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Nucleoid-associated Rok differentially affects chromosomal transformation on *Bacillus subtilis* recombination-deficient cells

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Summary

Rok, a *Bacillus subtilis* nucleoid-associated protein (NAP), negatively regulates competence development and silences xenogeneic genes. We show that *rok* inactivation increases *rpoB*482 natural intraspecies chromosomal transformation (CT) and plasmid transformation to a different extent. In Δ*addAB*, Δ*recO*, *recF15*, Δ*recU*, Δ*ruvAB* or *rec*$^+$ cells intraspecies CT significantly increases, but the Δ*recD2* mutation reduces, and the Δ*recX*, Δ*radA* or Δ*dprA* mutation further decreases CT in the Δ*rok* context when compared to *rok*$^+$ cells. These observations support the idea that *rok* inactivation, by altering the topology of the recipient DNA, differentially affects the integration of homologous DNA in *rec*-deficient strains, and in minor extent the competent subpopulation size. The impairment of other NAP (Hbsu or LrpC) also increased intra- and interspecies CT (nonself-DNA, ∼8% nucleotide sequence divergence) in *rec*$^+$ cells, but differentially reduced both types of CTs in certain *rec*-deficient strains. We describe that *rok* inactivation significantly stimulates intra and interspecies CT but differentially reduces them in transformation-deficient cells, perhaps by altering the nucleoid architecture. We extend the observation to other NAPs (Hbsu, LrpC).

Introduction

Horizontal gene transfer (HGT), *i.e.*, the nonsexual exchange of genes, is crucial for the acquisition of genetic diversity in bacterial populations. HGT has a central role in evolution and a major impact on the transfer of antibiotic resistance, on the emergence of bacterial pathogens and on vaccine escape (Gogarten *et al*., 2002; Fraser *et al*., 2007; Syvanen, 2012). HGT is a complex event: from one side includes the widespread acquisition of mobile genetic elements from even different taxonomic groups by conjugation, viral transduction or natural transformation. From another side, HGT refers to the narrow exchange of chromosomal markers among members of the same *Genus*. These markers become integrated *via* homologous recombination (HR) into the highly compacted recipient genome *via* natural chromosomal transformation (CT), viral generalized transduction or Hfr conjugation (Matic *et al*., 1996; Fraser *et al*., 2007).

To reduce any deleterious effect on the new host introduced by the acquired DNA, bacteria have developed barriers to HGT, but their relevance vary with the DNA transfer mechanism used (Fraser *et al*., 2007; Sorek *et al*., 2007; Takeuchi *et al*., 2014). First, adaptive immune systems (as CRISPR-Cas) and restriction-modification systems provide barriers to nonself-transfer via conjugation and viral transduction. However, these systems are usually absent (*e.g.*, CRISPR-Cas) or are not effective (*e.g.*, restriction-modification systems) to fragment the internalized single-stranded DNA (ssDNA) in naturally non-replicating competent cells (as those of the Firmicutes Phylum) (Trautner and Spatz, 1973; Kidane *et al*., 2012; Oliveira *et al*., 2016). Second, the mismatch repair system strongly reduces gene exchange *via* transduction or conjugation (Rayssiguier *et al*., 1989; Matic *et al*., 1996; Fraser *et al*., 2007). Nevertheless, it poorly operates in non-replicating Firmicutes competent cells, or the system is easily saturated during natural CT (Humbert *et al*., 1995; Majewski and Cohan, 1998; Carrasco *et al*., 2019). Third, if the donor DNA is similar but not identical (homeologous DNA), nucleotide sequence divergence up to 15%–16% imposes a major barrier to gene acquisition *via* Hfr conjugation or natural CT (Rayssiguier *et al*., 1989; Zawadzki *et al*., 1995; Serrano *et al*., 2021). In natural *Bacillus subtilis* competent cells, beyond 15% sequence divergence only microhomologous DNA segments are integrated *via*

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RecA-facilitated microhomologous integration (Carrasco *et al*., 2016; Serrano *et al*., 2021). Finally, bacteria have evolved mechanisms that modulate the trade-off between benefit and cost of acquired foreign (xenogeneic) genes (Dillon and Dorman, 2010; Gordon *et al*., 2011; Singh *et al*., 2016). Here, a specialized family of nucleoid associated proteins (NAPs), which do not affect the DNA transfer process *per se*, prevent transcription (silencing) of the acquired xenogeneic genes, *via* conjugation and viral transduction. However, natural transformable bacteria lack xenogeneic silencers (Singh *et al*., 2016).

How natural competent bacteria balance the potential costs of inefficiently expressed newly acquired xenogeneic genes is still unknown. It has been hypothesized that some species of the *Bacillus* genus, in particular those of the *B. subtilis* and *B. pumilus* clades, have acquired the *rok* gene from an unidentified source *via* HGT, but others such as the species of the *B. cereus* or *B. megaterium* clades have not (Albano *et al*., 2005; Smits and Grossman, 2010). The narrowly distributed Rok NAP silences the expression of xenogeneic genes of a conjugative transposon, contributes to the nucleoid architecture and reduces gene transfer by limiting the size of the subpopulation that develops competence (Maamar and Dubnau, 2005; Smits and Grossman, 2010; Marbouty *et al*., 2015). Xenogeneic silencers use the fact that horizontally acquired genes tend to be more dA + dT-rich than the genome of the recipient host to recognize them. However, Rok differs from the prototype of xenogeneic silencers, *i.e*., H-NS-like proteins (found in bacteria of the α-, β- and γ-Proteobacteria *Classes*), MvaT-like proteins (γ-Proteobacteria *Class*) and Lsr2-like proteins (in bacteria of the Actinobacteria Phylum) (Castang *et al*., 2008; Dillon and Dorman, 2010; Gordon *et al*., 2011; Ali *et al*., 2014; *Ding et al*., 2015; Qin *et al*., 2019). While most xenogeneic silencers use an AT-hook (as H-NS- and LsrA-like) or an AT-pincer motif (as MvaT-like) (Gordon *et al*., 2011; Ali *et al*., 2014; Ding *et al*., 2015; Singh *et al*., 2016), *B. subtilis* Rok recognizes dA + dT regions from the DNA minor groove *via* a winged-helix motif and forms oligomers (Smits and Grossman, 2010; Duan *et al*., 2018). Rok, which has a higher preference for TACTA, AACTA, and ATATA motifs (Smits and Grossman, 2010; Duan *et al*., 2018), imposes transcription and translation limitations that may affect the persistence of xenogeneic genes in the new host (Smits and Grossman, 2010; Duan *et al*., 2018; Qin *et al*., 2019). The sequence motifs preferred by Rok are not frequent in the resident genome of *B. subtilis* cells, and this might help Rok to efficiently distinguish self from nonself-DNA (Smits and Grossman, 2010; Duan *et al*., 2018). Therefore, *rok* inactivation should affect interspecies CT, but not intraspecies CT.

In *B. subtilis* competent cells, if the internalized homeologous ssDNA shows more than one mismatch in an 8-bp interval within the minimal efficient processing segment (MEPS), recombination-mediated DNA strand exchange is blocked (Serrano *et al*., 2021). This confirms that sequence divergence provides a physical barrier for chromosomal gene transfer. Indeed, the HR system and DNA sequence divergence are the main barriers for CT (Carrasco *et al*., 2016; Serrano *et al*., 2021). Since natural competence is transiently developed in a minority of *B. subtilis* cells, detection of interspecies CT reached a plateau at a sequence divergence above 8% in *B. subtilis* wild-type (*wt*) cells (Zawadzki *et al*., 1995). To overcome such technical difficulty, Rok, which is also a repressor of the master transcriptional activator of natural competence ComK, was deleted (Albano *et al*., 2005). In the absence of Rok, which does not affect DNA uptake (Maamar and Dubnau, 2005), *comK* expression is deregulated and CT efficiency is significantly increased. This was interpreted to be due to an increasement of the size of the subpopulation that develops natural competence (Maamar and Dubnau, 2005), thereby increasing indirectly the sensitivity of the CT assay (Carrasco *et al*., 2016). The role of Rok, however, has been recently challenged, because intraspecies CT efficiency was lower than expected in certain *rec*-deficient strains in the Δ*rok* context (Serrano *et al*., 2021).

