCCTCAAGAGGGACTGTCAACGCGCTGCGCG CGACGCCGCCGACACCGCGTGGCGTTTCCAGTCCCATTGAGCCGCGGTGGTT GCTGTTGTTGTGTTTGAGTTTAGTGGTAGTGCGGTGCGGCGGCGCGCGCGCAGCGCGTTGTCAGCCCCTT
$VCR_{\Delta R''}$	**rev1** **rev2**	ACGCGTTGACAGTCCCTCTTGAGGCGTTTCAGATCTG GGGCTGACAACGCACTACCACTAAACTCAAACACAACAACAGCAACCACCGCGGCTCAATGGGACTGGAAACGCC
	fwd1 **fwd2**	AATTCAGATCTGAAACGCCTCAAGAGGGACTGTCAACGCGTGGCGTTTCCAGTCCCATTGAGC CGCGGTGGTTGCTGTTGTTGTGTTTGAGTTTAGTGGTAGTGCGTTGTCAGCCCCTT

B) Primers used to generate the pTSC derivative plasmids

Name		**Sequences**
VCR	**VCR rev1**	ACGCGTTGACAGTCCCTCTTGAGGCGTTTGTTATAACAGA
	VCR rev2	CCGGTTATAACGCCCGCCTAAGGGGCTGACAACGCACTACCACTAAACTCAAACACAACAACAGCAACCACCGCGGCTCAATGGGAC TGGAAACGCC
	VCR fwd1	TCTGTTATAACAAACGCCTCAAGAGGGACTGTCAACGCGTGGCGTTTCCAGTCCCATTGAGC
	VCR fwd2	CGCGGTGGTTGCTGTTGTTGTGTTTGAGTTTAGTGGTAGTGCGTTGTCAGCCCCTTAGGCGGGCGTTATAACCGG
attC*aadA7*	***attC*$_{aadA7}$ rev**	TGCCTAACGCTTGAATTAAGCCGCGCCGCGAAGCGGCGTCGGCTTGAATGAATTGTTAGGCA
	***attC*$_{aadA7}$ fwd**	TGCCTAACAATTCATTCAAGCCGACGCCGCTTCGCGGCGCGGCTTAATTCAAGCGTTAGGCA

C) Primers used to generate the nicked pSW::*aadA7* derivatives plasmids

Name		**Sequences**
Nb-BtsI (MfeI)	**rev**	AATTGGGCACTGCGCTAGCC
	fwd	AATTGGCTAGCGCAGTGCCC
Nb-BtsI (NaeI)	**rev**	GGCGGCACTGCGCTAGCGCC
	fwd	GGCGCTAGCGCAGTGCCGCC

D) Primers used to confirm the *attC* x *attI* insertion and the *attC* x *attC* deletion events and to map the S1 cleavage sites.

Name	Sequences
MFD	CGCCAGGGTTTTCCCAGTCAC
MRV	AGCGGATAACAATTTCACACAGGA
Sw23beg	CCGTCACAGGTATTTATTCGGCG
Sw23end	CCTCACTAAAGGGAACAAAAGCTG
AAP	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG

Table S3: Description of the used *attC* sites

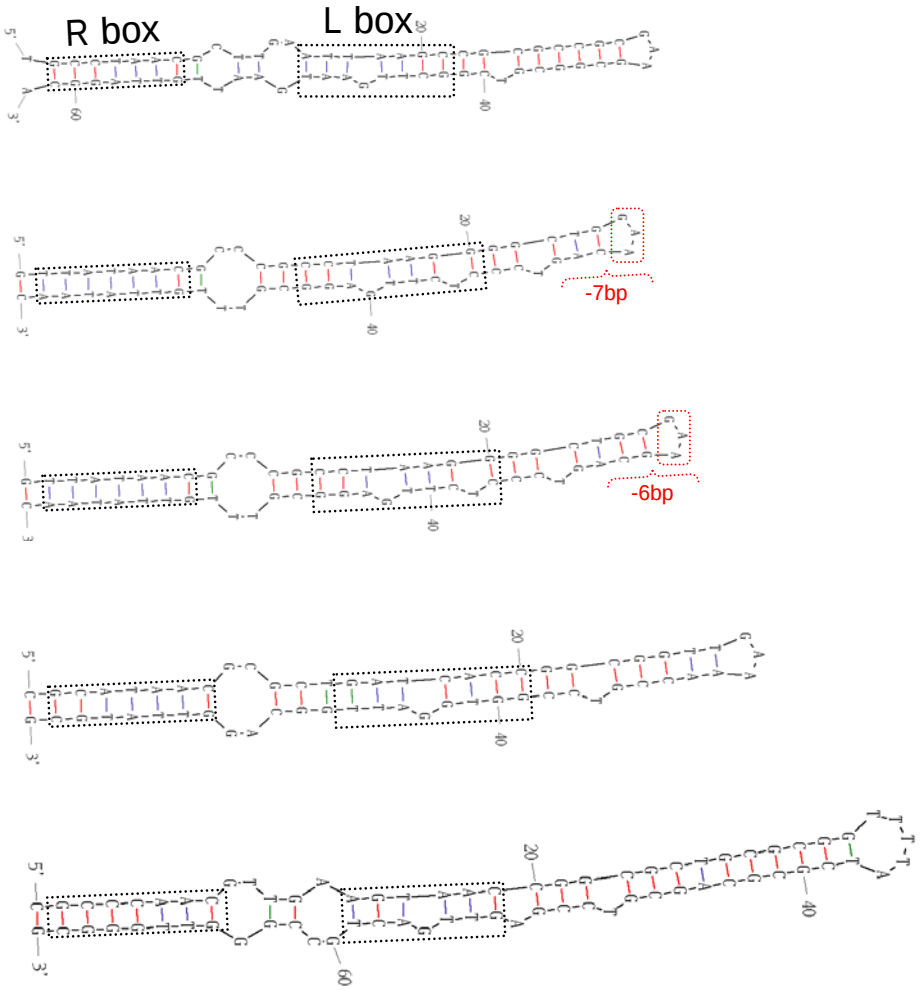
Sequences of the bottom strand of each *attC* sites are presented. Frequencies of recombination of the *attC* derivative sites during the suicide conjugation assay (Conj) (see also Figure 3A) and during the replicative assay (Rep) (see also Figure 4A) are indicated. The VTS size of the *attC* sites and the probability to fold a recombinogenic *attC* site (Pfold) are also indicated. b=base

