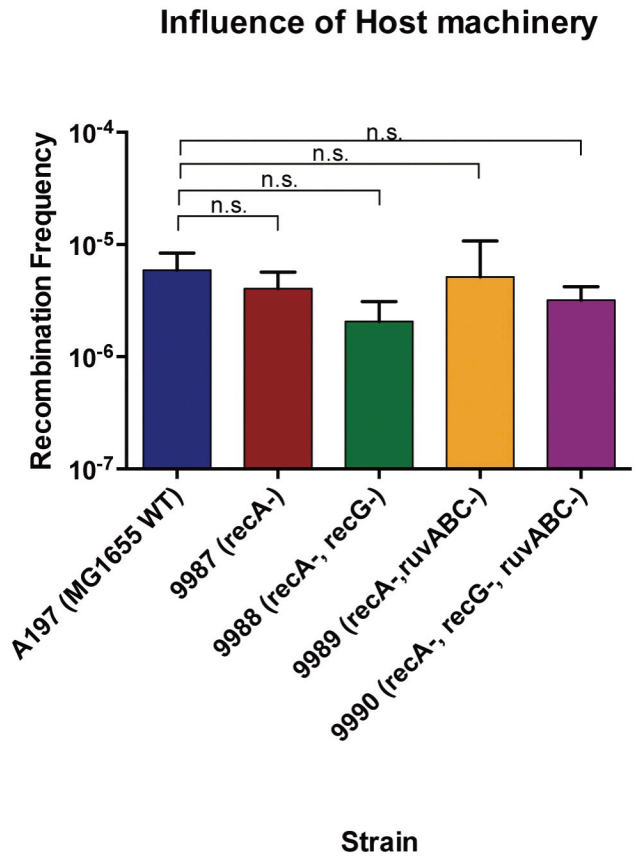
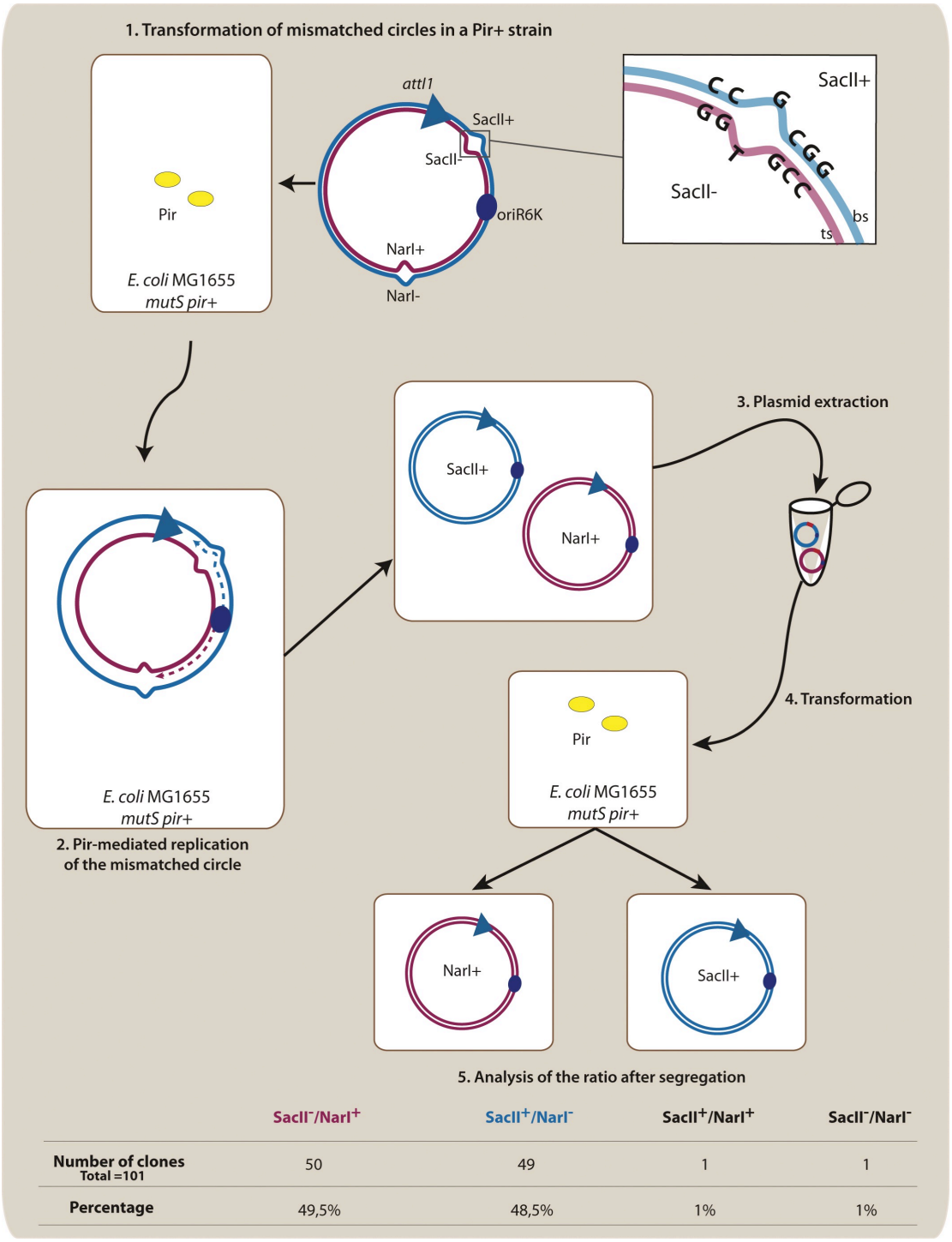