The maintenance of the integrated genetic information might depend on a biological barrier derived from the balance between the fitness advantage provided by the new gene and the cost linked to its expression. Recently, it has been shown that Rok controls large- and small-scale DNA structures that influence DNA transactions, including replication and transcription (Smits and Grossman, 2010; Marbouty *et al*., 2015). In *B. subtilis* cells there are ∼20 statistically significant dynamically distinct topological domains [chromosomal interaction domain (CID)] (Marbouty *et al*., 2015). The majority of them coincide with highly expressed genes (12/20) that block the diffusion of free supercoils, but a significant number of regions (6/20) correlate with genomic positions bound by NAPs (*e.g*., Rok) or with AT-rich sequences of mobile genetic elements (*ICEBs*1 and the SPβ). Two non-mutually exclusive model have been proposed to explain the role of Rok. First, Rok binds to promoter regions of xenogeneic genes, which are more AT-rich than their associated ORFs, and silences the expression of genes acquired *via* HGT, thus its absence should affect natural interspecies CT by decreasing the cost of acquiring homeologous genes (Smits and Grossman, 2010; Duan *et al*., 2018; Qin *et al*., 2019). Second, Rok in concert with AbrB creates an opportunity for competence within the sporulation window (Schultz, 2009). Rok contributes to repress ComK expression and reduces competence development (Albano *et al*., 2005; Maamar and Dubnau, 2005), thus, inactivation of *rok*

increases the size of the subpopulation that develops competence and indirectly might increase the acquisition of xenogeneic genes.

When *B. subtilis* cells enter in the competence state, which is induced in response to different types of stress at the onset of stationary phase, the expression of a large set of genes (~5% of total genes, being *recA*, *ssbA*, *ssbB* and *dprA* among them) is altered (Berka *et al*., 2002; Hamoen *et al*., 2002; Ogura *et al*., 2002). During competence development, ongoing DNA replication is halted, transcript–translation is significantly reduced, (p)ppGpp levels are increased, the interdependent actions of partially redundant pathways that together determine the DNA topological state is reshaped, the overall negative supercoiling of the DNA is decreased, and the DNA uptake apparatus is built at one cell pole (Hahn *et al*., 2005; Kaufenstein *et al*., 2011; Kalamara *et al*., 2018; Dubnau and Blokesch, 2019; Maier, 2020). In contrast, during vegetative growth, the coordinated interplay between transcription and DNA replication dynamically regulates the supercoiling level and the nucleoid structure, with the NAPs exerting a rheostatic control both locally and globally (Rimsky and Travers, 2011). Since growth is transiently arrested during competence, the integration of the incoming ssDNA is independent of this replication–transcription interplay, and the mechanism for uncoupling negative supercoiling from incoming ssDNA integration during natural transformation is still poorly understood. However, the competence recombination machinery has the ability to utilize this 'special' environment (see Supplementary material Annex I) (Serrano *et al*., 2020; Serrano *et al*., 2021).

The contribution of Rok and the HR machinery to intra- and interspecific genetic exchange *via* natural *B. subtilis* CT is of significant interest and was studied here. Rok might differentially affect DNA integration by altering the DNA topology rather than by limiting the competent subpopulation size. We propose that *rok* inactivation facilitates the integration of incoming DNA in transformation-proficient cells but significantly reduces CT in certain *rec*-deficient cells (*e.g*., *recX*), perhaps by the accumulation of a torsional stress that might affect the RecA-mediated DNA strand exchange efficiency, and we extend the observation to other NAPs, as Hbsu and LrpC.

Results and discussion

Absence of Rok increases CT and to a lesser extent the subpopulation of cells that develops competence

To test whether the increase in the size of the *B. subtilis* subpopulation that develops natural competence in the absence of Rok also drives to an increase in natural CT, we selected the homologous 2997-bp *rpoB*482 DNA [bearing a single centrally located point mutation at codon 482 (nucleotide position 1443)], which confers resistance to rifampicin (Rif^R). Natural CT requires active DNA uptake and the HR machinery to catalyse homology-directed RecA-mediated integration of the incoming ssDNA in the recipient duplex chromosome (see Supporting Information Annex I) (Alonso *et al*., 1988). On the other hand, natural plasmid transformation requires active DNA uptake, internal homology (*e.g*., an oligomeric plasmid molecule), a replication origin and single strand annealing proteins (*e.g*., DprA, RecO) and a negative RecA modulator [*e.g*., RecX (or RecU in the Δ*recX* context)] to reconstitute an active replicon in a RecA-independent manner (Canosi *et al*., 1978, 1981; Yadav *et al*., 2012, 2013; Serrano *et al*., 2020). Since cells competent for CT are also competent for plasmid transformation, the heterologous plasmid pHP14 was used as a reporter of the size of the subpopulation that develops natural competence in the Δ*rok* context.

The selected donor DNAs have two other particularities. First, the dA + dT content of the homologous *Bsu* 168 *rpoB*482 DNA (dA + dT 54.7%) is slightly lower and of the heterologous plasmid pHP14 DNA is significantly higher (dA + dT content 60.2%), than the average of the species (dA + dT 56.5%). Second, both donor DNAs (*Bsu* 168 *rpoB*482 and pHP14 DNA) lack the high affinity Rok target site (TACTA), and the occurrence of the non-cognate AAAAA site is ~7- and ~ 4-times, respectively, more frequent than the Rok high avidity site.

The BG214 *wt* strain and its isogenic derivatives lacking Rok (BG1359), RecA (BG190) or Rok and RecA (BG1633) (Table 1) were developed into competence state as described (see Materials and methods), and incubated with limiting concentrations of *Bsu rpoB*482 DNA or oligomeric pHP14 DNA (0.1 $\mu g \cdot ml^{-1}$) to test chromosomal and plasmid transformation efficiency, respectively. Then, the transformation reactions were plated on LB agar plates containing Rif (8 $\mu g \cdot ml^{-1}$) or Ery (2 $\mu g \cdot ml^{-1}$), for the selection of chromosomal and plasmid transformants, respectively (Alonso *et al*., 1988; Carrasco *et al*., 2016). The plasmid DNA, which shares no significant degree of identity with the host genome, was artificially oligomerized (linearized and self-ligated under high DNA concentration conditions) to optimize the transformation frequency (see Canosi *et al*., 1978).

The frequency of intraspecies CT, with homologous *Bsu* 168 *rpoB*482 DNA, increased ~15-fold ($p < 0.01$) in competent Δ*rok* cells when compared to the parental *wt* (rok^+) control (Fig. 1; Supporting Information Table S1) (Carrasco *et al*., 2016). As expected, *recA* inactivation blocks CT, to levels comparable to the spontaneous mutation rate (Fig. 1, Table S1) (Carrasco *et al*., 2019).

As reported, plasmid transformation proceeds with ~10-fold ($P < 0.01$) lower efficiency than CT in *wt* cells

Table 1. *rec*-deficient strains used in this study.