$attC_{aadA7}$	**Conj**=3.35x10^{-3} **Rep**=3.53x10^{-1} **VTS size**=3b **Pfold**=0.184
TGCCTAACGCTTGAATTAAGCCGCGCCGCGAAGCGGCGTCGGCTTGAATGAATTGTTAGGCA	
VCR_{56}	**Conj**=3.08x10^{-3} **Rep**=3.96x10^{-1} **VTS size**=3b **Pfold**=0.0157
GTTATAACGCCCGCCTAAGGGGCTGGAACAGTCCCTCTTGAGGCGTTTGTTATAAC	
VCR_{58}	**Conj**=3.08x10^{-3} **Rep**=1.08x10^{-1} **VTS size**=3b **Pfold**=0.0156
GTTATAACGCCCGCCTAAGGGGCTGCGAAGCAGTCCCTCTTGAGGCGTTTGTTATAAC	
$attC_{ereA2}$	**Conj**=1.46x10^{-3} **Rep**=3.39x10^{-1} **VTS size**=3b **Pfold**=0.247
CGCATAACGCGCTGATCACCGGCGGTTGAAAACCGTCCGGTGGATTGGCAGGTTATGCG	
$attC_{oxa2}$	**Conj**=3.40x10^{-3} **Rep**=1.85x10^{-1} **VTS size**=6b **Pfold**=0.47
CGCCCAACGTTGAAGTAACCGGCGCTGCGCGGTTTTATCGCGCAGCGTCCGAGTTGACTGCCGGGT TGGGCG	
VCR_{GAA}	**Conj**=3.04x10^{-3} **Rep**=2.16x10^{-1} **VTS size**=3b **Pfold**=0.0156
GTTATAACGCCCGCCTAAGGGGCTGACAACGCCTTGCGTTGACAGTCCCTCTTGAGGCGTTTGTTAT AAC	
VCR_{TTC}	**Conj**=1.93x10^{-3} **Rep**=1.34x10^{-1} **VTS size**=3b **Pfold**=0.0156
GTTATAACGCCCGCCTAAGGGGCTGACAACGCGAAGCGTTGACAGTCCCTCTTGAGGCGTTTGTTA TAAC	
VCR_{TA}	**Conj**=3.47x10^{-3} **Rep**=1.35x10^{-1} **VTS size**=8b **Pfold**=0.0156
GTTATAACGCCCGCCTAAGGGGCTGACAACGCTAAAAAATGCGTTGACAGTCCCTCTTGAGGCGTT TGTTATAAC	
VCR_{GC}	**Conj**=2.38x10^{-3} **Rep**=1.01x10^{-1} **VTS size**=8b **Pfold**=0.0156
GTTATAACGCCCGCCTAAGGGGCTGACAACGCGCCCCCCGGCGTTGACAGTCCCTCTTGAGGCGTT TGTTATAAC	
VCR_{97a}	**Conj**=9.65x10^{-4} **Rep**=1.66x10^{-3} **VTS size**=24b **Pfold**=0.00731
GTTATAACGCCCGCCTAAGGGGCTGACAACGCACTACCGCTCAATGGGACTGGAAACGCCACGCG TTGACAGTCCCTCTTGAGGCGTTTGTTATAAC	
VCR_{97b}	**Conj**=9.46x10^{-4} **Rep**=2.81x10^{-2} **VTS size**=24b **Pfold**=0.0156
GTTATAACGCCCGCCTAAGGGGCTGACAACGCACTACCACTAAACTCAAACACAACAACAGCGCG TTGACAGTCCCTCTTGAGGCGTTTGTTATAAC	
VCR_{100}	**Conj**=1.56x10^{-3} **Rep**=1.29x10^{-2} **VTS size**=27b **Pfold**=0.0156
GTTATAACGCCCGCCTAAGGGGCTGACAACGCACTACCACTAAACTCAAACACAACAACAGCCCG GCGTTGACAGTCCCTCTTGAGGCGTTTGTTATAAC	
VCR_{116a}	**Conj**=3.53x10^{-3} **Rep**=2.65x10^{-4} **VTS size**=43b **Pfold**=0.0045
GTTATAACGCCCGCCTAAGGGGCTGACAACGCACTACCACTAAACTCAAACACAACAACTCAATG GGACTGGAAACGCCACGCGTTGACAGTCCCTCTTGAGGCGTTTGTTATAAC	
VCR_{116b}	**Conj**=3.32x10^{-3} **Rep**=1.01x10^{-2} **VTS size**=43b **Pfold**=0.0156

GTTATAACGCCCGCCTAAGGGGCTGACAACGCACTACCACTAAACTCAAACACAACAACAGCAAC CACCGCGGCTCAATGGGCGTTGACAGTCCCTCTTGAGGCGTTTGTTATAAC
VCR_a **Conj**=1.89x10^{-3} **Rep**=1.11x10^{-3} **VTS size**=64b **Pfold**=0.000159
GTTATAACGCCCGCCTAAGGGGCTGACAACGCACTACCACTAAACTCAAACACAACAACAGCAAC CACCGCGGCTCAATGGGACTGGAAACGCCACGCGTTGACAGTCCCTCTTGAGGCGTTTGTTATAAC
VCR_b **Conj**=1.02x10^{-3} **Rep**=2.09x10^{-3} **VTS size**=62b **Pfold**=0.00896
GTTATAACGCCCGCCTAAGGGGCTGACAACGCGCTACCACTAAACTCAAACACAACAACAGCAAC CACCGCGGCTCAATGGGACTGGAAACGCCGCGCGTTGACAGTCCCTCTTGAGGCGTTTGTTATAAC
VCR_{139} **Conj**=2.13x10^{-3} **Rep**=9.96x10^{-3} **VTS size**=64b **Pfold**=0.0155
GTTATAACGCCCGCCTAAGGGGCTGACAACGCGCTGACTACCACTAAACTCAAACACAACAACAG CAACCACCGCGGCTCAATGGGACTGGAAACGCCACCAGCGCGTTGACAGTCCCTCTTGAGGCGTTT GTTATAAC
VCR_{147a} **Conj**=5.65x10^{-3} **Rep**=1.77x10^{-2} **VTS size**=64b **Pfold**=0.0156
GTTATAACGCCCGCCTAAGGGGCTGACAACGCGCTGCGCGACTACCACTAAACTCAAACACAACA ACAGCAACCACCGCGGCTCAATGGGACTGGAAACGCCACCGCGCAGCGCGTTGACAGTCCCTCTTG AGGCGTTTGTTATAAC
VCR_{147b} **Conj**=3x10^{-4} **Rep**=2.42x10^{-4} **VTS size**=81b **Pfold**=0.0000912
GTTATAACGCCCGCCTAAGGGGCTGACAACGCACTACCACTAAACTCAAACACAAGCGTTAAACTA CCGAACAACAGCAACCACCGCGGCTCAATGGGACTGGAAACGCCACGCGTTGACAGTCCCTCTTG AGGCGTTTGTTATAAC
VCR_{147c} **Conj**=3.47x10^{-6} **Rep**=4.37x10^{-5} **VTS size**=87b **Pfold**=0.0000105
GTTATAACGCCCGCCTAAGGGGCTGACAATTTACTACCACTAAACTCAAACACAAGCGTTAAACTA CCGAACAACAGCAACCACCGCGGCTCAATGGGACTGGAAACGCCACGCGTTGACAGTCCCTCTTG AGGCGTTTGTTATAAC
VCR_{147d} **Conj**=1.08x10^{-3} **Rep**=5.63x10^{-3} **VTS size**=78b **Pfold**=0.000331
GTTATAACGCCCGCCTAAGGGGCTGACAACGCGACTACCACTAAACTCAAACACAACGTTAAACTA CCGACAACAGCAACCACCGCGGCTCAATGGGACTGGAAACGCCACCGCGTTGACAGTCCCTCTTGA GGCGTTTGTTATAAC
VCR_{147e} **Conj**=3.11x10^{-4} **Rep**=2.23x10^{-4} **VTS size**=83b **Pfold**=0.0000319
GTTATAACGCCCGCCTAAGGGGCTGACAACGTACTACCACTAAACTCAAACACAAGCGTTAAACTA CCGAACAACAGCAACCACCGCGGCTCAATGGGACTGGAAACGCCACGCGTTGACAGTCCCTCTTG AGGCGTTTGTTATAAC
VCR_{147f} **Conj**=1.11x10^{-4} **Rep**=6.20x10^{-5} **VTS size**=85b **Pfold**=0.0000109
GTTATAACGCCCGCCTAAGGGGCTGACAACTTACTACCACTAAACTCAAACACAAGCGTTAAACTA CCGAACAACAGCAACCACCGCGGCTCAATGGGACTGGAAACGCCACGCGTTGACAGTCCCTCTTG AGGCGTTTGTTATAAC
VCR_{164} **Conj**=3.82x10^{-3} **Rep**=2.06x10^{-3} **VTS size**=64b **Pfold**=0.0156
GTTATAACGCCCGCCTAAGGGGCTGACAACGCGCTGCGCGCGCGCCGCACTACCACTAAACTCAAA CACAACAACAGCAACCACCGCGGCTCAATGGGACTGGAAACGCCACGCGGCGTCGCGCGCAGCGC GTTGACAGTCCCTCTTGAGGCGTTTGTTATAAC
VCR_{180} **Conj**=1.51x10^{-3} **Rep**=1.72x10^{-2} **VTS size**=64b **Pfold**=0.0156
GTTATAACGCCCGCCTAAGGGGCTGACAACGCGCTGCGCGCGGCCGCCGCACCGCACTACCACTAA ACTCAAACACAACAACAGCAACCACCGCGGCTCAATGGGACTGGAAACGCCACGCGGTGTCGGCG GCGTCGCGCGCAGCGCGTTGACAGTCCCTCTTGAGGCGTTTGTTATAAC