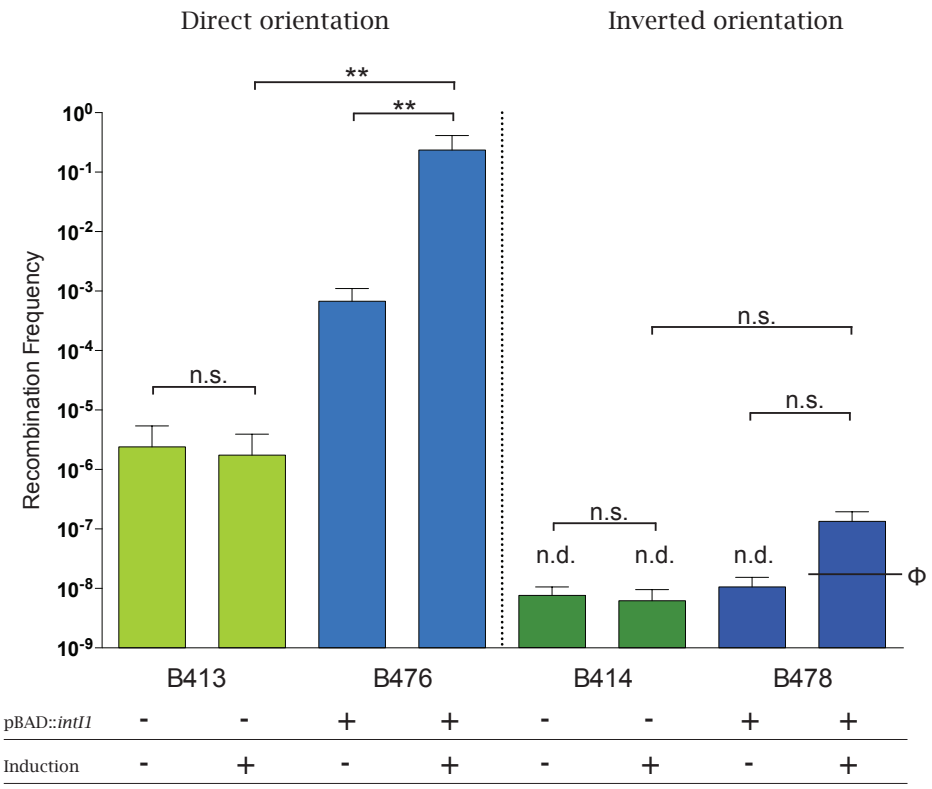



