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The cell pole: The site of cross talk between the DNA uptake and genetic recombination machinery

Dawit Kidane^{1,†}, Silvia Ayora^{2,†}, Joann Sweasy¹, Peter L. Graumann³, and Juan C. Alonso^{2,*}

¹Departments of Therapeutic Radiology and Genetics and The Yale Comprehensive Cancer Center, 333 Cedar Street, New Haven, CT 06520, USA

²Centro Nacional de Biotecnología, CSIC, 3 Darwin, 28049 Madrid, Spain

³Universität Freiburg, Schänzle Straße 1, 79104 Freiburg, Germany

Abstract

Natural transformation is a programmed mechanism characterized by binding of free double-stranded (ds) DNA from the environment to the cell pole in rod-shaped bacteria. In *Bacillus subtilis* some competence proteins, which process the dsDNA and translocate single-stranded (ss) DNA into the cytosol, recruit a set of recombination proteins mainly to one of the cell poles. A subset of single-stranded binding proteins, working as “guardians”, protect ssDNA from degradation and limit the RecA recombinase loading. Then, the “mediators” overcome the inhibitory role of guardians, and recruit RecA onto ssDNA. A RecA-ssDNA filament searches for homology on the chromosome and, in a process that is controlled by “modulators”, catalyzes strand invasion with the generation of a displacement loop (D-loop). A D-loop resolvase or “resolver” cleaves this intermediate, limited DNA replication restores missing information and a DNA ligase seals the DNA ends. However, if any step fails, the “rescuers” will repair the broken end to rescue chromosomal transformation. If the ssDNA does not share homology with resident DNA, but it contains information for autonomous replication, guardian and mediator proteins catalyze plasmid establishment after inhibition of RecA. DNA replication and ligation reconstitute the molecule (plasmid transformation). In this review, the interacting network that leads to a cross talk between proteins of the uptake and genetic recombination machinery will be placed into perspective.

Keywords

horizontal gene transfer; natural competence; homologous recombination; chromosomal transformation; plasmid transformation; viral transfection

Introduction

Natural transformation is a mode of horizontal gene transfer (HGT) in bacteria that contributes to the maintenance and evolution of bacterial genomes. Since the discovery of

Address for correspondence: Juan C. Alonso, Centro Nacional de Biotecnología (CSIC), Departamento de Biotecnología Microbiana, Darwin Str. 3, Cantoblanco, E-28049 Madrid, Spain. Tel (+34) 91585 4546. jcalonso@cnb.csic.es.

[†]These authors contributed equally to this work

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natural transformation of *Streptococcus pneumoniae* competent cells, by Griffith in 1928 (Griffith, 1928), and the identification of DNA as the transforming principle by Avery, MacLeod and McCarty in 1944 (Avery et al., 1944), more than 90 bacterial species have been shown to be naturally competent (Lorenz and Wackernagel, 1994, Claverys et al., 2000, Dubnau, 1999, Johnsborg and Håvarstein, 2009, Sørensen et al., 2005, Stewart and Carlson, 1986). Competence is a programmed physiological state in which the bacterium may take up DNA from the external environment and incorporates it into its genome. This DNA uptake occurs in three stages: binding to the double-stranded (ds) DNA in the external solution, degrading one strand to render the DNA single-stranded (ss), and transport it into the cell (Chen and Dubnau, 2004, Claverys et al., 2000, Claverys et al., 2009, Dubnau, 1999, Smith et al., 1995, Stewart and Carlson, 1986). If the internalized ssDNA is recombined into the genome, the bacterium is considered naturally transformed by genetic recombination (GR). Transformation entails recombination of genes between similar or different lineages, representing a form of bacterial sex that increases standing genetic variation, and the recombination that occurs as part of this process is called chromosomal transformation. If the incoming ssDNA shares no homology with recipient, but has a self-replication potential (an episome), it can be reconstituted into its dsDNA form using recombination and replication function. The recombination that occurs as part of this process is called plasmid transformation or viral transfection (Viret et al., 1991, Chen and Dubnau, 2004, Claverys et al., 2000, Claverys et al., 2009, Dubnau, 1999, Smith et al., 1995, Stewart and Carlson, 1986).

In several bacterial species, such as *Neisseria gonorrhoeae*, natural competence is constitutively expressed, while in others it is stochastically induced and time-limited (Claverys et al., 2000, Dubnau, 1999). The mechanism that controls this induction of natural competence varies among bacteria and is not regulated in direct response to the occurrence of free dsDNA in the microenvironment. Competence may be induced in response to specific environmental conditions, such as in *Bacillus subtilis*, that is caused by intrinsic noise in competence gene expression (bistability) (Solomon and Grossman, 1996, Macfadyen, 2000, Maamar and Dubnau, 2005), by the presence of antibiotics or DNA damage, as occurs in *S. pneumoniae*, *Helicobacter pylori* or *Legionella pneumophila*, or by the presence of biofilms on chitin surfaces, like in *Vibrio cholera* (Claverys et al., 2000, Claverys et al., 2006, Dubnau, 1999, Meibom et al., 2005, Dorer et al., 2010, Charpentier et al., 2011, Lo Scrudato and Blokesch, 2012). This versatility suggests that specific, not yet characterized, requirements for the occurrence of competence could exist in many other species, so that many more bacteria may be able to undergo HGT via this pathway.

At the stage of competence, the metabolic activity of *B. subtilis* competent cells is reduced (Nester and Stocker, 1963), DNA replication is halted and cell division is inhibited (Haijema et al., 2001, Johnsen et al., 2009, Briley et al., 2011), but the SOS system, which is induced when replication is inhibited (see Sassanfar and Roberts, 1990), is weakly induced (see below). Natural competence is found in a broad spectrum of bacterial Phyla, and it is clinically relevant because several human pathogens are naturally competent and can acquire drug resistances by this mechanism of GR, making it a serious health problem. The most thoroughly studied species are *B. subtilis* and *S. pneumoniae* of the Firmicutes Phylum and *Haemophilus influenza*, *Neisseria gonorrhoeae* and *H. pylori* of the Proteobacteria Phylum (Claverys et al., 2000, Dubnau, 1999, Smith et al., 1995, Stewart and Carlson, 1986).