Strains[a]	Relevant genotype	Source	Strains[b]	Relevant genotype	Source
BG214	*wt* (*rec*$^+$)	Lab. strain	BG1359	+ Δ*rok*	Carrasco *et al*. (2016)
BG190	+ Δ*recA*	Ceglowski *et al*. (1990)	BG1555	+ Δ*recA* Δ*rok*	Carrasco *et al*. (2019)
BG1245	+ Δ*radA*	Gándara and Alonso (2015)	BG1373	+ Δ*radA* Δ*rok*	(Torres *et al*. (2017))
BG439	+ Δ*recO*	Fernndez *et al*. (1999)	BG1641	+ Δ*recO* Δ*rok*	Serrano *et al*. (2021)
BG129	+ *recF15*	Alonso *et al*. (1988)	BG1611	+ *recF15* Δ*rok*	Serrano *et al*. (2021)
BG1337	+ Δ*addAB*	Vlasic *et al*. (2014)	BG1631	+ Δ*addAB* Δ*rok*	Serrano *et al*. (2021)
BG703	+ Δ*ruvAB*	Sanchez *et al*. (2005)	BG1485	+ Δ*ruvAB* Δ*rok*	Serrano *et al*. (2021)
BG855	+ Δ*recU*	Fernndez *et al*. (1998)	BG1653	+ Δ*recU* Δ*rok*	Serrano *et al*. (2021)
BG675	+ Δ*recJ*	Sanchez *et al*. (2006))	BG1813	+ Δ*recJ* Δ*rok*	Serrano *et al*. (2021)
BG1455	+ Δ*recD2*	Torres *et al*. (2017)	BG1549	+ Δ*recD2* Δ*rok*	Serrano *et al*. (2021)
BG1065	+ Δ*recX*	Crdenas *et al*. (2012)	BG1397	+ Δ*recX* Δ*rok*	Serrano *et al*. (2021)
BG1163	+ Δ*dprA*	Gándara and Alonso (2015)	BG1811	+ Δ*dprA* Δ*rok*	Serrano *et al*. (2021)
BG1879	+ *hbs*55	This work	BG1871	+ *hbs*55 Δ*rok*	This work
BG1893	+ Δ*lrpC*	This work	BG1901	+ Δ*lrpC* Δ*rok*	This work
BG1899	+*hbs*55 Δ*recX*	This work	BG1909	+ *hbs*55 Δ*lrpC*	This work
BG955	+ Δ*topB*	This work	BG1903	+ Δ*lrpC* Δ*recX*	This work

All *B. subtilis* strains are isogenic with BG214 (*trpCE metA*5 *amyE1 ytsJ*1 *rsbV*37 *xre*1 *xkd*A1 *att*$^{SP\beta}$ *att*^{ICEBs1}).

(Table S1) (Canosi *et al*., 1978). In competent Δ*rok* and Δ*rok* Δ*recA* cells, oligomeric plasmid DNA transformation was slightly increased (up to threefold, $P > 0.05$) when compared with *wt* and Δ*recA* cells (Fig. 1, Table S1). Since *rok* inactivation does not compromise DNA uptake (see Maamar and Dubnau, 2005), but CT is increased by ∼15-fold while xenogenic plasmid transformation only by threefold when compared to the *rok*$^+$ control, we have to assume that Rok must be performing other functions during CT rather than simply increasing the size of the subpopulation that develops competence. However, from the two non-mutually exclusive hypotheses enunciated in the Introduction, and the data presented in Fig. 1 and Table S1, we considered it unlikely that Rok works as a xenogeneic silencer during intraspecies CT. First, the *rpoB* gene maps into a discrete CID that is not influenced by Rok (Marbouty *et al*., 2015), and Rok neither interacts with nor regulates *rpoB* expression (Seid *et al*., 2017). Second, the donor *Bsu* 168 *rpoB*482 DNA, which only includes the coding region of the *rpoB*482 gene, has a single mismatch difference (the Rif^R mutation) with the recipient DNA, and this mutation maps in a region with a dA + dT content (53.9%) below the average of the species (dA + dT 56.5%). Third, the presence or absence of the mismatch repair system marginally affects (<3-fold) the efficiency of intraspecies CT, suggesting that the single mismatch on the donor *Bsu* 168 *rpoB*482 DNA is corrected with lower efficiency in natural competent *B. subtilis* cells (Carrasco *et al*., 2019). Finally, there is no spurious effect of Rif on RNA polymerase transcription, because similar CT frequencies were observed when the concentration of Rif was increased or decreased by factor 2 Carrasco *et al*., 2016). Then, Rok cannot impose a cost to acquire a self *rpoB*482 DNA, and the differential increase in chromosomal *vs* plasmid transformation might have a different answer. The most plausible assumption is that Rok, in addition to regulate ComK expression, provides a physical barrier to intraspecies CT different from gene silencing.

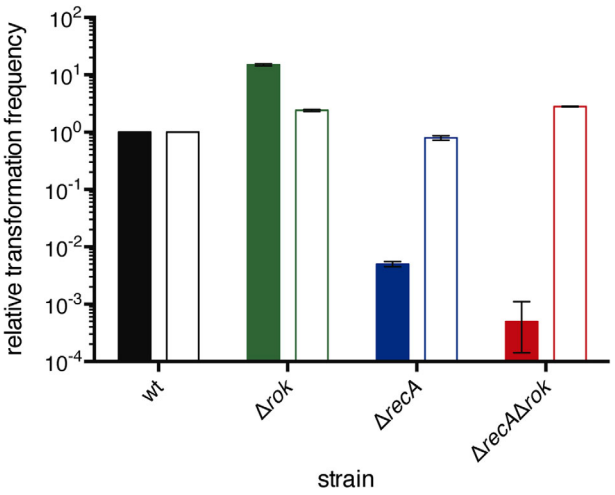

Fig 1. Absence of Rok increases CT and to a lesser extent the subpopulation of cells that develops competence. Natural competence development and transformation was performed as described (see Material and methods and Table S1). Competent cells were transformed with 0.1 μg·ml^{-1} of *rpoB*482 DNA (Rif^R, 8 μg·ml^{-1}) or pHP14 plasmid DNA (Ery^R, 2 μg·ml^{-1}) as described (Alonso *et al*., 1988). The yields of Rif^R (chromosomal transformants, filled bars) and Ery^R (plasmid transformants, empty bars) *cfu*s were normalized relative to that of the *wt* strain, recorded as 1. The results are shown as mean ± SEM of at least four independent experiments. The data used in the graph can be found in Table S1. [Color figure can be viewed at wileyonlinelibrary.com]

Inactivation of rok *inhibits intraspecies CT in* ΔrecD2, ΔrecX, ΔradA *and* ΔdprA *cells*

To evaluate whether the inactivation of *rok* affects a presynaptic stage, homology search or the processing of the

recombination intermediates, and if there is a crosstalk of Rok with HR functions during CT, the single and double mutant strains listed in Table 1 were made competent and CT frequency was measured. In a first step, the frequency of pre-existing or untargeted Rif^R mutants in the strains listed in Table 1, in the absence of *Bsu* 168 *rpoB*482 DNA, was measured. As previously described (Serrano *et al*., 2020; Serrano *et al*., 2021), the frequency of untargeted Rif^R mutants in the *rec*$^-$ strains was similar to the frequencies observed in competent *rok*$^+$ or Δ*rok* cells (6 to 8 x 10^{-9}), whereas in competent Δ*recD2* or Δ*recD2* Δ*rok* cells the frequency of Rif^R mutants increased by ∼threefold (Serrano *et al*., 2020, 2021).