**Supplementary Figure 1.** Influence of host machinery involved in HJ resolution, in the *attI* x *attI* reaction. Bar graph representing the recombination frequency of an MG1655 parental strain and *recA*$^{-}$, *recG*$^{-}$, *ruvABC*$^{-}$ and the triple mutant derivatives thereof. Columns represent the mean and error bars represent standard deviation (n=3). Significance testing was performed with Kolmogorov-Smirnov test (P value < 0.05; n.s. not significant.)

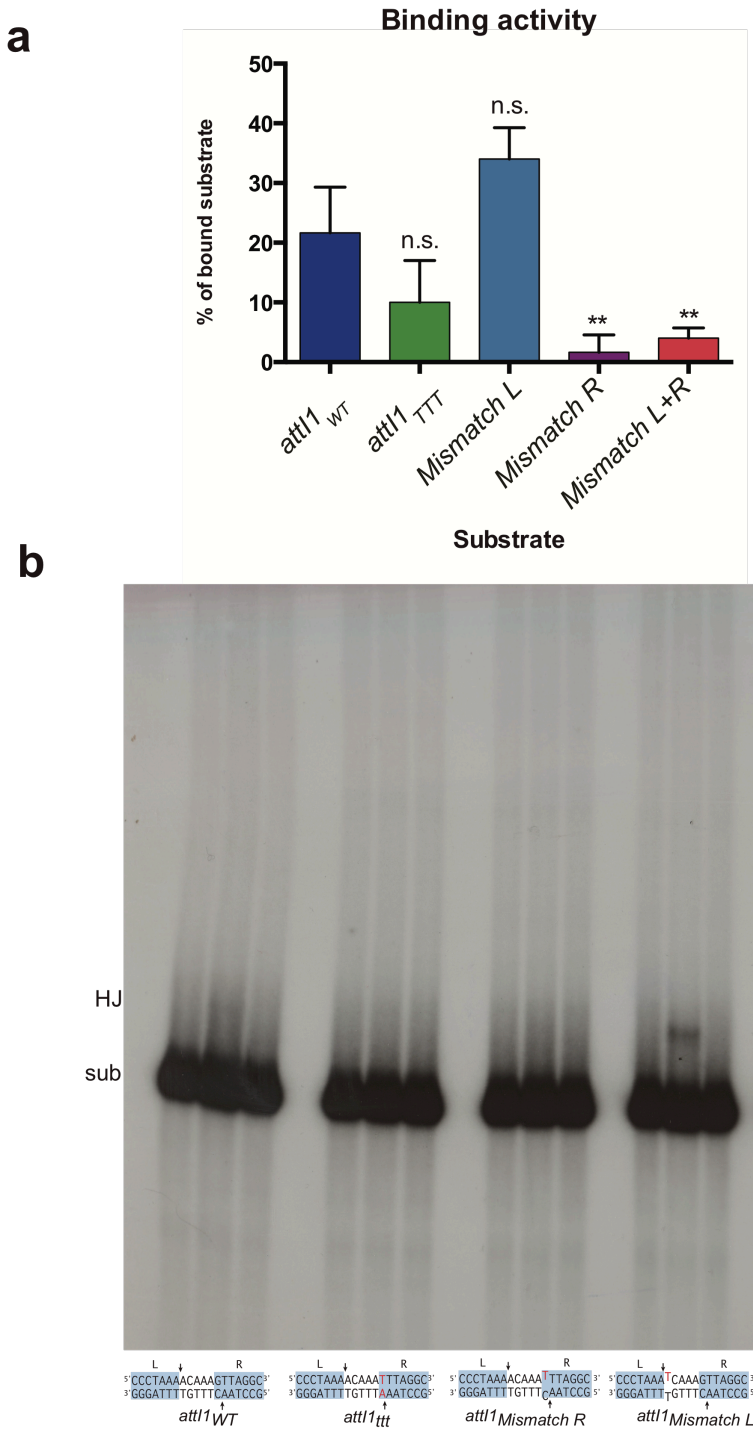



| | SacII⁻/NarI⁺ | SacII⁺/NarI⁻ | SacII⁺/NarI⁺ | SacII⁻/NarI⁻ |
|---|---|---|---|---|
| Number of clones Total =101 | 50 | 49 | 1 | 1 |
| Percentage | 49,5% | 48,5% | 1% | 1% |