Most naturally competent bacteria, as *B. subtilis*, *S. pneumoniae*, *H. pylori*, *Acinetobacter baylyi* and *Pseudomonas stutzeri* can take up DNA of any source (Claverys et al., 2000, Dubnau, 1999, Meier et al., 2002), although these cells usually acquire DNA from their microenvironment, where closely related organisms grow. Other bacteria, such as *H. influenzae* and *N. gonorrhoeae* only take up DNA from their own clade (Mathis and Scocca,

1982, Sisco and Smith, 1979, Lorenz and Wackernagel, 1994). This selective uptake, which depends on the presence of a sequence-specific DNA binding protein, allows efficient transport into the periplasm of a DNA that contains an uptake signal sequence (USS, also known as DNA uptake sequences, DUS), that exerts a negative control for interspecies recombination (Chen and Dubnau, 2004). The USS (9- to 11-bp in length in *H. influenzae*) or the DUS motifs (10- to 12-bp in Neisserial species) are highly over-represented sequences (one USS or DUS every 3.0-kb or 2.2-kb, respectively) when compared with the probability of presenting a USS motif in a random sequence (once every ~ 260- to 4,000-kb per genome) or a DUS motif (once every ~ 1,000- to 16,000-kb), respectively (Danner et al., 1980, Goodman and Scoocca, 1988, Redfield et al., 2006, Treangen et al., 2008). However, the level of transformation due to the presence of a DUS motif, and the ability of excess DUS to inhibit transformation in Neisserial species are strain dependent (Duffin and Seifert, 2010), suggesting that this difference is not due solely to differential DNA uptake.

In all natural competent bacteria, DNA transformation is also controlled at the level of formation of a stable displacement loop (D-loop). In *B. subtilis*, a short segment of ~ 30-nt in length of identity is the minimum length required for RecA-mediated DNA strand invasion and D-loop formation (Majewski and Cohan, 1999), but RecA accessory factors might control the stability of the D-loop.

Some species of Cyanobacteria, Chlorobi, Actinobacteria and of the *Deinococcus-Thermus* Phylum have been shown to be also naturally transformable. With exception of the *Deinococcus-Thermus* Phylum, the few studies published are all focused on the description of transformation phenomenon rather than the description of mechanisms of DNA processing, hence natural transformation in these species will not be further discussed. In this review we will summarize the fate of the imported ssDNA and the mechanisms of chromosomal integration or plasmid establishment that lead to a new phenotype (Dubnau, 1999, Lorenz and Wackernagel, 1994).

Different barriers control HGT, and among these the restriction-modification (RM) systems prevent artificial transformation (Ca^{2+} -treated or electroporated cells) and viral transduction, and reduce plasmid conjugation (Arber, 2000). The RM systems are abundant in bacteria and are traditionally associated with a cellular protection mechanism against incoming foreign dsDNA, mainly of bacteriophage origin. The restriction enzyme is not harmful to the host cell, because its dsDNA is protected from cleavage by methylation, but incoming dsDNA that carry a cognate site for the enzyme will be cleaved (Arber, 2000). The incoming linear ssDNA, which is ready to initiate RecA-mediated recombination, overcomes the host restriction barriers by its ssDNA nature. Therefore, the potential negative effect of restriction on interspecies transformation generally is not significant in naturally competent cells. Furthermore, in some bacterial species (e.g., *S. pneumoniae*) the methylation of the incoming ssDNA actively protects it from the endonuclease and allows transformation without any potential restriction (Lacks et al., 2000). In *H. pylori*, however, restriction enzymes limit the frequency of transformation with unmodified plasmid DNA, although the restriction barrier on chromosomal DNA is a matter under discussion (Ando et al., 2000, Aras et al., 2002, Humbert and Salama, 2008, Pinto et al., 2005, Humbert et al., 2011). *H. pylori* also takes up ssDNA into the cytosol (Stingl et al., 2010) and to explain the limitation by restriction it was hypothesized that the restriction endonucleases might act before dsDNA is converted to ssDNA (Humbert et al., 2011). Alternatively the partial sensitivity of the taken up ssDNA (Stingl et al., 2010) to restriction endonucleases could be explained if there is no sufficient SsbA_{Hpy} to inhibit the spontaneous annealing of the plasmid ssDNA or the disassembly of all potential DNA secondary structures, because both processes would lead to transient dsDNA regions sensitive to restriction endonucleases.

Are competent cells efficiently transformed with ssDNA? There are few reports supporting that ssDNA can transform *N. gonorrhoeae* cells (at similar levels) or *H. influenzae* (50-fold reduced) when compared to dsDNA (Stein, 1991). Recently it was shown that *P. stutzeri* competent cells take up ssDNA with a 20- to 60-fold lower transformation efficiency than dsDNA (Meier et al., 2002). In Firmicutes transformation with exogenously added ssDNA was not detected, and when detected the result has been challenged because purification of the ssDNA has not been rigorous enough to remove all dsDNA (reviewed in Dubnau, 1999, Stewart and Carlson, 1986).