The intraspecies CT frequency in the Δ*recO*, *recF*15, Δ*addAB*, Δ*ruvAB* or Δ*recU* strains was not significantly affected, and it was 15- to 20-fold higher ($P < 0.01$) in the Δ*rok* context when compared to *rok*$^+$ cells (Fig. 2 and Table S2). However, inactivation of *recJ*, *recX*, *radA* or *dprA*, significantly decreased *Bsu* 168 *rpoB*482 DNA intraspecies CT frequency in the *wt* (*rok*$^+$) context ($P < 0.01$) (Fig. 2 and Table S2). Similar results were observed in the *wt*, Δ*recX* or Δ*rok* strains using the *metA*5 chromosomal mutation, which also contains a single point mutation, with *met*$^+$ selection (Crdenas *et al*., 2012; Carrasco *et al*., 2016). Competent Δ*recJ* cells decreased *Bsu* 168 *rpoB*482 intraspecies CT to a similar extent in *the rok*$^+$ or Δ*rok* context (Fig. 2 and Table S2). Unexpectedly, *recD2* inactivation marginally reduced (twofold to threefold; $P > 0.05$) the efficiency of intraspecies CT in the *wt* (*rok*$^+$) control, but reduced CT ∼900-fold ($P < 0.01$) in the Δ*rok* background (Fig. 2 and Table S2). The absence of RecX, RadA/Sms and DprA reduced CT in the *rok*$^+$ background ($P < 0.01$), but further impaired CT in the Δ*rok* context ($P < 0.01$) (Fig. 2 and Table S2). Similar CT frequencies were previously reported for the Δ*rok* Δ*recD2*, Δ*rok* Δ*recX*, Δ*rok* Δ*radA* or Δ*rok* Δ*dprA* strain (Serrano *et al*., 2021). This enhanced impairment in the *recD2*, *recX*, *radA* or *dprA* cannot be attributed to poor competence developments, to a defect on DNA uptake or to an early exit of the competent state, because the competent Δ*rok* Δ*radA*, Δ*rok* Δ*recD2*, Δ*rok* Δ*recX* or Δ*rok* Δ*dprA* cells had a similar level of uptake of radiolabelled double-stranded DNA (dsDNA) (Serrano *et al*., 2020; Serrano *et al*., 2021). Furthermore, plasmid transformation is not significantly impaired in competent Δ*radA* Δ*rok* cells ($P > 0.05$) (Torres *et al*., 2019) and impaired to a similar extent in the presence or absence of Rok in the Δ*recX* context (Fig. 3 and Table S3) or in the Δ*dprA* or Δ*recD2* context (data not shown). It is likely that *rok* inactivation increases CT of transformation-proficient strains (namely in the Δ*recO*, *recF*15, Δ*addAB*, Δ*ruvAB* or Δ*recU* context) as well as of *wt* cells, but significantly decreases CT in the transformation-deficient strains (in the Δ*recX*, Δ*recD2*, Δ*radA* or Δ*dprA* context) (Fig. 2 and Table S2). As shown in the previous section, this paradox cannot be explained by the absence of the xenogenic silencer or by an increase in the size of the subpopulation that develops competence in the Δ*rok* context.

Since the intraspecies CT frequency is significantly increased when compared to the plasmid transformation one, we can envision that Rok exerts its effect on CT by affecting the architectural basis of the bacterial chromosome (either reducing or increasing the supercoils, by

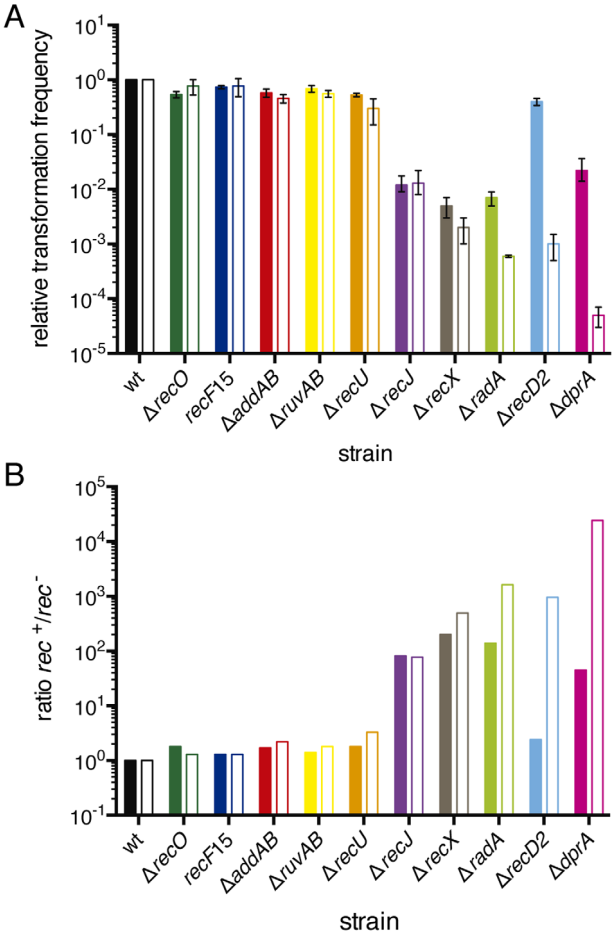

Fig 2. Inactivation of *rok* inhibits intraspecies CT in Δ*recD2*, Δ*recX*, Δ*radA* and Δ*dprA* cells.

A. The rec-deficient mutants, both in the presence (filled bars) or in the absence (empty bars) of the *rok* gene, were made competent and were transformed with 0.1 μg·ml^{-1} of *rpoB*482 DNA (Rif^R) derived from *B. subtilis* 168 (1 single mismatch) as described (see Material and methods and Table S2). The yield of Rif^R (CT) *cfu*s was normalized relative to that of the *rok*$^+$ or the Δ*rok* strain, recorded as 1 (in parentheses, number of transformants/total number of cells).

B. The ratio of transformants between the *rec*$^+$ strain and each *rec*$^-$ mutant, both in the presence (filled bars) or in the absence (empty bars) of the *rok* gene, was calculated from the data represented in panel A. The results are shown as mean ± SEM of at least four independent experiments. The data used in the graph can be found in the Supporting Information Table S2. [Color figure can be viewed at wileyonlinelibrary.com]

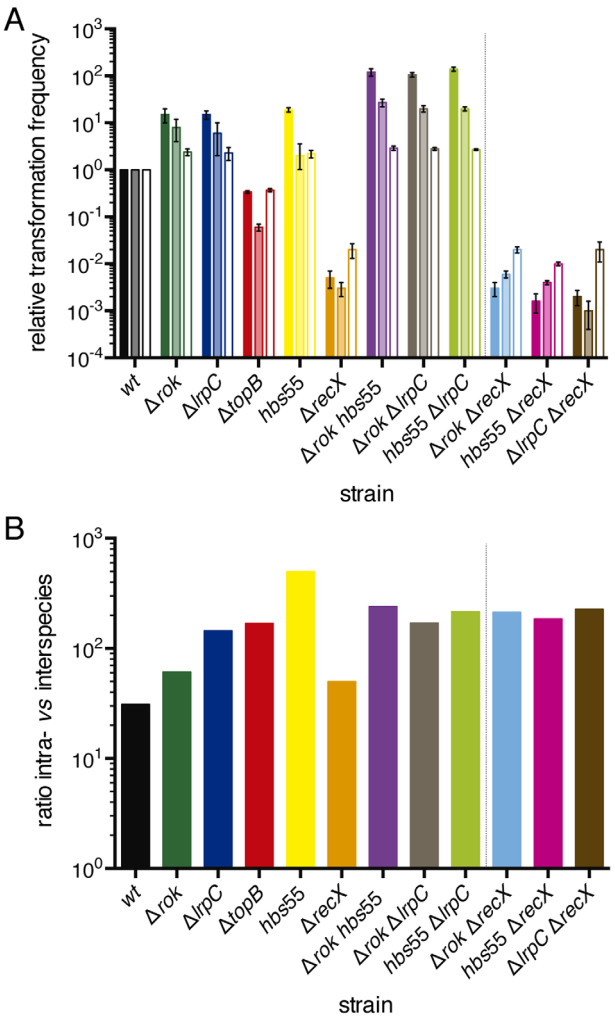

Fig 3. Inactivation of Hbsu or LrpC also show a dual activity during natural CT.