Figure S1: Proposed secondary structures of the used *attC* sites

Secondary structures were determined using the UNAFOLD online interface of the Institut Pasteur. G:C and A:T base pairs are marked by red and blue dashes respectively. The 5'and 3'ends are indicated and the bases (b) are numerated. The *attC* sites are classified according to their size (smallest to largest). The natural *attC* sites (WT: Wild Type) are indicated. The modifications made from the wild type VCR site are described for all the VCR derivatives.



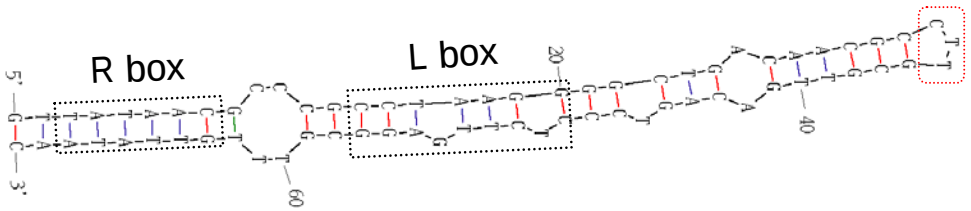
attC$_{aadA7}$bs (WT)

VCR$_{56}$bs (substitution of the VTS by 3b)
(7bp deletion in the stem)

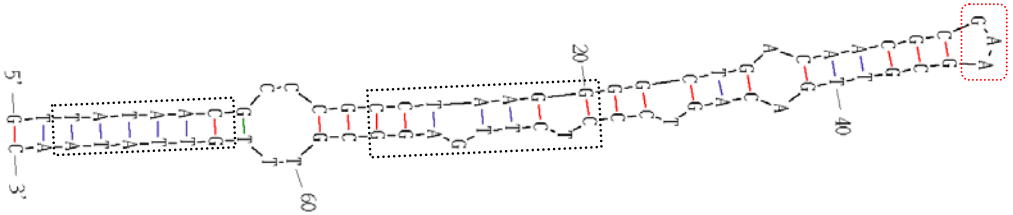
VCR$_{58}$bs (substitution of the VTS by 3b)
(6bp deletion in the stem)

attC$_{ere2}$bs (WT)

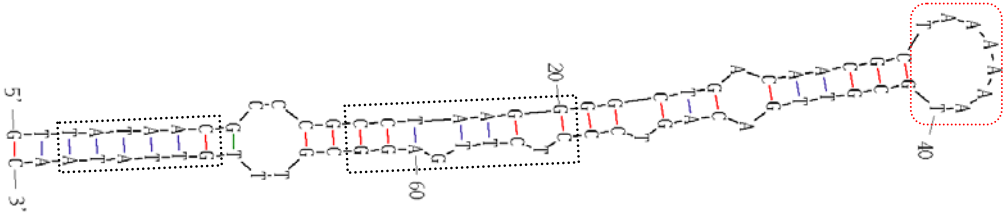
attC$_{oxa2}$bs (WT)



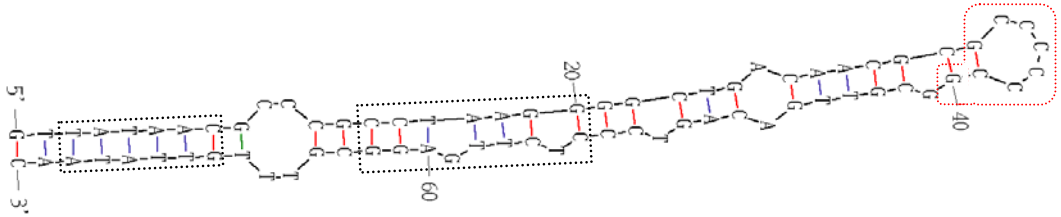
$VCR_{GAA}bs$
(substitution of the VTS by 3b)



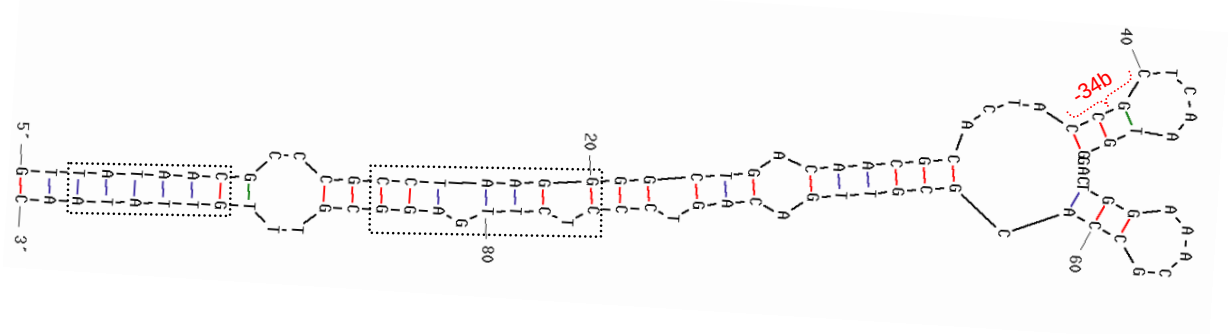
$VCR_{TTC}bs$
(substitution of the VTS by 3b)