Competence development coordinates the expression of proteins involved in DNA uptake and recombination

Programmed competence coordinates the expression of proteins involved in DNA uptake and translocation with the expression of some proteins of the recombination machinery (Kidane et al., 2009, Stohl and Seifert, 2001). In Firmicutes, natural competence is induced by a “quorum sensing” mechanism that up regulates “early” competence (*com*) genes (Dubnau, 1999, Morrison and Lee, 2000, López et al., 2009, Shank and Kolter, 2011). Competence induction in both *B. subtilis* and *S. pneumoniae* uses a two-component signal transduction system, ComP-ComA and ComD-ComE, respectively, to respond to the extracellular peptide(s) ComX and the competence and sporulation factor (CSF) in *B. subtilis* and to competence-stimulating peptide (CSP) (encoded by *comC*) in *S. pneumoniae* (Dubnau, 1999, Håvarstein and Morrison, 1999). (Note that in the remaining of this review, unless otherwise stated, the indicated genes and products are of *B. subtilis* origin). The processed, modified and secreted peptide ComX or the processed and secreted CSP of *S. pneumoniae* (CSP_{*Spn*}) stimulates a receptor histidine kinase (ComP, ComD_{*Spn*}) that activates a response regulator transcription factor (ComA, ComE_{*Spn*}), with the CSF peptide contributing to the activation of ComA (Dubnau, 1999, Morrison and Lee, 2000). In *B. subtilis*, expression of over 20 genes appears to be controlled directly by this signalling pathway (“early” genes), and expression of over 150 additional genes, including those involved in competence development, appears to be controlled indirectly (Comella and Grossman, 2005), whereas ComE_{*Spn*} activates transcription of a limited set of genes, including the *comCDE*_{*Spn*} operon (Martin et al., 2010). Post-translational modifications also control competence development; specifically through phosphorylation of arginine residues in ComK, ComGA, ComFA and ComFC (Elsholz et al., 2012). The underlying mechanisms for this phosphorylation, however, remained to be addressed.

In some bacterial species, competence-induced murein hydrolases (*e.g.*, LytA_{*Spn*}, LytC_{*Spn*}, LytF_{*Spn*}, CbpD_{*Spn*}) promote heterolysis of the non-competent siblings. In contrast, the immunity protein (*e.g.*, ComM_{*Spn*}) prevents autolysis of competent cells (Guiral et al., 2005, Håvarstein et al., 2006, Berg et al., 2012a). This fratricidal effect facilitates intra- and cross-species specific transformation by taken up the DNA of siblings or closely related streptococci sharing the same microenvironment (Johnsborg and Håvarstein, 2009, Claverys et al., 2009, Berg et al., 2012b).

One of the early *com* induced genes encodes the master regulator (ComK, ComX_{*Spn*}) that subsequently regulates the expression of the “late” genes. The ComK regulon drives expression of > 150 genes in wild type (wt) cells (Berka et al., 2002, Hamoen et al., 2002, Ogura et al., 2002). From those, ~23 ComK-dependent genes are necessary for building up the DNA uptake apparatus, and only ~5 genes are necessary for the recombination machinery (reviewed by van Sinderen et al., 1995, Chen and Dubnau, 2004, Claverys et al., 2006, Dubnau, 1999, Burton and Dubnau, 2010). Of those necessary for recombination, DprA (also termed Smf or CilB), which recruits RecA onto ssDNA, SsbB (YwpH), which protects the incoming ssDNA from the nuclease attack, and CoiA (YjbF), which has an

unknown activity, are specifically induced during competence development, whereas expression of SsbA, which protects the incoming ssDNA and limits the RecA recombinase loading onto ssDNA, and expression of RecA are transiently enhanced during competence (Berka et al., 2002, Hamoen et al., 2002, Ogura et al., 2002) (Table 1 and Figure 1).

From several studies, it has been demonstrated that during the competence stage, ComK could directly activate a subset of genes (*recA*, *dinB*, *tagC*, *yneAB*, *yhjB*) whose expression is also regulated within the SOS response. This could be attributed either to a limited induction of the SOS response during competence development (halted DNA replication) or to ComK-dependent activation of a small subset (18%, 6/33) of LexA and RecA regulated genes (SOS response). We favor the latter hypothesis because some of these genes might have a ComK cognate binding site, and induction is a LexA-independent event (Hamoen et al., 2002, Berka et al., 2002, Ogura et al., 2002). Only the absence of one of them, RecA, decreased the ability to be transformed by chromosomal DNA (Love et al., 1985, Hamoen et al., 2001, Hamoen et al., 2002, Berka et al., 2002, Ogura et al., 2002, Au et al., 2005). It is likely that programmed competence and the SOS response are not intimately connected. Furthermore, *S. pneumoniae*, *H. pylori* or *Neisseria* spp, lack an SOS response (Claverys et al., 2009).

During competence development the induction of essential genes such as those coding the type I DNA topoisomerase and for enzymes involved in cell shape as Mbl, MreB, MreC and MreD was observed in certain bacterial species, but not in others (Table 1) (Carballido-López, 2012). In certain bacteria, the *topA* gene is part of the *dprA* operon, and its induction might be by a guilty-by association event. Furthermore, increased expression of other *B. subtilis* homologous recombination (HR) genes (e.g., *addB* and *addA*) was only observed by *lacZ* fusion experiments (Haijema et al., 1995), and increased ComK-dependent expression of the *sbcD* and *sbcC* genes, which are part of the *addB addA sbcD sbcC* operon, was observed in one (Ogura et al., 2002) of the three independently performed microarray experiments (Berka et al., 2002, Hamoen et al., 2002, Ogura et al., 2002).