A. Competent cells were transformed with 0.1 μg·ml^{-1} of homologous *rpoB*482 DNA (intraspecies transformation, dark filled bars) or homeologous *rpoB*482 DNA (interspecies transformation, clear filled bars) (Rif^R, 8 μg·ml^{-1}) or pUB110 plasmid DNA (empty bars) (Nm^R, 5 μg·ml^{-1}) as described (see Material and methods and Table S3). The yields of Rif^R (chromosomal transformants) and Nm^R (plasmid transformants) *cfu*s were normalized relative to that of the *wt* strain, recorded as 1 (in parentheses, number of transformants / total number of cells) as described (Alonso *et al*., 1988). For the Δ*rok* Δ*recX*, *hbs*55 Δ*recX* and Δ*lrpC* Δ*recX*, the transformation efficiency is expressed with respect to their most efficient parental strain (Δ*rok*, *hbs*55 or Δ*lrpC* strain).

B. The ratio of transformants between intraspecies and interspecies transformation for each mutant was calculated from the data represented in panel A. The results are shown as mean ± SEM of at least four independent experiments. The data used in the graph can be found in Table S3. [Color figure can be viewed at wileyonlinelibrary.com]

bending, bridging or by folding the genome into a more condensed structure). This way, Rok might negatively counter RecA-mediated capture of a homologous segment and/or DNA strand invasion. In contrast, Rok only partially affects the requirement of RadA, RecX, RecD2 and DprA proteins during intraspecies CT (Fig. 2 and Table S2). Furthermore, inactivation of *rok* might increase interspecies CT transformation by reducing the biological barrier imposed by sequence divergence in the *wt* strain, but interspecies CT is significantly aggravated in the Δ*recX* context (Fig. 3 and Table S3). A nucleotide sequence analysis revealed that all Rif^R clones were genuine transformants in the *wt* strain, but this number was reduced to ∼50% in the Δ*recX* mutant, and even to ∼25% in the Δ*recD2* strain (Serrano *et al*., 2021). To test the hypotheses, we have tested determined CT frequencies upon inactivation of other NAPs.

Inactivation of hbs *or* lrpC *also show a dual activity during natural CT*

The presence of the *rok* gene is limited only to some *Bacillus* species (Albano *et al*., 2005; (Smits and Grossman, 2010). Thus, it is likely that the Rok-dependent genome organizing function is redundant and it can be compensated by another NAP(s) in *Bacillus* species lacking Rok. To test whether another NAP might provide a given architecture that facilitates RecA dynamic filament formation and/or RecA-mediated DNA strand invasion and strand exchange in the *rec*$^+$ context but limit it in certain *rec*$^-$ transformation-deficient strains, we have searched for NAPs that show bending, wrapping, bridging, higher levels of compaction and bind DNA in a sequence-independent manner.

In bacteria of the Firmicutes Phylum, the number of NAPs and chromosome organizers is small when compared to bacteria of the Proteobacteria Phylum. For example, *Escherichia coli* possesses more than 12 different NAPs [Uαβ (or its variants HUα and HUβ), IFH, Fis, H-NS, StpA/Hha/YdgT/YmoA, Lpr, CRP, CbpA, CbpB, IciA and Dps], whose role could be more regulatory than architectural, three different macrodomains organizers (*i.e*. SeqA, SlmA and MatP) and a higher-order chromosome organizer (MukBEF) (Azam and Ishihama, 1999; Dillon and Dorman, 2010; Shen and Landick, 2019; Verma *et al*., 2019; Dame *et al*., 2020). In *B. subtilis*, Hbsu [(the gene is termed *hbs*) an orthologue of *E. coli* HU], LrpC (orthologue of *E. coli* Lrp), Rok, Dps, and perhaps AbrB contribute to chromatin architecture, and the SMC-ScpAB complex (counterpart of *E. coli* MukBEF) to chromosome organization (Klein and Marahiel, 2002; Beloin *et al*., 2003; Maamar and Dubnau, 2005; López-Torrejon *et al*., 2006; Thaw *et al*., 2006; Smits and Grossman, 2010; Duan *et al*., 2018; Shen and Landick, 2019; Dame *et al*., 2020). From those, three candidates (AbrA, Dps, and Smc-ScpAB) were not further analysed (see Supporting Information Annex II).

From the remaining two candidates, it can be said that Hbsu is an essential very-abundant homodimeric protein that binds, bends and kinks DNA in a sequence-independent manner, compacting the genome at low protein concentrations and re-extending it at higher concentrations (Tanaka *et al*., 1984; Kohler and Marahiel, 1997; Klein and Marahiel, 2002; Song *et al*., 2016). The Hbsu concentration significantly increases at the early stationary phase as does its *E. coli* heterodimer HU counterpart (Claret and Rouviere-Yaniv, 1997; Klein and Marahiel, 2002). In exponentially growing Firmicutes cells, a twofold reduction in the amount of Hbsu causes a∼20% increase in DNA relaxation, which is at the limit of cell survival (Ferrandiz *et al*., 2018). A leaky *hbs*55 mutation, on residue R55 in the β-arm of Hbsu (R55A), crucial for DNA binding, renders cells impaired in repair-by-recombination when compared to the parental strain (Fernndez *et al*., 1997).

The other candidate, LrpC, is a medium-abundance homo-octameric protein whose concentration increases ∼threefold at the early stationary phase when compared to exponentially growing cells (García-Tirado, 2011). Cytological studies revealed that the pattern of localization of GFP-LrpC in exponentially growing cells is similar to that of SMC-GFP (Britton *et al*., 1998); García-Tirado, 2011; Schibany *et al*., 2020). In the absence of LrpC, the DAPI-stained nucleoid increases in volume (García-Tirado, 2011). LrpC binds, bends and compacts DNA, constrains positive supercoils, and also wraps DNA around it in a right-handed superhelix, to yield a nucleosome-like structure in a sequence-independent manner (Beloin *et al*., 1997, 2003; Tapias *et al*., 2000; López-Torrejon *et al*., 2006; Thaw *et al*., 2006). The *lrpC* gene forms an operon with *topB*, whose product belongs to the subfamily of type I DNA topoisomerases (Beloin *et al*., 1997). Like high eukaryotes, certain bacteria of the *Bacilli* Class encode for two TopB-like enzymes (Topo IIIα and IIIβ). Topo IIIα is efficient in decatenating interlinked DNA dimers, as its $TopB_{Eco}$ counterpart, but Topo IIIβ is not a decatenase Li *et al*., 2006). *B. subtilis* only encodes for Topo IIIβ (García-Tirado, 2011), whose role is poorly understood.

To analyse whether these candidates contribute to CT, the leaky *hbs*55, the Δ*lrpC* or the Δ*topB* mutation was moved into the *wt* background by SPP1-mediated viral transduction (Table 1). Previously, it has been shown that interspecies CT decreases log-linearly, and at ∼8% sequence divergence CT is at the limit of detection in rec^+ cells, but it is detected up to 15% divergence in the Δ*rok* context (Serrano *et al*., 2021).

The donor *rpoB*482 DNA for intraspecies CT was *Bsu* 168 *rpoB*482. For interspecies CT, the 2997-bp *B. atrophaeus* 1942 *rpoB*482 DNA (*Bat* 1942 *rpoB*482) of the *B. subtilis* clade with 91.65% identity at the nucleotide level and 250 mismatches nearly homogeneously distributed was selected (Carrasco *et al*., 2016). The *non-self Bat* 1942 *rpoB*482 has a dA + dT content of 54.8%, similar to the dA + dT content of the *B. subtilis rpoB* gene (54.7%). The *Bat* 1942 *rpoB*482 lacks the high avidity Rok site (TACTA), and the occurrence of the non-cognate AAAAA site is ∼7-times higher than the high avidity Rok sites (Duan *et al*., 2018). For the plasmid transformation control, we have used the 4.548-bp pUB110 DNA, which has a significantly higher dA + dT content (64.3%) when compared to the average host chromosome (dA + dT 56.5%). Plasmid pUB110 DNA has a low number of copies of the Rok high avidity site, and the occurrence of the AAAAA site is ∼8-times higher than that of the Rok high avidity site (Duan *et al*., 2018). The plasmid DNA, which shares no significant degree of identity with the host genome, was artificially oligomerized (linearized and self-ligated under high DNA concentration conditions) to optimize the transformation frequency (see Canosi *et al*., 1978).