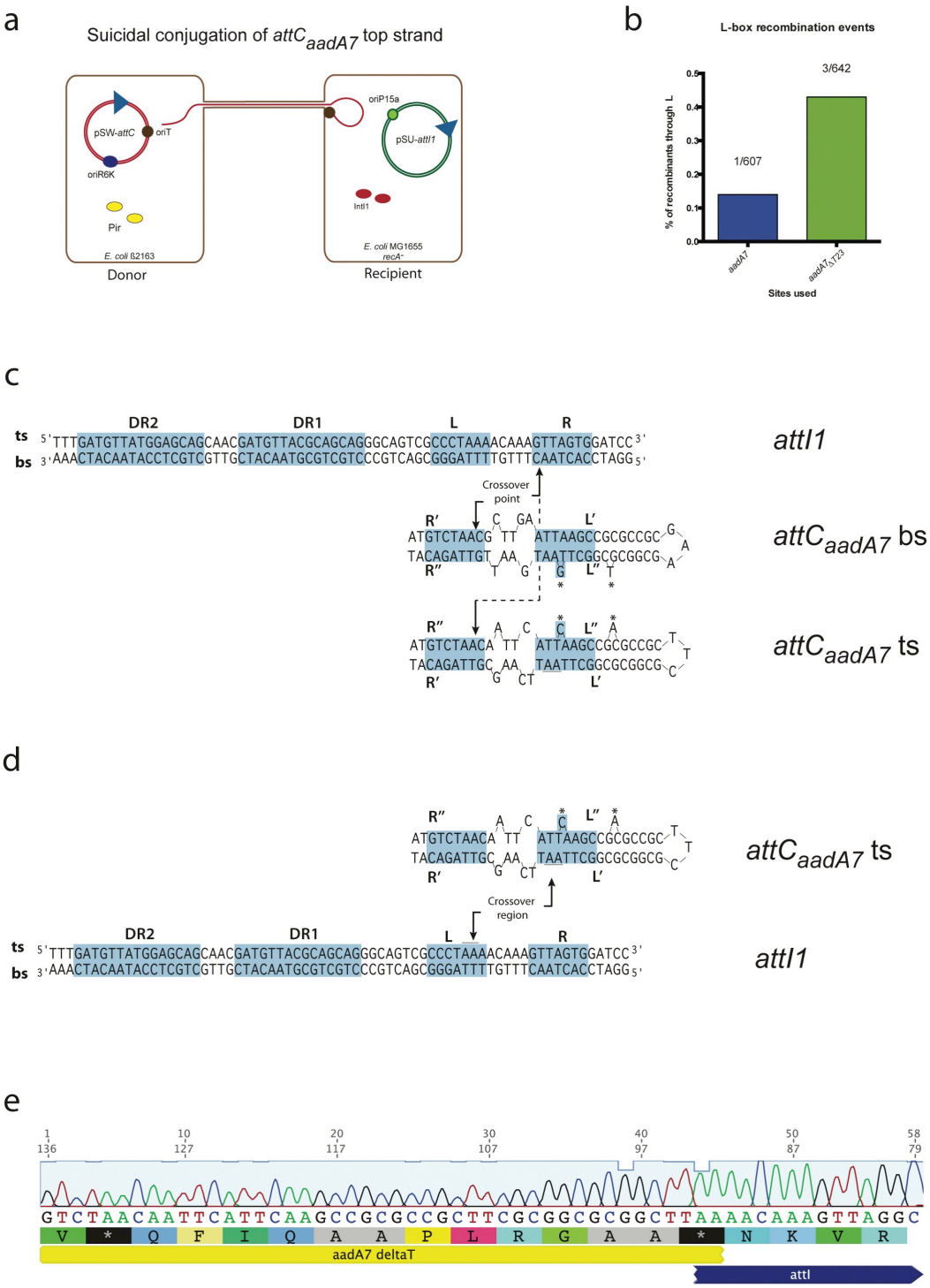
**Supplementary Figure 2.** Verification of mismatched covalent circles. **1**, After production and hybridization of the two strands (see "materials and methods"), the mismatched covalent circles were transformed into an MG1655 *mutS*⁻, *pir*⁺ strain. In this genetic background circles are theoretically not repaired (*mutS*⁻) and can be replicated (*pir*⁺). **2**, Each strand is hence used as a template for replication, leading to a mixed population of plasmids within the cell. If the construction of the circles is correct and the 1:1 stoichiometry between strands is respected, each profile should represent 50% of the plasmid content within the cell. **3**, To analyze this, plasmids were extracted, diluted and segregated again by transformation in the same strain (**4**). **5**, Transformants were analyzed by PCR amplification and restriction, giving a close to 50/50 distribution of profiles.




**Supplementary Figure 3.** Recombination frequency of *aadA7* x *ereA2 attC* sites in direct and inverted orientation. Columns represent the mean and error bars represent standard deviation (n≥3). Significance testing was performed with an ANOVA test (**: P value < 0.01; n.s: not significant; n.d.: not detected). In the reactions where no recombinants were detected the column represents the limit of detection of the experiment. Φ represents the limit of detection ($1{,}67 \times 10^{-8}$) for recombination events in which the *attC* could have hypothetically been recognized and processed as a double strand (not detected).




**Supplementary Figure 4.** Activity of IntI1 on mismatched sites **(a)** Binding activity of IntI1 to wt *attI1* site and mismatched sites. Binding is measured as the percentage of substrate retained on nitrocellulose filters after incubating 5 pmoles of integrase with 1 pmol of radiolabeled substrate. Values represent the mean and error bars the standard deviation (n=3). Statistical analysis was performed using Dunnett's test with the binding to the wt site as the control (n.s.: not significant; **: significant (α=0,01)). **(b)** Uncut gel from Figure 5. Sub: substrate.




**Supplementary Figure 5.** L-box recombination in the *attI* x *attC* reaction. (**a**) Diagram of the suicidal conjugative delivery of the top strand of $attC_{aadA7}$ and a $\Delta T_{23}$ variant. (**b**) Frequency of L-box recombination events (described in **d**) for wild type and $\Delta T_{23}$ mutant site. (**c**) Diagram of the known recombination events involving the R box of $attC_{aadA7}$ (bs and ts) and of *attI*. (**d**) Recombination events through the L-boxes detected in this experiment. The crossover region is marked based on **e**. (**e**) Example of the sequence of the *attI1* x $attC_{aadA7\Delta T23}$ recombination product after left-box recombination. The exact crossover point cannot be determined due to adenine repetitions.