DNA uptake and recombination machineries assemble at the cell pole

In general, Proteobacteria and Firmicutes share a common set of competence proteins. The critical step in the uptake mechanism is the transport of DNA from the environment into the cytosolic compartment of the bacteria, which is an energy-demanding task. With few exceptions (e.g. *H. pylori* uses a conjugation like system for DNA transport) (Hofreuter et al., 2000, Hofreuter et al., 2001) the DNA uptake machinery is related to type II secretion systems or to type IV pili, to acquire naked dsDNA from the extracellular environment (Chen and Dubnau, 2004, Claverys et al., 2006, Dubnau, 1999, Stewart and Carlson, 1986). In *B. subtilis* and *H. pylori* at least 16 competence-specific (Com) proteins and few competence-independent proteins have been localized at the cell pole (Chen et al., 2005, Stingl et al., 2010, Kidane et al., 2009). In *B. subtilis* these proteins generally work at three different levels based on their function. The proteins of the first group participate in a sequential step of DNA binding and processing of environmental dsDNA. They include the ones that form the competence pseudopilus (ComG proteins), which facilitates the pulling of DNA towards the translocation machinery in the cytoplasmic membrane, and the NucA endonuclease that generates double strand breaks (DSBs), and fragmentation of one strand with its subsequent degradation into the extracellular environment by an unknown enzyme. The ComG proteins include the ComGA ATPase, ComGB polytopic membrane protein, ComGC major pre-pilin like protein, and ComGD, ComGE, and ComGG that are three minor pre-pilin proteins. The pre-pilin proteins integrate into the cytoplasmic membrane, and when processed by the peptidase ComC, these subunits translocate to the exterior of the membrane (Albano et al., 1989, Briley et al., 2011, Chen and Dubnau, 2004, Chen et al.,

2006, Chung and Dubnau, 1995, Chung et al., 1998, Craig and Li, 2008, Provvedi et al., 2001, Puyet et al., 1990). The proteins of the second group participate in the transport of ssDNA across the cell membrane into the cytosol of the bacterium. They include ComFA, ComEA and ComEC proteins, which form the membrane transport apparatus. (Meima et al., 2002, Inamine and Dubnau, 1995, Londono-Vallejo and Dubnau, 1993, Draskovic and Dubnau, 2005, Provvedi and Dubnau, 1999, Kaufenstein et al., 2011). The proteins of these two groups, which include only competence-induced proteins, form the DNA uptake machinery. The ComFB, ComFC and ComEB, of unknown function, also co-assembled with the polar uptake machinery (Kaufenstein et al., 2011).

ComGA (first group) and ComFA (second group) coordinate the interaction of the DNA uptake apparatus with proteins of the recombination machinery (third group) (Kramer et al., 2007). Post-translational modifications also control the localization and/or delocalization of membrane-bound competence protein and cytosolic proteins; specifically through phosphorylation of arginine (e.g., ClpC, ClpP, McsB, ComGA, ComFA, ComFC, RecA, SsbA and SsbB) and/or tyrosine (SsbA, SsbB) residues (Elsholz et al., 2012, Mijakovic et al., 2006). The underlying mechanisms for this phosphorylation, however, remain undefined.

In Proteobacteria, as *Neisseria gonorrhoeae* and *H. pylori*, the DNA uptake machinery has another membrane that needs to be crossed (Figure 2B and 2C). The dsDNA is bound on the cell surface and transported as intact dsDNA into the periplasm through a ring-shaped assembly of proteins of a type IV pili known as secretins (e.g., PilQ_{Ngo}) (Figure 2B) (Mattick, 2002, Collins et al., 2004), or through a type IV secretion system as the one used to transport dsDNA into the periplasmic space in *H. pylori* used (Figure 2C) (Hofreuter et al., 2001). Once dsDNA has crossed the outer membrane and is in the periplasmic region of the cell, the transport of the ssDNA into the cytosol is based on the DNA uptake machinery, similar to the one described for Firmicutes (Figure 2A) (Danner et al., 1980, Elkins et al., 1991, Stingl et al., 2010). In *H. pylori* it has been shown that the uptake machinery also assembles preferentially at the cell pole and takes up ssDNA into the cytosol by a two-step mechanism (Stingl et al., 2010) (Figure 2C). However, the fate of incoming ssDNA and the recruitment of RecA onto ssDNA are not yet clear in these bacteria and need further study.

The third group of proteins, which localize outside the membrane and at the cell pole in competent cells, are cytosolic in nature, they do not affect the uptake of ssDNA and belong to the recombination machinery (Figure 2A) (Sanchez et al., 2007b, Claverys et al., 2009, Kidane et al., 2009, Kidane and Graumann, 2005). The recombination machinery, which protects, processes and recombines the incoming ssDNA will be the topic of this review. This includes five competence-induced (DprA, SsbA, SsbB, CoiA and RecA) and at least two competence-independent (RecU, RecX) proteins (Figure 1) that transiently and specifically localize at the cell membrane prior to the internalization of ssDNA (Figure 2A).

ComGA, ComFA and SsbB, which localize at the cell pole in rod-shaped bacteria with a rod-shaped form, appear to promote the localization of CoiA, DprA and RecA (Figure 1 and 2A). In some cases the assembly might occur away from the pole and these complexes diffuse until they are captured at the pole (see Hahn et al., 2009, Kaufenstein et al., 2011). For example, cytosolic SsbB can associate with the pole independently of other Com proteins and its absence does not seem to affect ComGA and RecA localization, but decreased DprA localization (Kramer et al., 2007). In other cases the protein associated with the cell pole (e.g., ComGA) facilitates the localization of a cytosolic protein (e.g., RecA), and this in turn facilitates the localization of second cytosolic protein (e.g., DprA, RecU). However, DprA, which forms polar assembly in the absence of RecA, is loosely associated with other uptake proteins (e.g., SsbB, RecA) (Kidane and Graumann, 2005, Hahn et al.,

2009, Kramer et al., 2007). RecU (a RecA modulator and a Holliday junction, HJ, resolving enzyme) (Ayora et al., 2004, McGregor et al., 2005) only forms a polar assembly in the presence of RecA (Figure 2A, Table 1) (Kidane et al., 2009). All these proteins mainly assemble at a single cell pole, but assembly at both poles was also observed (Hahn et al., 2005). In those cells containing two DNA uptake machineries, one at each cell pole, RecA might assemble preferentially at one of them (Kaufenstein et al., 2011). These results suggest that: i) the complex is dynamic, ii) not all associations might coexist simultaneously or their stoichiometry might vary, and iii) one and rarely two uptake machineries per cell might be competent for DNA transfer. The association of recombination proteins with the uptake machinery is usually dynamic: they accumulate at the poles as transformability develops and then delocalize as transformability proceeds and/or declines (Hahn et al., 2009, Tadesse and Graumann, 2007).