A newly acquired gene might function inefficiently within the new background, so the transfer event has potential to disrupt the physiology of the host. To test whether *hbs* or *lprC* inactivation differently affect intra- and interspecies CT, competent *wt*, *hbs*55 or Δ*lrpC* cells were incubated with the different DNAs, and then plated on LB agar plates containing Rif (8 $\mu g \cdot ml^{-1}$) or Nm (5 $\mu g \cdot ml^{-1}$) for the selection of chromosomal and plasmid transformants, respectively (Alonso *et al*., 1988; Carrasco *et al*., 2016). In competent *wt* cells, intra- and interspecies CT efficiency was of $1.5 \times 10^{-5} \pm 1 \times 10^{-6}$ and $4.8 \times 10^{-7} \pm 7 \times 10^{-8}$, respectively, showing that ∼8% sequence divergence decreased the CT efficiency by ∼30-fold ($P < 0.01$) (Fig. 3 and Table S3). The *hbs*55 mutation or the inactivation of *lrpC* increased both intra- (by 19- and 15-fold, $P < 0.01$) and interspecies CT (by ∼twofold and ∼sixfold, $P < 0.05$), respectively, when compared with the *wt* strain (Fig. 3 and Table S3). The CT efficiency decreased by ∼500-fold and ∼145-fold at ∼8% sequence divergence in the *hbs*55 and Δ*lrpC* context, respectively ($P < 0.01$) (Fig. 3 and Table S3), suggesting that a physical barrier decreases interspecies CT. It is worth mentioning that the *Bsu* 168 *rpoB*482 or *Bat* 1942 *rpoB*482 gene is transcribed from the native promoter of the recipient strain, and neither Rok nor Hbsu affects the expression of the *rpoB* promoter (Smits and Grossman, 2010; Seid *et al*., 2017), thus no silencing of the *rpoB* gene is expected. As described in the Supporting Information Table S1 for plasmid pHP14 transformation in the Δ*rok* context, pUB110 plasmid transformation was increased twofold to threefold ($p > 0.05$) in the Δ*rok*, *hbs*55 or Δ*lrpC* context when

compared with the *wt* strain (Fig. 3 and Table S3). This suggests that plasmid transformation frequency is not affected by a significant increase in dA + dT content.

Finally, since *lrpC* maps in an operon with *topB*, to discard that *topB* inactivation affects CT and is responsible for the observed effect on CT upon *lrpC* inactivation, competent Δ*topB* cells were analysed. Unlike Δ*lrpC* cells, *topB* inactivation significantly decreased the CT efficiency ($P < 0.01$) (Fig. 3 and Table S3), allowing us to rule out any polar effect in the Δ*lrpC* strain. It will be of significant interest to understand which NAP counters the negative effect of *topB* inactivation in the efficiency of CT (to be published elsewhere).

Inactivation of NAPs synergistically increases intraspecies and to a lesser extent interspecies CT

The expression of genes encoding NAPs is controlled by interdependent feedback loops (Rimsky and Travers, 2011). To learn how Hbsu or LrpC increases intraspecies CT and if they contribute to CT by increasing the activity of another NAP(s), the Δ*rok* mutation was moved onto the *hbs*55 or Δ*lrpC* backgrounds and the Δ*lrpC* mutation onto the *hbs*55 host by SPP1-mediated generalized transduction (table 1).

The absence of Rok synergistically increased the intra- (by ∼120- and ∼100-fold, $p < 0.01$) and interspecies (by ∼27- and ∼ 20-fold, $P < 0.01$) CT, respectively, in the *hbs*55 and Δ*lrpC* context, when compared to the *wt* control (Fig. 3 and Table S3). In the *hbs*55 Δ*lrpC* strain, intra- and interspecies CT increased by ∼140- and ∼ 20-fold ($P < 0.01$), respectively, when compared to the *wt* control (Fig. 3 and Table S3). These data altogether suggest that: (i) the absence of Hbsu or LrpC increases intraspecies and in minor extent interspecies CT in the Δ*rok* context; (ii) the *rok*, *hbs* and *lrpC* genes are not epistatic in response to intra- and interspecies CT (Supporting Information Table S3); (iii) the plasmid transformation for the single *rok*, *hbs* or *lrpC* or double mutant strains, however, was only slightly increased when compared to the *wt* control (Supporting Information Tables S1 and S3), suggesting that *rok* inactivation may be compensated by changes in a second NAP; and (iv) Rok, Hbsu and LrpC provide redundant activities that are structural rather than regulatory. Here, we have focused in understanding how inactivation of a NAP affects the transformation frequency plating the transformants on rich medium. It will be of significant interest to determine the fitness cost through comparisons of growth dynamics and head-to-head competition in those strains.

To test whether the different NAPs are tightly interdependent of HR and analyse the effect of sequence divergence in *rec*-deficient cells, we have constructed the *hbs*55 Δ*recX* and Δ*lrpC* Δ*recX* strains by SPP1-mediated viral transduction (Table 1). The *recX* background was selected because the role of RecX on competent cells has been previously characterized (Crdenas *et al*., 2012). Inactivation of *recX* decreased both intra- (by ∼200-fold, $P < 0.01$) and interspecies CT (by ∼300-fold, $P < 0.01$), and plasmid transformation efficiency (by ∼50-fold, $P < 0.01$), when compared with the *wt* strain (Fig. 3 and Table S3) (Crdenas *et al*., 2012; Serrano *et al*., 2021). Intraspecies CT in the double Δ*recX* Δ*rok*, Δ*recX hbs*55 or Δ*recX* Δ*lrpC* mutant strain was significantly reduced when compared to the CT efficiency of their respective Δ*rok*, *hbs*55 or Δ*lrpC* parental strain ($P < 0.01$). However, it was still 9- to 11-fold ($P < 0.01$) above the CT efficiency of the Δ*recX* parental strain (Fig. 3 and Table S3). The efficiency of interspecies CT at ∼8% sequence divergence was strongly reduced in the Δ*recX* context ($P < 0.01$) but increased only twofold to threefold ($p > 0.05$) in the double Δ*recX* Δ*rok*, Δ*recX hbs*55 or Δ*recX* Δ*lrpC* mutant strain when compared to the Δ*recX* parental strain (Fig. 3 and Table S3). Nevertheless, the efficiency of interspecies CT at ∼8% sequence divergence in the Δ*recX* context might be over-estimated, because the number of transformants is similar to the spontaneous mutation rate. Here, only 40%–50% of the Rif^R transformants were genuine transformants as detected by nucleotide sequence analyses (Serrano *et al*., 2021). In contrast, the plasmid transformation efficiency was similar to that of the Δ*recX* mutant strain ($P > 0.1$) (Supporting Information Table S3).

The absence of NAPs does not affect the MIC of Novobiocin

The long-range and higher-order architectural organization of the bacterial nucleoid is regulated in a supercoiling-independent manner by poorly characterized factors, whereas the short-range structural organization, that might be important in restraining DNA in a negative-supercoiled state, is influenced by the NAP proteins (Dillon and Dorman, 2010;Shen and Landick, 2019; Verma *et al*., 2019; Dame *et al*., 2020). In this context, novobiocin (Nov) is an useful tool for understanding the maintenance of an appropriate DNA topology that is essential for different DNA transactions (such as transcription and DNA replication, recombination and chromosomal segregation) (Gellert, 1976b; Cozzarelli, 1977; Cozzarelli, 1980). Nov binds with high affinity to the GyrB subunit of DNA gyrase and with significantly lower affinity to the ParE subunit of Topo IV and blocks ATP hydrolysis by these enzymes (Gellert, 1976a; Peng and Marians, 1993). In *Streptococcus pneumoniae*, which lacks other NAPs (LrpC, Rok, Fis, IHF, or H-NS), treatment with Nov at the minimal inhibitory concentration (1× MIC) induces an initial relaxation and triggers a homeostatic response to restore the

native level of DNA supercoiling. However, Nov treated cells at 40× MIC suffer a 25% increase in DNA relaxation, with no restoration of the supercoiling level after 30 min, and inhibition of cell proliferation (Ferrandiz, 2010). A two-fold reduction in the amount of Hlp (counterpart of Hbsu), which is at the limit of cell survival, triggered a 21% increase in DNA relaxation, but if those cells are treated also with 1× MIC of Nov, DNA relaxation increases a 35%, and cells can only grow if Hlp levels are restored (Ferrandiz *et al*., 2018).