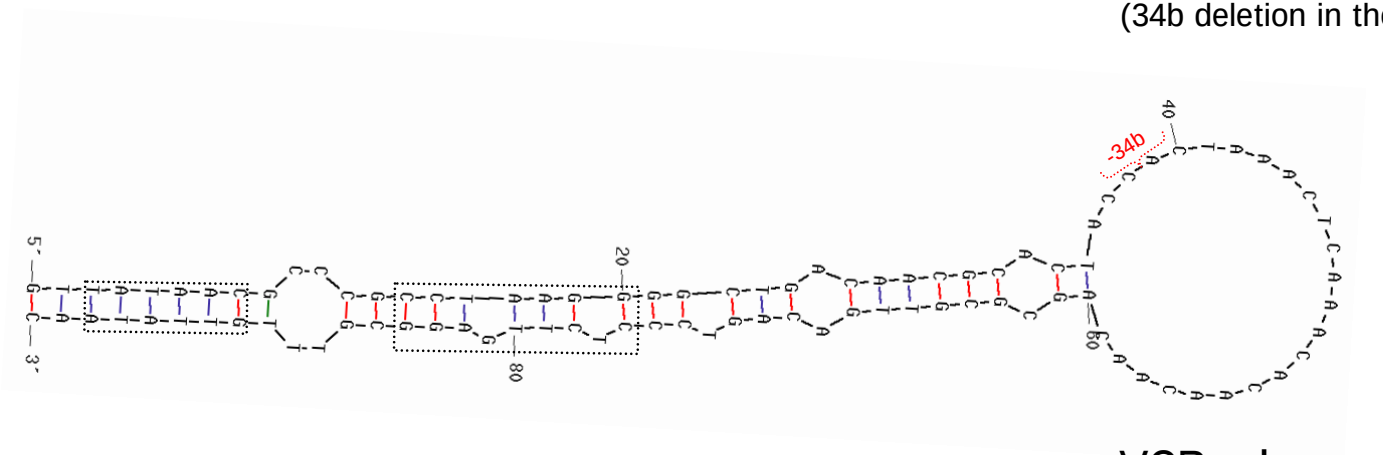
$VCR_{TA}bs$
(substitution of the VTS by a stretch of TA)



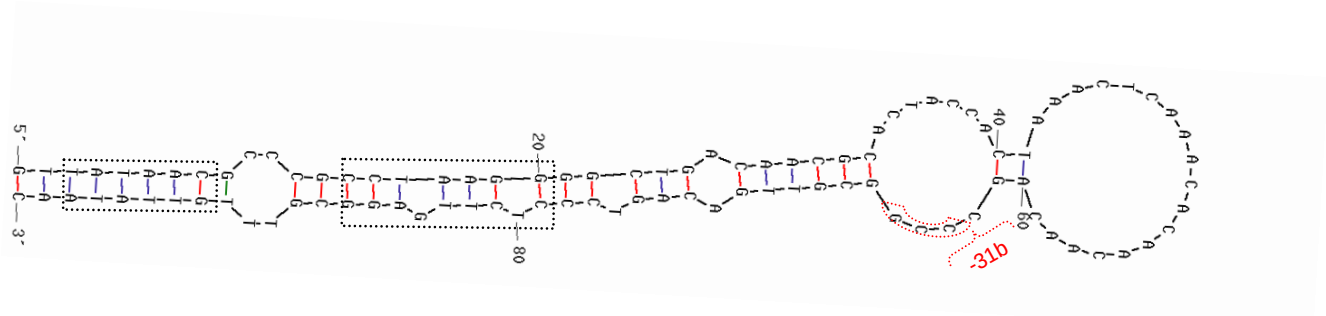
$VCR_{GC}bs$
(substitution of the VTS by a stretch of GC)



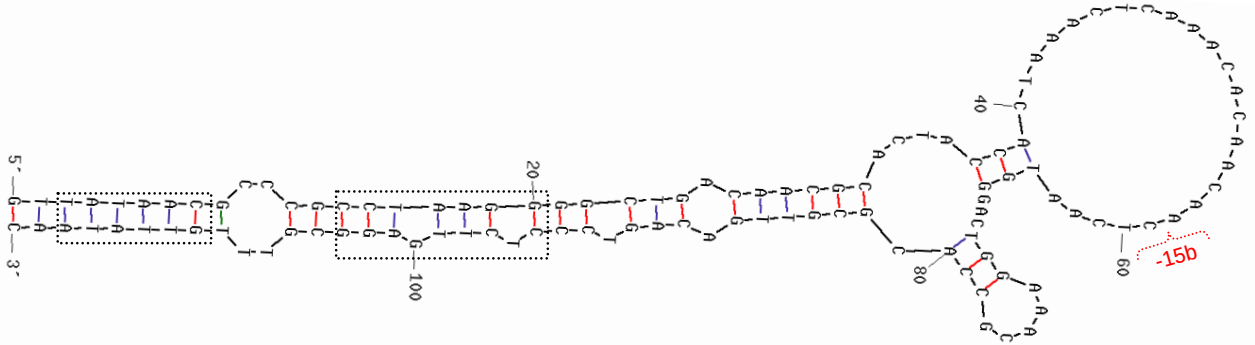
$VCR_{97a}bs$
(34b deletion in the VTS)



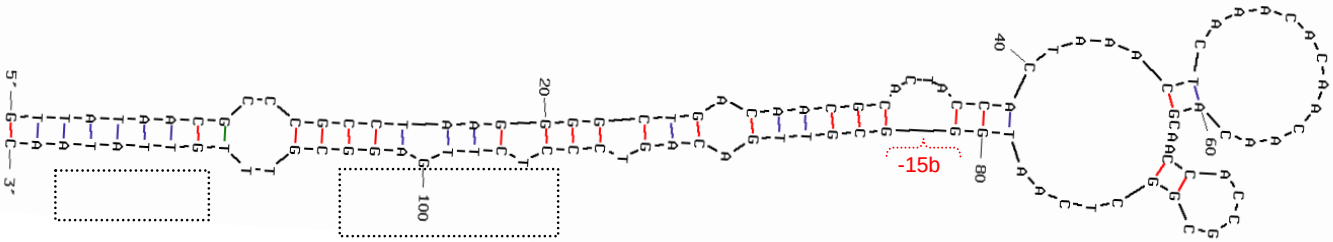
$VCR_{97b}bs$
(34b deletion in the VTS)
(modification of a part of the 30 remaining bases)



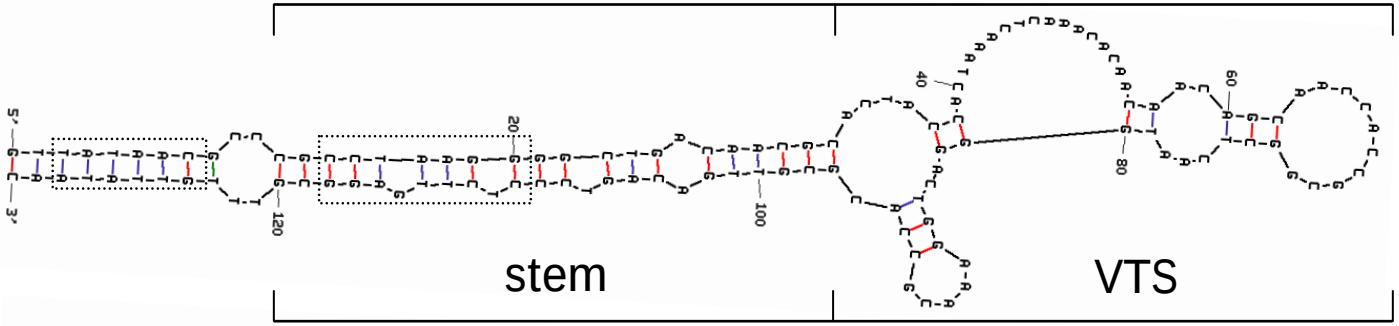
$VCR_{100}bs$
(31b deletion in the VTS)
(modification of 3b among the 33 remaining bases)