There is another set of recombination proteins, such as RecN or SbcE, which belong to the family of structural maintenance of chromosomes (SMC)-like proteins (Graumann and Knust, 2009), that dynamically oscillate from cell pole to cell pole scanning for the incoming ssDNA (Figure 2A). Once the ssDNA is inside, RecN and SbcE lose their dynamic oscillation and bind to the incoming DNA ends (Kidane and Graumann, 2005, Krishnamurthy et al., 2010). The internalization of ssDNA segments with self-annealing potential (*e.g.*, during plasmid transformation) promotes the localization of RecO, which is modulated by the presence of RecN (Kidane et al., 2009).

Different recombination avenues exist for different DNA substrates

In this review, the mechanism and efficiency of recombination of various DNA types, as substrates, are summarized. When the incoming ssDNA shows complementarity with recipient dsDNA two different events might take place: If the ssDNA recombines and integrates into the chromosome or into a recipient plasmid the event is called chromosomal transformation (Figure 3A), but if the homologous segments are used to reconstitute an autonomous replicating molecule (without integration) the event is termed plasmid facilitation (Figure 3B).

Chromosomal transformation is an intermolecular recombination process that leads to the formation of a D-loop intermediate, due to the exchange of a strand of the recipient molecule with the newly internalized ssDNA molecule. Then the D-loop is resolved, and the replaced strand degraded (Figure 3A). Like chromosomal transformation, plasmid facilitation is a transient intermolecular recombination process due to homology with recipient DNA (the chromosome or another resident plasmid). It is believed that in plasmid facilitation, the newly internalized plasmid ssDNA molecule interacts with its complementary recipient strand, which is used as a template to circularize, the D-loop is then disrupted after DNA synthesis, and next, DNA replication reconstitutes an autonomously replicating plasmid (Figure 3B). Chromosomal transformation and plasmid facilitation are RecA-dependent events (Fernández et al., 2000, Lacks, 1988).

When the incoming ssDNA is an autonomous replication molecule that shows no homology with recipient, two different events might take place: if this imported ssDNA molecule is oligomeric the event is termed plasmid transformation (Figure 3C), but if it only contains internal repeats the event is called monomeric activation (Figure 3D). In Figure 3 the fate of an incoming monomeric plasmid ssDNA that contains an autonomous replication origin, but neither shows homology with recipient nor with itself (monomeric plasmid transformation) will not be depicted, because this ssDNA is inactive in transforming *B. subtilis* cells (Canosi and Trautner, 1979, Dubnau et al., 1980). Monomeric plasmid ssDNA, however, transformed competent cell of some species (*e.g.*, *S. pneumoniae*) (Saunders and Guild,

1981). Monomeric transformation, which is a RecA-dependent event (Fernández et al., 2000, Lacks, 1988), showed a two hit kinetic, where DNA fragments from two or more plasmid molecules had to enter the cell and recombine (see below).

Plasmid transformation and monomeric activation are intra-molecular recombination processes in which the internalized strand(s) recombines to reconstitute a circular dsDNA molecule. Depending on the type of plasmid substrate this could be partially RecA-dependent (monomeric activation) or a RecA-independent process (plasmid transformation) (Canosi et al., 1978, Michel et al., 1982, Lorenz and Wackernagel, 1994). A plasmid carrying direct internal repeats transform competent cells (monomeric activation). The efficiency of transformation varied with the square of the length of the repeats and plasmid rearrangements were observed (see below) (Michel et al., 1982).

There is another complex recombination event, viral transfection, that it is poorly understood. Viral transfection is an inter- and intra-molecular recombination process (Sanchez et al., 2007b, Kidane et al., 2009). We will discuss the requirements and mechanisms of plasmid and chromosomal transformation. Beyond sharing the same binding pathway, the use of surface nucleases that are active during competence and the type of repair proteins associated with the DNA uptake machinery, the mechanism of processing of the incoming DNA is different between chromosomal and plasmid transformation and will be discussed below.

Genetic analysis of the recombination machinery

Genetic analyses have allowed us to gain insight into the contribution of DNA repair and competence specific functions in chromosomal and plasmid transformation. Among the proteins that localize with the DNA uptake apparatus and are competence-induced, the absence of RecA and DprA shows the strongest phenotype (Table 3). Except in *D. radiodurans* that chromosomal transformation is reduced only 40- to 100-fold (Bouthier de la Tour et al., 2011, Satoh et al., 2012), the absence of RecA abolishes chromosomal transformation (reduced $> 10^4$ -fold). In general plasmid transformation is a RecA-independent event (2- to 4-fold reduction), but in some species (e.g., *S. pneumoniae*) plasmid transformation is indirectly abolished in the *recA* context. Here, RecA is required to protect incoming transforming ssDNA (Berge et al., 2003), suggesting that in this bacterium RecA also plays a role as a “guardian” of the incoming ssDNA.