To test whether the absence of NAPs impacts in the DNA topology and indirectly in cell viability, we measured the MIC to Nov as described in Materials and methods. We found that Nov treatment did not significantly affect the MIC in the absence of the indicated NAP when compared to the *wt* strain or the most affected single mutant strain (Supporting Information Table S4). Thus, it seems that the negative-supercoiled state might be still regulated in the absence of Rok, Hbsu or LrpC (Supporting Information Table S4). Alternatively, an uncharacterized NAP might contribute to the architectural organization of the bacterial nucleoid and the maintenance of DNA topology in the Δ*roK*, *hbs*55 or Δ*lrpC* context.

Conclusions

The present work leads to five main conclusions. First, *rok* inactivation increases up to threefold the frequency of plasmid transformation when compared to the *rok*$^+$ background (Fig. 1). Similar results were observed in the *hbs*55 or Δ*lrpC* context (Fig. 3), suggesting that Rok directly limits (Maamar and Dubnau, 2005), and Hbsu or LprC might indirectly decrease the size of the subpopulation that develops competence. Second, *rok* inactivation significantly increases (>10-fold) the frequency of intraspecies CT in the *rec*$^+$ control, as well as in the *recO*, *recF*, *addAB*, *recU* and *ruvAB* context (Serrano *et al*., 2021). Similarly, the *hbs*55 or Δ*lrpC* mutation significantly increases the frequency of intraspecies CT in the *rec*$^+$ control (Fig. 3). Since the absence of RecO, RecF, AddAB, RecU or RuvAB do not affect the frequency of CT in otherwise *rec*$^+$ cells, we assumed that Rok, Hbsu or LprC, by maintaining the proper nucleoid volume, limits CT by affecting RecA-mediated capture of a homologous segment and/or DNA strand invasion (Fig. 4A and B). Third, inactivation of *rok* reduces intraspecies CT in the *recD*2 context and further inhibits CT in the Δ*recX*, Δ*radA* and Δ*dprA* context when compared to their respective *rok*$^+$ control (Figs 2 and 4A and C). Similar results were observed in the *hbs*55 Δ*recX* and Δ*lrpC* Δ*recX* strains (Fig. 3). Since DprA and RecX act before and during RecA-mediated homology search, respectively, and the RecD2 or RadA/Sms accessory proteins during RecA-mediated DNA strand exchange, we assumed that the Rok, Hbsu or LrpC NAPs are necessary for RecA-mediated DNA strand exchange (Fig. 4C). Fourth, inactivation of *rok* in the Δ*lrpC* or *hbs*55 context synergistically increases intraspecies CT, but the effect was less manifest when the interspecies CT efficiency was analysed, suggesting that Rok, Hbsu or LrpC, by modulating the DNA topology of the centrally located non-replicating nucleoid through their different binding modes (bending, wrapping, bridging, condensing, cooperative filamenting, etc.) compacts it and differentially enhances CT in otherwise *rec*$^+$ cells. Finally, inactivation of *rok*, *lrpC* or the *hbs*55 mutation in the Δ*recX* context reduces interspecies CT when compared with the *rec*$^+$ control (Fig. 3).

Rok is associated with a large subset of chromosomal domain boundaries, suggesting that it contributes to the genome organization of *B. subtilis* (Marbouty *et al*., 2015). We propose that Rok exerts a dual and competitive contribution in CT as in other DNA transactions (*e.g*., bacterial transcription) in a fail-safe manner (Shen and Landick, 2019; Verma *et al*., 2019; Dame *et al*., 2020). It is poorly understood how NAPs work *in vivo* and how they link nucleoid architecture with CT efficiency in non-replicating competent cells. Based on extensive *in vitro* studies, it appears that the NAPs affect chromosome architecture, thus aiding in DNA condensation and in the structural organization of the nucleoid (Marbouty *et al*., 2015; Shen and Landick, 2019; Verma *et al*., 2019; Dame *et al*., 2020). The most likely assumptions are: first, Rok, and perhaps Hbsu and LrpC, which preferentially binds with high-affinity to structurally distorted DNA, constrains supercoils and plays a dual role by transiently condensing the volume of the nucleoid through their different interactions with the chromosomal DNA. Then, the dsDNA regions are only transiently exposed to RecA, and homology finding through many random, weak, and transient interactions should be less efficient, but more accurate, in otherwise *rec*$^+$ cells (Fig. 4A). Here, with the help of its accessory proteins, RecA correctly interacts with the short homologous regions and performs homology search and three-strand exchange with the transiently untwisted recipient duplex. If the RecA accessory proteins are missing or sequence divergence is present, RecA interaction with short homologous regions should render random and persistent unproductive interactions with the dsDNA, and as a result the three-strand exchange is less efficient (Fig. 4C). For instance, in the absence of RecX, the RecA threads persist, and such long-living threads impairs CT (Crdenas *et al*., 2012). Second, when cells are proficient in CT, the search for the homologous locus should be enhanced if the genomic dsDNA is less convoluted, as it occurs in the Δ*rok*,

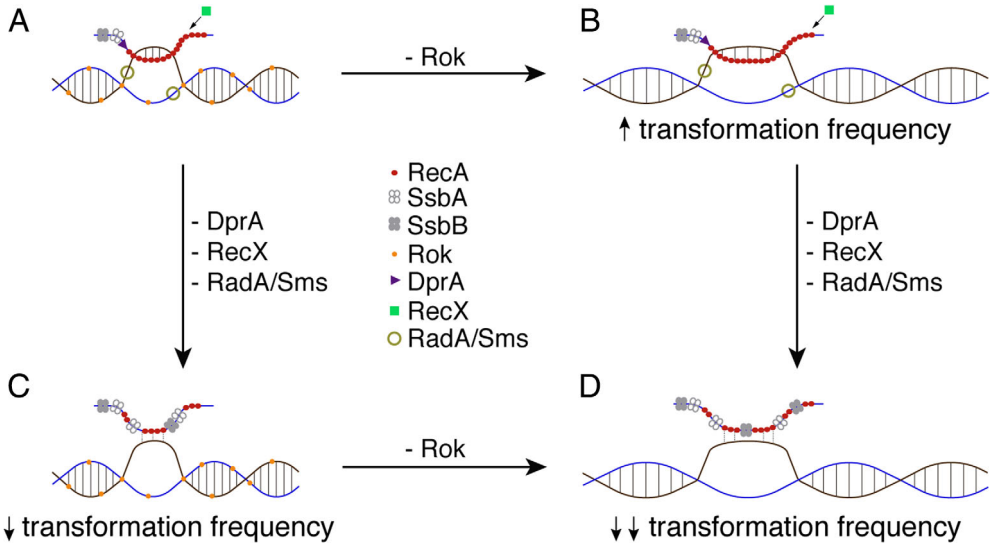

Fig 4. Model for the action of NAPs and recombination functions in natural chromosomal transformation.
A. Rok (or Hbsu, LrpC) transiently condenses the nucleoid through its different interactions with the chromosomal dsDNA. A RecA-ssDNA filament, through transient interactions with the recipient dsDNA, undergoes homology search through many random and weak interactions that become more efficient in the presence of the RecA accessory functions (DprA, RecX, RadA/Sms).
B. In the absence of Rok (or Hbsu, LprC), the genomic dsDNA should be less convoluted and homology search should be enhanced, allowing a more efficient RecA-mediated homology search without compromising the reaction fidelity.
C. In the absence of the accessory functions (DprA, RecX, RadA/Sms) RecA interaction with short homologous regions should render random and persistent unproductive interactions (dotted lines) with the dsDNA resulting in a less efficient DNA strand invasion and three-strand exchange.
D. In the absence of Rok (or Hbsu, LprC), the nucleoid should be expanded, and if the accessory functions are not operative, the efficiency of CT is further reduced since the unproductive interactions are facilitated. [Color figure can be viewed at wileyonlinelibrary.com]