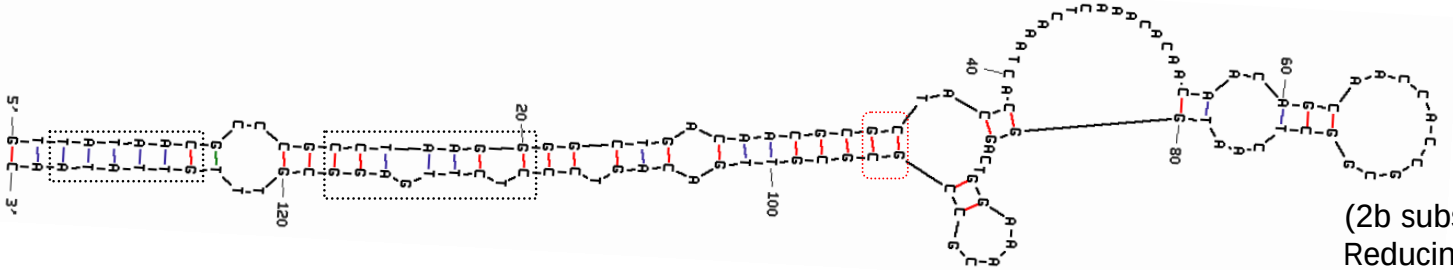
$VCR_{116a}bs$
(15b deletion in the VTS)



$VCR_{116b}bs$
(15b deletion in the VTS)

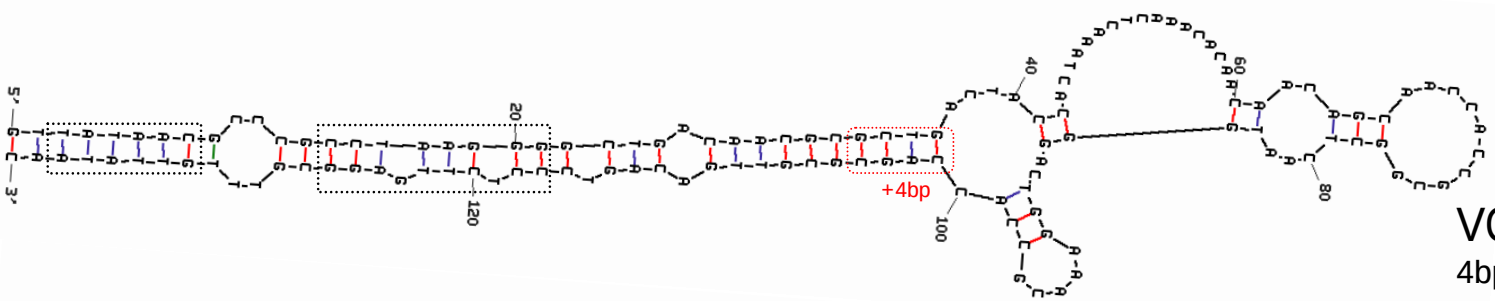


$VCR_{a}bs$ (WT)

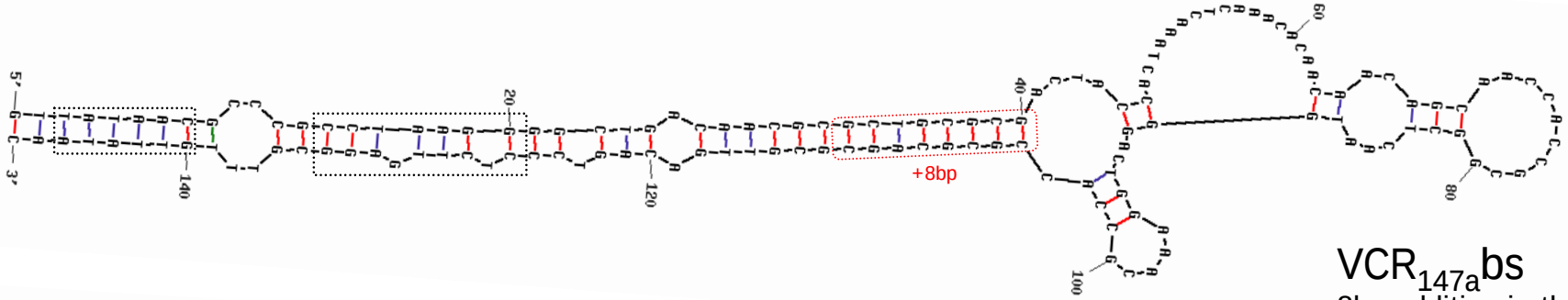


$VCR_{b}bs$

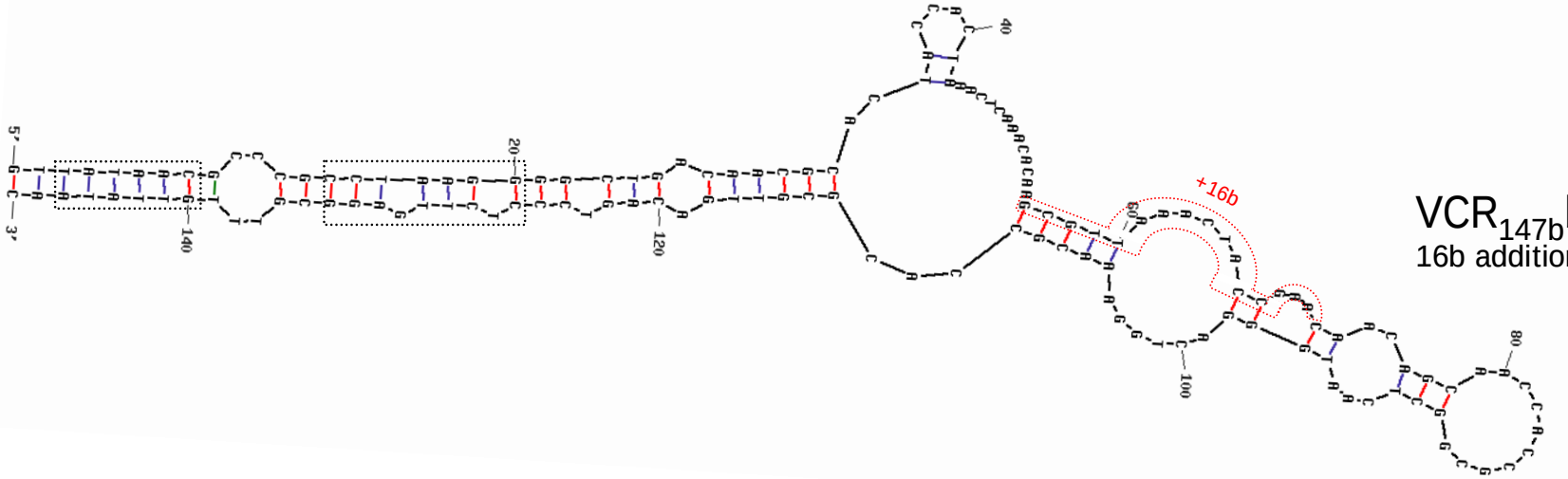
(2b substitution in the VTS
Reducing it by 4b and
extending the stem by 2bp)