Supplementary Table 1. *Escherichia coli* strains used in this work

| Strain number | Relevant genotype | Reference |
|---|---|---|
| | **Basic Strains** | |
| ω55 | *E. coli* K12 MG1655 | Laboratory collection |
| ω162 | DH5α | Laboratory collection |
| ω1628 | Π1 DH5α *ΔthyA*::(erm-pir) | Demarre *et al*. 2005[1] |
| ω72 | β2163 | Demarre *et al*. 2005[1] |
| ω87 | β2150 Δ*dapA*::(*erm*,*pir* ) *thrB1004, pro, thi, strA, hsdS, lacZ* D*M15*,(F' *lacZ* D*M15 lacIq, traD36, proA+, proB+*) | Demarre *et al*. 2005[1] |
| ω8488 | MG1655 Δ*dapA* recA269::Tn10 (p3153) strain used for the integration of pSW plasmids into the attB site of the chromosome through lambda recombination mediated by plasmid p3153 | This work |
| | **Host Machinery** | |
| ωA197 | MG1655 (p3938) (p4884) | This work |
| ω9987 | MG1655 recA269::Tn10 (N4279) with plasmids p3938 and p4884 | RG. Lloyd and this work. |
| ω9988 | MG1655 recA269::Tn10 recG263::kan (N5059) (p3938) (p4884) | RG. Lloyd and this work. |
| ω9989 | MG1655 recA269::Tn10 ruvABC::cm (N5091) (p3938) (p4884) | RG. Lloyd and this work. |
| ω9990 | MG1655 recA269::Tn10 ruvABC::cm recG263::Km (N5070) (p3938) (p4884) | RG. Lloyd and this work. |
| | **Mismatched Covalent Circles** | |
| | **Single strand production** | |
| ω8675 | β2150 (p8669) | This work |
| ω8676 | β2150 (p8670) | This work |
| | **Transformed strains** | |
| ω7120 | MG1655*mutS*215 (p1177) | Loot *et al*. 2012[2] |
| ω7994 | MG1655*mutS*215 (p3938) (p929) | Loot *et al*. 2012[2] |
| ωA266 | MG1655*mutS*215 (p929) | Loot *et al*. 2012[2] |
| ω1628 | Π1 DH5α *ΔthyA*::(erm-pir) | Demarre *et al*. 2005[1] |
| | **Chromosome recombination assay** | |
| ωA642 | DH5α (pA642) plasmid from GeneArt with *attI* sites in direct (head to tail) orientation. | This work |
| ωA749 | DH5α (pA749) inversion of the left *attI* site in pA642 (*attI* sites in tail to tail orientation) | This work |
| ωA873 | Π1 (pA873) cloning of $attI1_{WT}$-$attI1_{STOP}$-*dapA* in direct orientation (from pA642) in a pSW plasmid | This work |
| ωA874 | Π1 (pA874) cloning of $attI1_{WT}$-$attI1_{STOP}$-*dapA* in inverse orientation (from pA749) in a pSW plasmid | This work |
| ωB36 | ω8488 attB::$attI1_{WT}$-$attI1_{STOP}$-*dapA* [$Sp^R$] (integration of plasmid pA873 in the chromosome). Direct orientation of the sites. | This work |
| ωB37 | ω8488 attB::$attI1_{WT}$-$attI1_{STOP}$-*dapA* [$Sp^R$] (integration of plasmid | This work |