The degree of involvement of DprA is species-dependent. The absence of DprA abolishes GR in *S. pneumoniae* competent cells (Table 3), decreases both chromosomal and plasmid transformation in *H. pylori* or *B. subtilis* cells, and only decreases chromosomal transformation in *H. influenzae* (Karudapuram and Barcak, 1997, Ando et al., 1999, Smeets et al., 2000, Berka et al., 2002, Ogura et al., 2002, Tadesse and Graumann, 2007, Hamoen et al., 2002, Marsin et al., 2008). One reason for such discrepancy is that DprA_{Spn} might play also as a “guardian” of the incoming ssDNA (Berge et al., 2003), whereas in *B. subtilis*, *H. pylori* and *H. influenzae* different proteins may protect the incoming ssDNA. DprA is not restricted to naturally transformable bacteria and is ubiquitous among free-living bacteria. The absence of DprA abolishes electro-transformation of plasmid DNA into non-naturally competent variants of *Campylobacter jejuni* (Takata et al., 2005). *Escherichia coli* contains two DprA family members (Smf and DprA), but the K12 strain only contains the *smf* reading frame. The absence of Smf does not affect Ca²⁺-mediated plasmid transformation or HFR conjugation in *E. coli* K12 cells (Smeets et al., 2006). Thus, the role fulfilled by DprA in non-natural competent bacteria remains to be unraveled.

The absence of SsbB or CoiA, in both *B. subtilis* and *S. pneumoniae* competent cells, moderately reduces (3- to 20-fold) chromosomal transformation (Table 3, and references

therein), suggesting that both proteins play a minor role in transformation, or that the redundancy of the system masks the outcome. Indeed, at high dsDNA concentrations ($>0.2 \mu\text{g}/10^8$ cells) the absence of SsbB_{Spn} shows two unexpected outcomes: i) it markedly decreases (~ 170 -fold) chromosomal co-transformation of two independent markers, and ii) it facilitates plasmid transformation (Attaiech et al., 2011). In the former case SsbB_{Spn} plays a role in optimizing chromosomal transformation, whereas in the latter case SsbB_{Spn} seems to antagonize plasmid transformation (Attaiech et al., 2011). This result, however, might not be easily extrapolated to competent cells from other species because in many cases the DNA processing and translocation machinery becomes saturated above $0.2 \mu\text{g}$ of dsDNA per $\sim 10^8$ *B. subtilis* cells (Dubnau, 1999). *In vitro*, SsbA overcomes the inhibitory effect exerted by SsbB in RecO-mediated DNA strand annealing (Yadav et al., 2012). *In vivo*, the contribution of the essential SsbA in natural transformation has not yet been reported, but *H. pylori* is naturally transformed and it encodes for a single SSB protein (SsbA_{Hpy}), suggesting that SsbA should have a relevant role in chromosomal transformation.

The Sms (also termed RadA) protein, which is distantly related to bacterial RecA and Lon protease, is induced in *S. pneumoniae* competent cells (Table 1), but not in *B. subtilis* competent cells. The role of Sms is poorly understood, but its absence partially suppresses the DNA repair defect of functions involved in the processing of RecA-mediated recombination intermediates (e.g., *recH*, ΔrecU) (Carrasco et al., 2002) (Table 3). Indeed, the absence of Sms reduces 10- and 100-fold chromosomal transformation in *B. subtilis* and *S. pneumoniae* competent cells, respectively (Burghout et al., 2007, Carrasco et al., 2002) (Table 3).

The *B. subtilis* proteins involved in recombinational repair, other than RecA, have been classified into 9 different epistatic groups (α to κ) (Ayora et al., 2011) (Table 1). Except for ΔrecX and ΔsbcE cells, chromosomal transformation did not change more than four-fold relative to the *rec*⁺ value when *B. subtilis* cells had a single *rec*⁻ function of the α to η epistatic groups (Fernández et al., 2000) (Table 3). The absence of RecX reduced chromosomal transformation ~ 50 -fold (Table 3), but only 5-fold reduced in *N. gonorrhoeae* (Stohl and Seifert, 2001, Cardenas et al., 2012). The absence of SbcE reduced chromosomal transformation ~ 20 -fold, but the absence of both SMC-like proteins, SbcE and RecN, decreased chromosomal transformation > 80 -fold (Krishnamurthy et al., 2010). Previously, it was proposed that different pathways act redundantly to promote chromosomal transformation (Alonso et al., 1993). Supporting the redundancy hypothesis it was shown that the absence of both pathways, RecF and AddAB (counterpart of the RecBCD enzyme), reduced natural transformation > 500 -fold (Table 3).

The RecG and RuvAB proteins are branch migration translocases that act after RecA-mediated D-loop formation (Ayora et al., 2011, Kowalczykowski et al., 1994). The absence of RecG or RuvAB, marginally reduced chromosomal transformation in *B. subtilis* or *N. gonorrhoeae* (Cañas et al., 2008, Sechman et al., 2006), but the lack of both branch migration translocases is synthetically lethal in those bacteria (Sanchez et al., 2005, Sanchez et al., 2007c, Sechman et al., 2006). In contrast, in *H. pylori* the absence of RecG showed no apparent defect, the absence of RuvB abolished transformation, but cells lacking both functions showed transformation levels similar to the wt strain, suggesting that the *recG*_{Hpy} mutation suppressed the *ruvB*_{Hpy} phenotype (Kang and Blaser, 2008). However, these observations differ from another published study that showed no significant difference of the single mutants as compared with wt (Humbert and Salama, 2008). In *S. pneumoniae*, the absence of RecG (also termed MmsA) reduced chromosomal transformation by ~ 25 -fold (Table 3) (Mortier-Barriere et al., 1997).