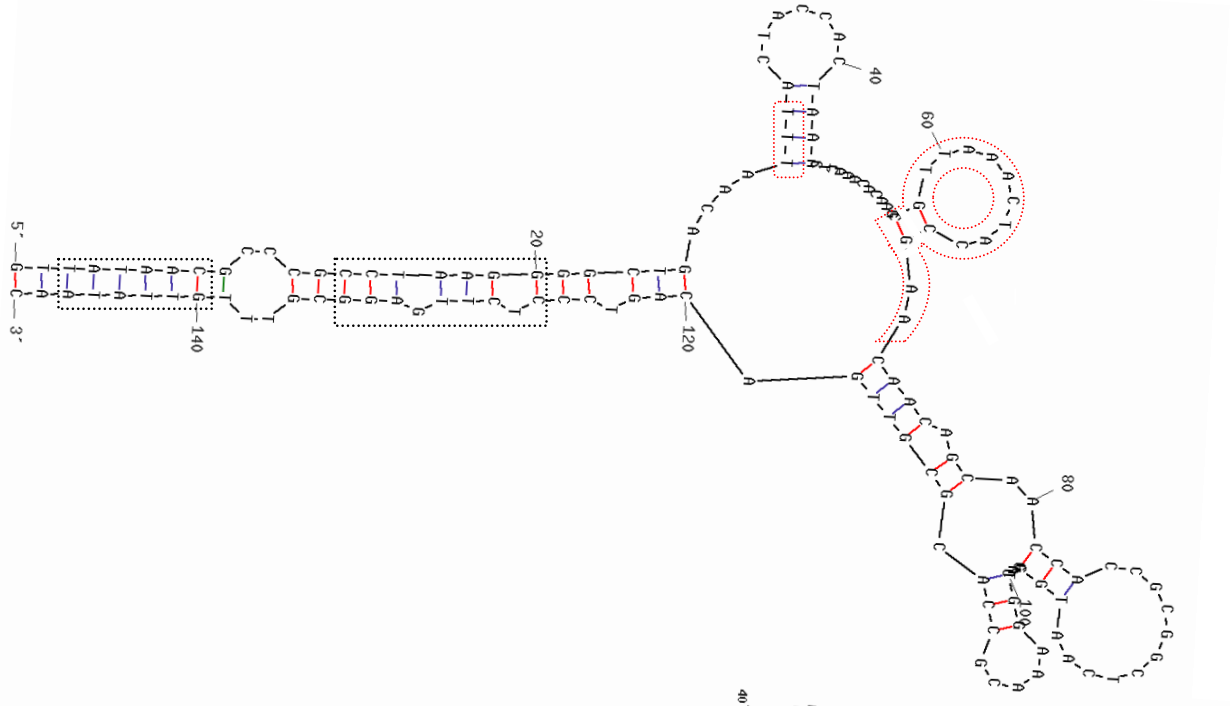
$VCR_{139}bs$
4bp addition in the stem



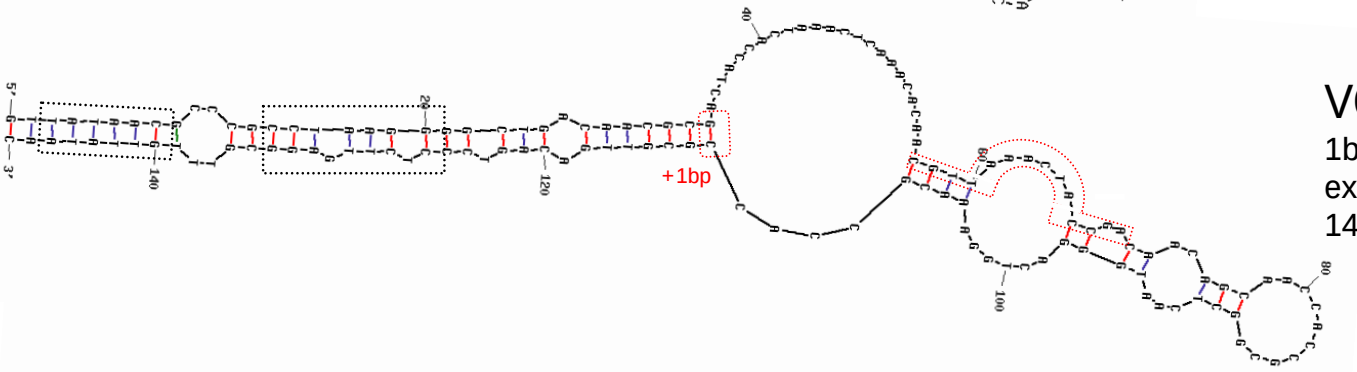
$VCR_{147a}bs$
8bp addition in the stem



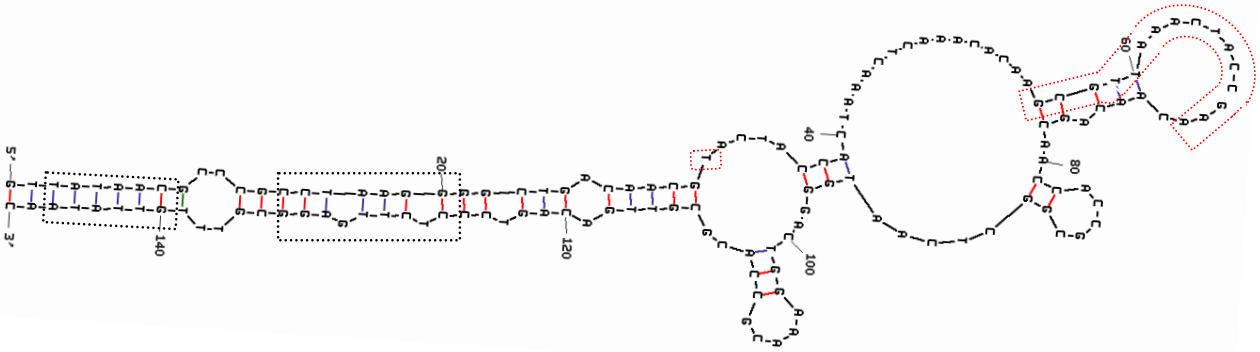
$VCR_{147b}bs$
16b addition in the VTS



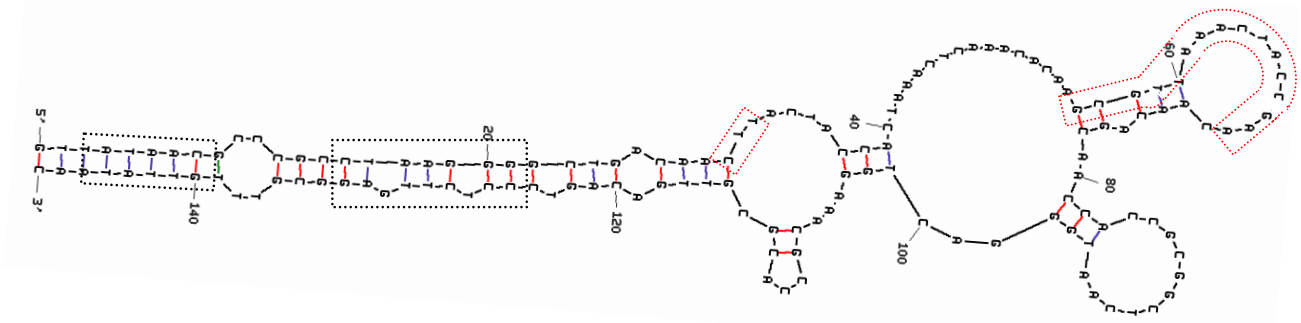
VCR_{147c}bs
3b substitution in the stem
reducing it by 14b
16b addition in the VTS



VCR_{147d}bs
1b addition in the stem
extending it by 1bp
14b addition in the VTS

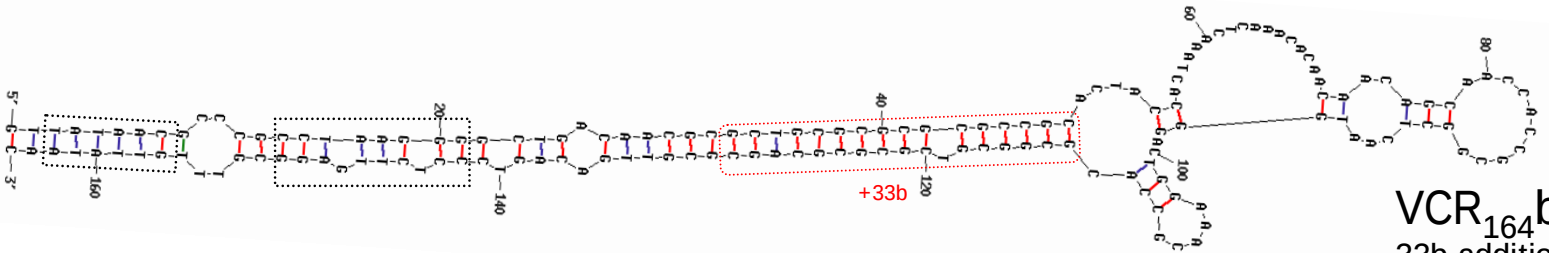


VCR_{147e}bs
1b substitution in the stem
reducing it by 2pb
16b addition in the VTS