| | | |
|---|---|---|
| | pA874 in the chromosome). Inverse orientation of the sites. | |
| ωB82 | B36 (p3938) | This work |
| ωB83 | B37 (p3938) | This work |
| ωC131 | Π1 (pC131) plasmid in which the $attI_{WT}$ of pA874 has been changed for an $attI_{AAA}$) | This work |
| ωC132 | Π1 (pC132) plasmid in which the $attI_{WT}$ of pA873 has been changed for an $attI_{AAA}$) | This work |
| ωC139 | ω8488 attB::$attI1_{AAA}$-$attI1_{STOP}$-dapA [$Sp^R$] (integration of plasmid pC132 in the chromosome). Direct orientation of the sites. | This work |
| ωC140 | ω8488 attB:: $attI_{AAA}$ -$attI1_{STOP}$-*dapA* [$Sp^R$] (integration of plasmid pC131 in the chromosome). Inverse orientation of the sites. | This work |
| ωC162 | ωC139 (p3938) | This work |
| ωC163 | ωC140 (p3938) | This work |
| ωB413 | ω8488 attB:: $attC_{aadA7}$ –$attC_{ereA2}$-*dapA* [$Sp^R$] (integration of plasmid pB350 in the chromosome). Direct orientation of the sites. | This work |
| ωB414 | ω8488 attB:: $attC_{aadA7}$ –$attC_{ereA2}$-*dapA* [$Sp^R$] (integration of plasmid pB340 in the chromosome). Inverse orientation of the sites. | This work |
| ωB476 | ωB413 (p3938) | This work |
| ωC478 | ωB414 (p3938) | This work |
| **Cleavage point determination through Deep Sequencing** | | |
| ωC351 | Π1 containing the library of $attI_N$ plasmids (pC351) | This work |
| ωC373 | β2163 containing pC351 library. | This work |
| ωC307 | MG1655 *recA* (p3938)(pC252) | This work |
| **IntI1-MBP purification** | | |
| ω9958 | Top10 strain bearing pMAL-CX5::$intI1_{Y312F}$ | This work |
| ωB335 | Top10 strain bearing pMAL-CX5::*intI1* | This work |
| ω888 | *E. coli* BL21 (DE3) strain containing the T7 polymerase and plasmid pLysS expressing T7 lysozyme | Studier *et al*. 1990[3] |
| **Left box recombination in the *attC* x *attI* reaction** | | |
| ωD060 | β2163 donor of pSW23T::$attC_{aadA7}$ bs | This work |
| ωD059 | β2163 donor of pSW23T::$attC_{aadA7}$ ts | This work |
| ωD805 | β2163 donor of pSW23T:: $\Delta T_{23}attC_{aadA7}$ bs | This work |
| ωD806 | β2163 donor of pSW23T:: $\Delta T_{23}attC_{aadA7}$ ts | This work |
| ω9669 | Recipient DH5α bearing p3938 (pBAD::*intI1)* and p929 (pSU38Δ::*attI1)* | This work |

## Supplementary Table 2. Plasmids used in this study

| **Plasmid Number** | **Relevant properties and construction** |
|---|---|
| | *General plasmids* |
| p929 | pSU38Δ::*attI1*, $ori_{p15A}$ [$Km^R$][4] |
| p3938 | pBAD::*intI1*, oriColE1 [$Ap^R$][5] |
| p4884 | pSU38Δ::*attI1*, $ori_{p15A}$ [$Sp^R$][2] |
| pSW23T | plasmid dependent on the pi protein for replication |
| pMAL-C5X | plasmid for protein purification using a maltose binding protein tag (commercial) |

| | |
|---|---|
| | *Mismatched covalent circles* |
| p8669 | pSW24:: *attI1*, *oriFd1*, SacII$^{+}$ NarI$^{-}$ *oriV$_{R6K}$,* [Cm$^{R}$]. EcoRI/BamHI substitution of the *attC* site of p7770[2] by an attI1 site. |
| p8670 | pSW24:: *attI1*, *oriFd1*, SacII$^{-}$ NarI$^{+}$ *oriV$_{R6K}$,* [Cm$^{R}$]. EcoRI/BamHI substitution of the attC site of p7771[2] by an attI1 site. |
| p1177 | pSB118::*pir116*[1] |
| | *Double cleavage. Chromosome.* |
| pA642 | pMK-RQ:: :: *attI1$_{WT}$-attI1$_{STOP}$-ΔdapA* (dir), *ColE1* (Km$^{R}$). Plasmid from GeneArt. |
| pA749 | pMK-RQ:: :: *attI1$_{WT}$-attI1$_{STOP}$-ΔdapA* (inv), *ColE1* (Km$^{R}$). Inversion of the left *attI* site in pA642by SmaI/XhoI digestión and religation. |
| pA873 | pSW23T:: *attI1$_{WT}$-attI1$_{STOP}$-dapA* (dir), *oriT$_{RP4}$*, *oriV$_{R6K}$*, *attP* [Sp$^{R}$]. Insertion (EcoRI/NruI) from pA642 into pSW23T. FRT (Flippase Recognition Target) added in EcoRI. |
| pA874 | pSW23T:: *attI1$_{WT}$-attI1$_{STOP}$-dapA* (inv), *oriT$_{RP4}$*, *oriV$_{R6K}$*, *attP* [Sp$^{R}$]. Insertion (EcoRI/NruI) from pA749 into pSW23T. FRT (Flippase Recognition Target) added in EcoRI. |
| pC131 | pSW23T:: *attI1$_{AAA}$-attI1$_{STOP}$-dapA* (dir), *oriT$_{RP4}$*, *oriV$_{R6K}$*, *attP* [Sp$^{R}$]. Modification of the left *attI* site from pA873 to avoid bottom-strand recombination. |
| pC132 | pSW23T:: *attI1$_{AAA}$-attI1$_{STOP}$-dapA* (inv), *oriT$_{RP4}$*, *oriV$_{R6K}$*, *attP* [Sp$^{R}$]. Modification of the left *attI* site from pA874 to avoid bottom-strand recombination. |
| pB350 | pSW23T:: *attC$_{aadA7}$-attC$_{ereA2}$-dapA* (dir), *oriT$_{RP4}$*, *oriV$_{R6K}$*, *attP* [Sp$^{R}$]. FRT (Flippase Recognition Target) added in EcoRI. |
| pB340 | pSW23T:: *attC$_{aadA7}$-attC$_{ereA2}$-dapA* (inv), *oriT$_{RP4}$*, *oriV$_{R6K}$*, *attP* [Sp$^{R}$]. FRT (Flippase Recognition Target) added in EcoRI. |
| | *Cleavage site: Deep sequencing* |
| pC351 | pSW23T::*attI1$_{N}$* Library of plasmids bearing random bases in *attI1* (XhoI/PstI). |
| pC252 | pSU38 bearing *attI$_{TTT}$*. |
| | *attC x attI recombination through the L box.* |
| pD060 | pSW23T::*attC$_{aadA7}$* bs |
| pD059 | pSW23T::*attC$_{aadA7}$* ts |
| pD805 | pSW23T:: *ΔT$_{23}$attC$_{aadA7}$* bs |
| pD806 | pSW23T:: *ΔT$_{23}$attC$_{aadA7}$* ts |