The HJs are cleaved, in concert with the RuvAB branch migration translocase, by a junction-resolving enzyme termed RecU in Firmicutes or RuvC in bacteria of other Phyla (Ayora et al., 2011, Declais and Lilley, 2008). The YrrK, YqgF_{Eco}, YqgF_{Ngo} or DprB_{Hpy} function is ubiquitous and predicted to possess an analogous catalytic activity to that of RuvC (Aravind et al., 2000). Although *yrrK*, *yqgF_{Eco}* and *yqgF_{Ngo}* are essential the *dprB_{Hpy}* gene is non-essential (Sanchez et al., 2007a, Humbert et al., 2011, Zhang and Blaser, 2012). The absence of RecU, RuvC_{Ngo}, RuvC_{Hpy} or DprB_{Hpy}, marginally reduces chromosomal transformation (Cañas et al., 2008, Loughlin et al., 2003, Zhang and Blaser, 2012, Sechman et al., 2006). However, the absence of both RuvC_{Hpy} and DprB_{Hpy} resolvases block chromosomal transformation (Humbert et al., 2011, Zhang and Blaser, 2012), suggesting that the essential YrrK might have a role in the processing of D-loop intermediates.

Plasmid transformation revealed an important role for some recombination proteins other than RecA in this process. The absence of RecX reduced plasmid transformation by ~ 200-fold and the absence of RecO or RecU by 30- to 40-fold (Table 3) (Fernández et al., 1999, Fernández et al., 1998, Kidane et al., 2009, Cardenas et al., 2012). The above requirement is not generally conserved among competent cells, for example plasmid transformation in *D. radiodurans* competent cells is not affected by the absence of RecO, but is reduced (> 100-fold) in the absence of the RecO associated partner, RecR, and of another species specific single strand annealing (SSA) protein (*e.g.*, DdrB) (Bouthier de la Tour et al., 2011, Satoh et al., 2012).

In certain bacteria, in the absence of transient diploidy (*e.g.*, competent cells, spores) or template-dependent HR, the two-ended DSBs are brought together and then joined via the non-homologous end-joining (NHEJ) process. The two main factors involved in NHEJ in bacteria are Ku and LigD (Gong et al., 2005, Weller et al., 2002, Shuman and Glickman, 2007). The transformation efficiency of chromosomal DNA is not affected in Δku cells but it is 6-fold reduced during plasmid or viral transformation (Kidane et al., 2009). This observation sheds light to the minor but significant contribution of protein factors that mediate NHEJ in plasmid or viral transformation (see below).

The contribution of the mismatch repair machinery to DNA transformation was also genetically analyzed. With few exceptions (*e.g.*, *S. pneumoniae*), the lack of mismatch repair genes does not affect chromosomal transformation (Majewski and Cohan, 1999, Sanchez et al., 2007b, Stewart and Carlson, 1986). The absence of *S. pneumoniae* HexAB (also termed MutSL) mismatch repair proteins positively affects transformation rates for donor DNA that contains one or few mismatches (Balganesh and Lacks, 1985, Claverys et al., 1984), but it is ineffective when transforming DNA that has ~5% of sequence divergence from the recipient DNA (Mortier-Barriere et al., 1997), suggesting that the HexAB system is easily saturated by DNA mismatches.

Functions involved in genetic recombination

Recombination reactions catalyzed by the RecA family of proteins form an integral part of DNA metabolism in all free-living bacteria, archaea and eukaryotes (reviewed in Cox, 2007a, San Filippo et al., 2008). In competent cells the major role of RecA is to form a helical nucleoprotein filament (visualized as dynamic threads structures across the nucleoid) on the internalized ssDNA and then to promote a search for homology with dsDNA recipient DNA, leading to integration into the genome of the competent cell (Kidane and Graumann, 2005, Sanchez et al., 2007b). The assembly of RecA onto the incoming ssDNA tracts is relatively infrequent and thus rate limiting. In addition, a set of RecA accessory factors is required to stimulate RecA nucleation and filament extension onto the incoming ssDNA. The accessory factors that act before RecA nucleation can be arbitrarily sub-divided

into two classes. The first class includes proteins that bind ssDNA that act as protectors (SsbA, SsbB, RecN, SbcE) and barriers (SsbA, SsbB) that limit RecA nucleation, and are tentatively called guardian proteins (Table 2). The second class includes proteins (as RecO, DprA) that promote the nucleation of RecA onto ssDNA and mediate annealing of complementary DNA strands coated by a single-stranded binding protein (SSB). These proteins were previously termed mediators (Beernink and Morrical, 1999, Cox, 2007b, Galletto and Kowalczykowski, 2007). The accessory factors that act during RecA-mediated homology search regulate the dynamic assembly/disassembly of the RecA-ssDNA filament (RecF, RecX, RecU), and are tentatively called RecA modulator proteins (Table 2). Genetic and biochemical data support the hypothesis that among the “guardians” there are proteins with two different roles: RecN or SbcE might protect the ends of the internalized linear ssDNA, whereas SsbA and/or SsbB coat the incoming ssDNA and remove secondary structures that hamper the downstream reaction (Kidane and Graumann, 2005, Krishnamurthy et al., 2010, Carrasco et al., 2008, Yadav et al., 2012) (Table 2). These guardian proteins might be less efficient in certain bacterial species and other proteins might perform similar functions, for example DprA_{Spn} and RecA_{Spn} are necessary to protect the internalized ssDNA (Berge et al., 2003).