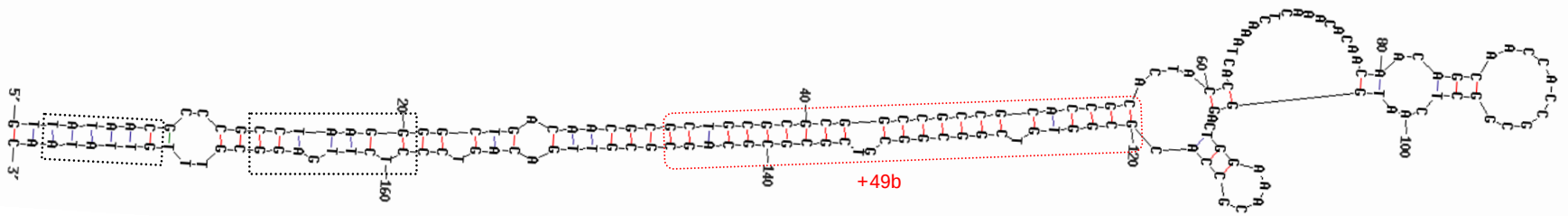
$VCR_{147f}bs$

3b substitution in the stem
reducing it by 4 bp
16b addition in the VTS



$VCR_{164}bs$

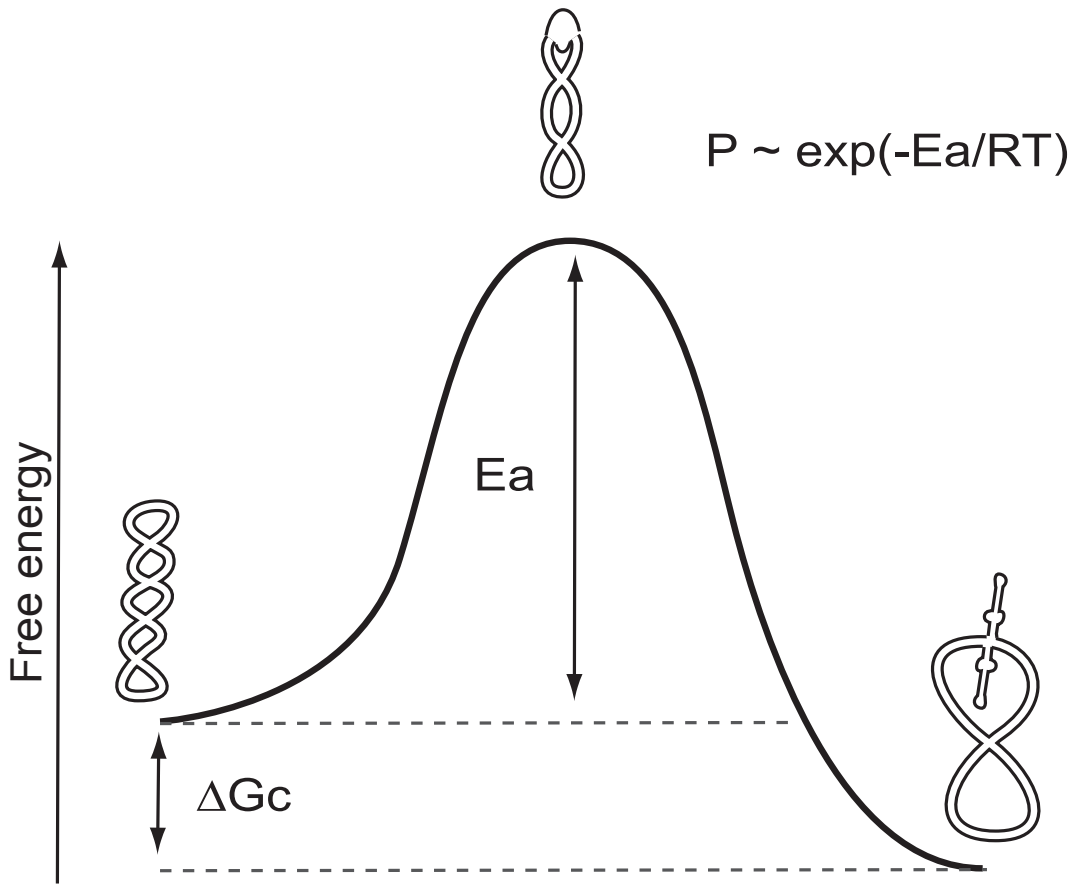
33b addition in the stem



$VCR_{180}bs$

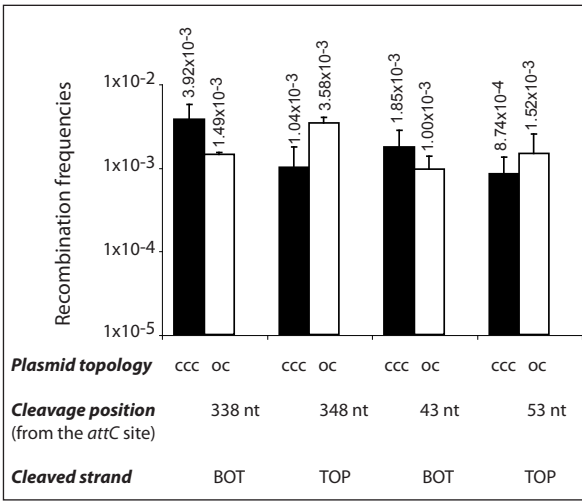
49b addition in the stem

Figure S2



Energy Path of cruciform formation in a supercoiled DNA molecule. In supercoiled DNA, melting at the dyad of a palindrome sequence can lead to intrastrand base-pairing and initiates cruciform formation. Branch migration elongating the cruciform is then energy driven. The energy required to realize the dyad melting can be viewed as the activation energy of cruciform formation and is directly linked to the probability of cruciform formation following the Arrhenius equation.

Figure S3

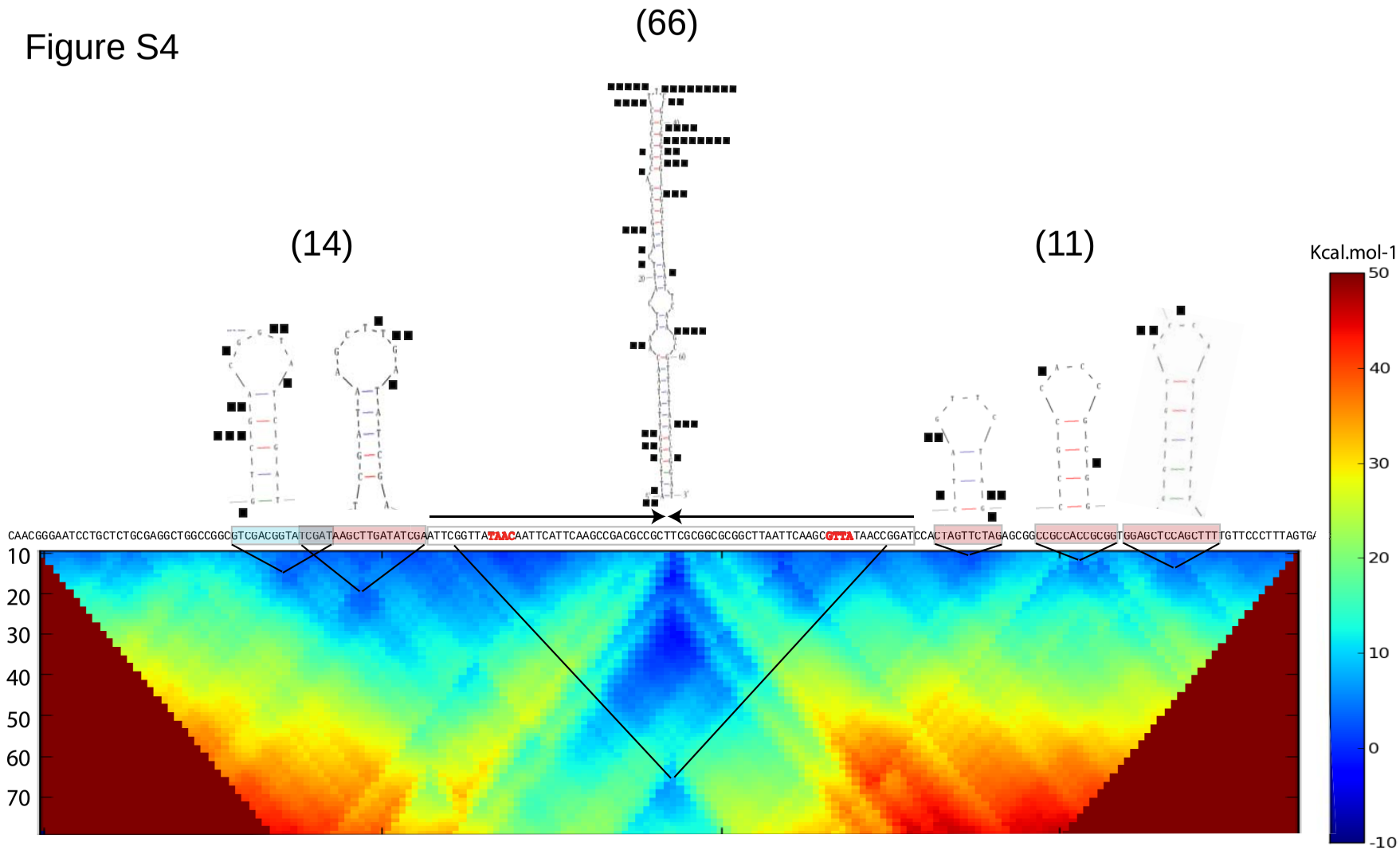


Effect of strand cleavage on recombination frequencies

The figure shows the recombination frequencies of different *attC*-containing plasmids (supercoiled or nicked) after transformation in non permissive recipient cell (see supplementary information). Error bars show standard deviations.

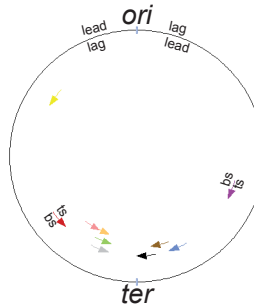
ccc, supercoiled plasmid; **oc,** open circular (nicked) plasmid;
BOT, bottom strand; **TOP,** top strand; **nt,** nucleotides.

Figure S4