## Supplementary Table 3. Oligonucleotides used in this study

| Number | Name | Sequence | Purpose |
|---|---|---|---|
| | | ***Host machinery*** | |
| 1897 | Swbeg | CCGTCACAGGTATTTATTCGGCG | Testing for recombination |
| 2420 | MFD | CGCCAGGGTTTTCCCAGTCAC | |
| | | ***Mismatched Covalent Circles*** | |
| 2391 | SeqattI1 | CACAGGAAACAGCTATGACC | Amplification of the region of the cointegrate including the mismatches. |
| 2393 | SeqNar | GCTTAATGAATTACAACAGTACTGC | |
| | | ***Double cleavage*** | |
| 1078 | MV143 | CCTCTTACGTGCCGATCAACGTCTC | Verification of the constructs in pSW plasmids |
| 1962 | DapA-R | GTGGTGCCAACAGAAACGATCGC | |

| | | | |
|---|---|---|---|
| 571 | lacI-F | CATTAATGCAGCTGGCACGA | Sequencing |
| 2263 | 5- Eco-FRT-Eco | AATTCGAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCG | Insertion of a FRT site in pSW plasmids |
| 2264 | 3- Eco-FRT-Eco | AATTCGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGAATAGGAACTTCG | |
| 1897 | Swbeg | CCGTCACAGGTATTTATTCGGCG | Verification of the monomeric/dimeric insertion of pSW plasmids in the chromosome |
| 1898 | Swend | CCTCACTAAAGGGAACAAAAGCTG | |
| 1319 | insertion lac-a | TTCAACTAGTGCGTGTGGAATGTGACGATCTTCGCGTCACC | |
| 1698 | inv1_attL6 | AATAATGATTTTATTTTGACTGATAGTGACCTGTTCGTTGC | |
| 1897 | Swbeg | CCGTCACAGGTATTTATTCGGCG | Verification of the inversion of the region between inverted *attI* sites (double cleavage) |
| 2933 | Directionel Rec DAP | CATACTCGTATGTTGTGTGG | |
| 930 | J23100-R | CACTGTACCTAGGACTGAGCTAGCC | |
| 1897 | Swbeg | CCGTCACAGGTATTTATTCGGCG | Verification of recombination between direct *attI* sites |
| 1962 | DapA-R | GTGGTGCCAACAGAAACGATCGC | |
| ***Cleavage site: deep sequencing*** | | | |
| 2915 | mutatti WT F | GCGCCTCGAGGTTCGGTTAATGTTATGGAGCAGCAACGATGTTACGCAGCAGGGCAGTCG | Amplification of *attI* sites with random bases to build the library of $attI_N$ |
| 2916 | mutatti WT R A1 | GCGCCTGCAGTCCCGTGGCGTAACTTTGNTTTAGGGCGACTGCCCTGCTGCGTAACATCG | |
| 2917 | mutatti WT R A2 | GCGCCTGCAGTCCCGTGGCGTAACTTTGTNTTAGGGCGACTGCCCTGCTGCGTAACATCG | |
| 2918 | mutatti WT R A3 | GCGCCTGCAGTCCCGTGGCGTAACTTTGTTNTAGGGCGACTGCCCTGCTGCGTAACATCG | |
| 2919 | mutatti WT R A4 | GCGCCTGCAGTCCCGTGGCGTAACTTTGTTTNAGGGCGACTGCCCTGCTGCGTAACATCG | |
| 2920 | mutatti WT R C1 | GCGCCTGCAGTCCCGTGGCGTAACTTTNTTTTAGGGCGACTGCCCTGCTGCGTAACATCG | |
| 2921 | mutatti WT R C2 | GCGCCTGCAGTCCCGTGGCGTAACTTTGTTTTANGGCGACTGCCCTGCTGCGTAACATCG | |
| 2922 | mutatti WT R C3 | GCGCCTGCAGTCCCGTGGCGTAACTTTGTTTTAGNGCGACTGCCCTGCTGCGTAACATCG | |
| 2923 | mutatti WT R C4 | GCGCCTGCAGTCCCGTGGCGTAACTTTGTTTTAGGNCGACTGCCCTGCTGCGTAACATCG | |
| 2924 | mutatti WT R Control (G) | GCGCCTGCAGTCCCGTGGCGTAANTTTGTTTTAGGGCGACTGCCCTGCTGCGTAACATCG | |
| 2925 | mutatti WT T | GCGCCTGCAGTCCCGTGGCGTAACTTTGTTTTNGGGCGACTGCCCTGCTGCGTAACATCG | |
| 3449 | attIN's right | TTTGATTTGAGCTCCACCGC | Amplification of the *attI* sites from the library and the |
| 1236 | MV202 | TCAACGGGAATCCTGCTCTG | |