*hbs*55 or Δ*lrpC* context, because unconstrained DNA should allow a more efficient RecA-mediated homology search on distal segments and strand exchange without affecting the fidelity of the reaction (Fig. 4B). However, on an expanded nucleoid and in the absence of RecA accessory proteins, the efficiency of CT is further reduced (Fig. 4D), since the unproductive interactions are facilitated and persist for longer. Then, in the Δ*recX* Δ*rok*, Δ*recX hbs*55 or Δ*recX* Δ*lrpC* cells, the global chromosome architecture and RecA-mediated DNA strand exchange are compromised, and it is unlikely that gene silencing can affect the CT efficiency by discriminating self-DNA.

It seems that different mechanisms modulate the trade-off between benefit and cost of acquired genes. In bacteria of the Proteobacteria or Actinobacteria Phyla specialized NAPs, being H-NS the prototype, silence the expression of the horizontally acquired xenogeneic genes. In contrast, in *B. subtilis* there are two non-mutually exclusive avenues that limit intra- and interspecies CT: the mechanism of homology-mediated RecA-dependent DNA integration itself, and the organization of the bacterial chromosome that is determined by NAPs and other proteins. It will be of significant interest to unravel the molecular basis of these interplay and how Rok, Hbsu and LrpC contribute to the speciation of the species.

Experimental procedures

Bacterial strains

The *B. subtilis* BG214 strain, which lacks AT-rich sequences acquired through HGT (as the conjugative transposon *ICEBs*1, the prophage SPβ, or plasmids), restriction-modification and CRISPR-Cas systems, and its isogenic derivatives are listed in Table 1. The isogenic derivatives with *hbs*47 (with a F47W mutation) and *hbs*4755 mutations were used, but since the phenotype of the *hbs*47 mutation is indistinguishable from the *wt* strain, for simplicity we made reference to the relevant *hbs*55 mutation (Table 1).

Donor DNAs

As donor DNA for intra- and interspecies CT assays, the coding region of the essential house-keeping *rpoB* gene, which encodes for the β subunit of RNA polymerase, was selected for different reasons. First, the *rpoB* gene lacks strand-biased architectural elements (Hendrickson *et al*., 2018). Second, the Rok repressor does not regulate *rpoB* expression (Seid *et al*., 2017). Third, a single C to T transition mutation at codon 482 confers resistance to rifampicin (Rif^R) (*rpoB*482 gene) (Carrasco *et al*., 2016). Fourth, Rif^R, which is an easily selectable marker, is centrally located (at position 1443) on the coding region of

the 2997-base pairs (bp) *rpoB*482 DNA (Carrasco *et al*., 2016). Fifth, the *rpoB*482 DNA for intraspecies, which is obtained from *B. subtilis* 168 (*Bsu* 168 *rpoB*482), and for interspecies CT, which is obtained from *B. atrophaeus* 1942 (*Bat* 1942 *rpoB*482), has a dA + dT content slightly lower (54.7% and 54.8, respectively) than the average of the species (56.5%). Sixth, the *Bsu* 168 *rpoB*482 has a single mismatch (0.03% sequence divergence), whereas *Bat* 1942 *rpoB*482 DNA has 250 mismatches homogeneously distributed and a sequence divergence of 8.35% when compared to the native *rpoB* gene (Carrasco *et al*., 2016). Finally, *B. subtilis* cells show no codon usage preferences, thus no impairment in translation is expected, and *B. atrophaeus* β subunit of RNA polymerase shares 99% amino acid sequence identity with its *B. subtilis* counterpart (Carrasco *et al*., 2016, 2019; Serrano *et al*., 2021).

For plasmid transformation, two plasmids with different replicons, selectable markers and dA + dT content were used. First, pHP14 DNA, that is a 4917-bp shuttle *E. coli/B. subtilis* plasmid DNA, which has a 60.2% dA + dT content and confers resistance to erythromycin (Ery^R). Second, pUB110 DNA, which has a 64.3% dA + dT content and confers resistance to neomycin (Nm^R). Only oligomeric plasmid DNA transforms *B. subtilis* competent cells, since monomeric plasmid DNA, which account to >90% of purified DNA, is inactive for transformation (Canosi *et al*., 1978). Thus, the isolated plasmid DNA was linearized and self-ligated under high DNA concentration conditions to artificially oligomerize it. About half of the molecules are ligated in a head-to-tail configuration (active form), whereas the other half of the ligated plasmid DNA is in the head-to-head, which is inactive in transformation. DNA was prepared by Qiagen Miniprep extraction. The '*self*' (*Bsu* 168 *rpoB*482 DNA), '*non-self*' (*Bat* 1942 *rpoB*482 DNA]) and plasmid pHP14 or pUB110 DNAs lack the high avidity Rok site (TACTA), and the occurrence of the AAAAA site is ∼7-, ∼7- and ∼ 4-times, respectively, more frequent than the Rok high avidity site (Duan *et al*., 2018).

Transformation assays

Natural competent *B. subtilis* cells were developed as previously described (Alonso *et al*., 1988). Competent cells (5–10 $\times 10^8$ *cfus*·ml^{-1}) were incubated with 0.1 μg·ml^{-1} of the 2.9-kb homologous *Bsu* 168 *rpoB*482, homeologous [a sequence that is similar (∼8% divergence) but not identical] *Bat* 1942 *rpoB*482 DNA or heterologous and oligomeric pHP14 or pUB110 DNA (60 min, 37°C). Then, the reaction mixture was plated on Rif (8 μg·ml^{-1}, CT), Ery (2 μg·ml^{-1}) or Nm (5 μg·ml^{-1}) containing plates, respectively, and incubated (overnight, 37°C) (Alonso *et al*., 1988, 1991).

The size of the subpopulation of BG214 (*rok*$^+$) and its isogenic derivatives that transiently develops natural competence might vary from 0.1% to 5% of total cells, and of BG1359 (Δ*rok*) and its isogenic derivatives up to ∼20% of total cells (Alonso *et al*., 1988; Carrasco *et al*., 2016). As a normalization factor for DNA uptake between strains, the proportion of competent cells was estimated measuring the uptake of radiolabelled donor DNA (determined as the DNase I-resistant radiolabelled linear dsDNA into cells grown to competence) and the total number of cells was estimated as described (Alonso *et al*., 1988; Alonso *et al*., 1991). These normalizations, however, show a certain degree of noise, thus we considered an increase/decrease in the number of transformants by <3-fold as poorly significant and t-tests were applied to analyse the statistical significance of the data.

Assay for novobiocin minimal inhibitory concentration

The minimal inhibitory concentration (MIC) of novobiocin (Nov) for the different *B. subtilis* mutants was estimated by exposing 1– 3 $\times 10^6$ *cfus*·ml^{-1} to increasing Nov concentrations for 16 h at 37° C in LB medium with shaking (240 rpm). Then, samples were collected, OD_{560} was measured, and the MIC was estimated, as described (Tabone *et al*., 2014).

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Author contributions

E.S. and J.C.A. conceived the project and designed the experiments, E.S. and R.T. performed the experiments, J.C.A. wrote the manuscript, and E.S., R.T. and J.C.A. edited the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1: Supplementary Information