The color graph represent the energy of cruciform formation along the sequence. On the y-axis is the size of the sequence window considered, and on the x-axis its position. Hairpins where S1 cleavage sites were found are represented above the graph and their position on the graph shown with black lines. Black squares by the hairpins represent the exact cleavage positions. Note that the large majority of S1 cleavage sites (66/91) are specific to the *attC* site (the central hairpin).

Figure S5



Pseudoalteromonas haloplanktis
Vibrio cholerae
Vibrio fisheri
Vibrio vulnificus YJ016
Vibrio splendidus
Photobacterium profundum
Vibrio vulnificus CMCP6
Vibrio harveyi
Vibrio parahaemolyticus
Treponema denticola

Location of the chromosomal integrons in sequenced bacterial genomes and relative orientation of the *attC* sites.

The position of the arrows along the circle represents the relative location of the different chromosomal integrons present in bacterial genomes. Each bacterial strain is identified by a specific color. Genomes analysis were made using the MAGE (MAgnifying GEnome) platform developped at the Genoscope (Evry, France, http://www.genoscope.cns.fr/agc/mage).
The arrows orientation indicates the cassettes orientation.
The origin *(ori)* and terminus *(ter)* regions of replication, and the respective leading (lead) and lagging (lag) strands of the two replichores are indicated.
ts, top strand; bs, bottom strand.