| | | | |
|---|---|---|---|
| 738 | MV84 | CGACCATATGAAGAGGATGCCGCT AGGACC | cointegrates. |
| 1896 | MRV | AGCGGATAACAATTTCACACAGGA | |
| **In vitro** | | | |
| 3885 | attI WT top | TTTGATGTTATGGAGCAGCAACGA TGTTACGCAGCAGGGCAGTCGCCC TAAAACAAAGTTAGGCATCA | Construction of *attI1* substrates for *in vitro* experiments. |
| 3886 | attI WT bot | TGATGCCTAACTTTGTTTTAGGGCG ACTGCCCTGCTGCGTAACATCGTTG CTGCTCCATAACATCAAA | |
| 3887 | attI TTT top | TTTGATGTTATGGAGCAGCAACGA TGTTACGCAGCAGGGCAGTCGCCC TAAAACAAATTTAGGCATCA | |
| 3888 | attI TTT bot | TGATGCCTAAATTTGTTTTAGGGCG ACTGCCCTGCTGCGTAACATCGTTG CTGCTCCATAACATCAAA | |
| 3889 | attI L:AAATC R:WT top | TTTGATGTTATGGAGCAGCAACGA TGTTACGCAGCAGGGCAGTCGCCC TAAATCAAAGTTAGGCATCA | |
| ***Influence of $T_{23}$ in the* attC *x* attI *reaction*** | | | |
| 3849 | $attC_{aadA7}$-Fw | AATTCGTCTAACAATTCATTCAAGC CGACGCCGCTTCGCGGCGCGGCTT AATTCAAGCGTTAGACG | Construction of the wild type *aadA7* site |
| 3850 | $attC_{aadA7}$-Rev | GATCCGTCTAACGCTTGAATTAAGC CGCGCCGCGAAGCGGCGTCGGCTT GAATGAATTGTTAGACG | |
| 3571 | $attC_{aadA7}$-$\Delta T_{23}$ Fw | GATCCGTCTAACGCTTGAATTAAGC CGCGCCGCGAAGCGGCGCGGCTTG AATGAATTGTTAGACG | Construction of the ΔT23 *aadA7* site |
| 3572 | $attC_{aadA7}$-$\Delta T_{23}$ Rev | AATTCGTCTAACAATTCATTCAAGC CGCGCCGCTTCGCGGCGCGGCTTA ATTCAAGCGTTAGACG | |
| 1897 | Swbeg | CCGTCACAGGTATTTATTCGGCG | Determination of the orientation and recombination box. |
| 1898 | Swend | CCTCACTAAAGGGAACAAAAGCTG | |
| 1895 | MFD | CGCCAGGGTTTTCCCAGTCAC | |
| 571 | lacI-F | CATTAATGCAGCTGGCACGA | |
| 3883 | RpLp | GGATCCGTCTAACGCTTG | |
| 3884 | RppLpp | GAATTCGTCTAACAATTC | |

Suplementary references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2. Loot, C., Ducos-Galand, M., Escudero, J.A., Bouvier, M. & Mazel, D. Replicative resolution of integron cassette insertion. *Nucleic Acids Res* **40**, 8361-70 (2012).
3. Studier, F.W., Rosenberg, A.H., Dunn, J.J. & Dubendorff, J.W. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol* **185**, 60-89 (1990).

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5. Demarre, G., Frumerie, C., Gopaul, D.N. & Mazel, D. Identification of key structural determinants of the IntI1 integron integrase that influence *attC* x *attI1* recombination efficiency. *Nucleic Acids Res* **35**, 6475-89 (2007).