RecN is a 576-residue long polypeptide chain (~ 64 kDa). *In vitro* RecN, which binds ssDNA and tethers the DNA ends, protects the 3'-OH end from ssDNA degrading enzymes (e.g., Exo III, PNPase) (Sanchez and Alonso, 2005, Sanchez et al., 2008, Cardenas et al., 2011). A similar role has been proposed for SbcE (a 963-residue polypeptide chain, ~ 110 kDa) (Krishnamurthy et al., 2010). With few exceptions (e.g., SsbA_{Hpy}) competent cells encode two SSB proteins (SsbA and SsbB) (Lindner et al., 2004). SsbA (a 172-residue polypeptide chain, ~ 18 kDa) is homologous to SsbB (a 113-residue polypeptide chain, ~ 12 kDa) and to SSB_{Eco} or SsbA_{Hpy} proteins. The essential SsbA and the non-essential SsbB form tetramers in solution (Yadav et al., 2012). *In vitro* SsbA (SsbA_{Spn}) and SsbB (SsbB_{Spn}) proteins co-assemble on ssDNA segments, where independent binding is possible (Salerno et al., 2011, Yadav et al., 2012), but the formation of heterotetramers was not observed (Yadav et al., 2012). The structure of SSB_{Eco}, SsbA_{Hpy} or SsbB complexed with ssDNA has been elucidated, and they have revealed a similar overall protein architecture and ssDNA-binding surface (Chan et al., 2009, Raghunathan et al., 2000, Yadav et al., 2012). SsbA and/or SsbB have three activities: (i) to protect ssDNA and disrupt DNA secondary structures; (ii) to inhibit RecA loading onto SsbA- or SsbB-coated ssDNA, and thereby repress unwanted RecA nucleation onto the incoming ssDNA (Cox, 2007b, Yadav et al., 2012); and (iii) to interact specifically with a large number of proteins (e.g., RecO, DprA, and replication proteins) (Costes et al., 2010, Lecointe et al., 2007, Shereda et al., 2008, Manfredi et al., 2010, Yadav et al., 2012), suggesting a more dynamic role than previously thought.

A mediator protein must reverse the negative effect exerted by the guardians and promote RecA nucleation onto SsbA- and/or SsbB-coated ssDNA (Table 2) (Ayora et al., 2011, Cox, 2007b, Yadav et al., 2012). Such a role as mediator has been demonstrated for *S. pneumoniae* DprA (a 297-residue polypeptide chain, ~ 32 kDa), but it remains unknown in *B. subtilis*. It was shown that purified DprA_{Spn} overcomes the inhibitory effect even exerted by heterologous SSB_{Eco} and facilitates nucleation of RecA_{Eco} onto SSB_{Eco}-coated ssDNA, suggesting that DprA_{Spn} is a RecA mediator albeit with relaxed species-specific requirement (Mortier-Barriere et al., 2007). It was proposed that DprA_{Spn} could interact with naked ssDNA, as soon as it exits from the trans-membrane channel, and in that case there would be no need to displace SsbB_{Spn} (Mortier-Barriere et al., 2007). *In vitro* data revealed the presence of mixed DprA_{Spn}-RecA_{Eco} nucleofilaments that are proficient for recombination (Mortier-Barriere et al., 2007). Furthermore, the findings that DprA_{Spn} could anneal complementary ssDNA segments suggested its role in plasmid transformation (Mortier-

Barriere et al., 2007). A physical interaction between DprA_{Spn} and RecA_{Spn} (Mortier-Barriere et al., 2007) and DprA with SsbB and RecA (Kramer et al., 2007) have been documented (see Figure 1). The rate-limiting step in RecA filament assembly is the nucleation step. Both events can be visualized *in vivo* as a focus (nucleation onto ssDNA?) or as dynamic threads (RecA-ssDNA filament?). *In vivo* RecA forms a focus and dynamics threads even in the absence of DprA in *B. subtilis* and *H. pylori* competent cells (Kidane and Graumann, 2005, Tadesse and Graumann, 2007, Orillard et al., 2011), suggesting that another mediator (*e.g.*, RecO) alone or in concert with a guardian protein (*e.g.*, SsbA, SsbB, RecN) might also recruit RecA onto SsbA- and/or SsbB-coated ssDNA (Figure 2A). Indeed, the absence of both DprA and RecO reduce both chromosomal and plasmid transformation > 1000-fold (Table 3) (Yadav et al., 2012).

RecO (a 255-residue polypeptide chain, ~ 29.3 kDa) has two activities: to recruit RecA onto SsbA-coated ssDNA, and to mediate annealing of two SsbA-coated complementary DNA strands (Kidane et al., 2009, Manfredi et al., 2008, Manfredi et al., 2010, Yadav et al., 2012). The mediator activity is essential to displace the SSB proteins from ssDNA and to load RecA onto SsbA- and/or SsbB-coated ssDNA (Manfredi et al., 2008, Manfredi et al., 2010, Yadav et al., 2012). Physical interactions between RecO and SsbA, and a biochemical one between RecO and RecA have been documented (Figure 1) (Manfredi et al., 2008, Manfredi et al., 2010, Yadav et al., 2012).

Both RecA (a 347-residue polypeptide chain, ~ 37 kDa) and RecA_{Spn} catalyze DNA strand exchange preferentially in the presence of dATP (Carrasco et al., 2008, Lovett and Roberts, 1985, Steffen and Bryant, 1999, Steffen et al., 2002). Based on cytological and biochemical analyses, the role of RecA during chromosomal transformation can be divided into several discrete stages. In the first step, RecA associates with ComGA, SsbB, DprA and RecU at the cell pole membrane (Figure 3A) (Kramer et al., 2007, Kidane et al., 2009, Kidane and Graumann, 2005). Secondly, RecA displaces RecN from the ssDNA ends (Sanchez et al., 2008). Third, SsbA or SsbB inhibits RecA nucleation onto ssDNA (Yadav et al., 2012). The presence of RecO (or perhaps DprA) counteracts the negative effect exerted by SsbA or by the SsbA and SsbB proteins in RecA loading onto ssDNA (Yadav et al., 2012). This is consistent with the observations that RecA foci formation upon DNA addition is reduced in the absence of RecN or RecO (Kidane et al., 2009, Kidane and Graumann, 2005). Fourth, the modulators (*e.g.*, RecF, RecX, RecU, *etc.*) facilitate the formation of a RecA-ssDNA filament (RecA thread) with an effectual length, both *in vivo* (Kidane and Graumann, 2005, Cardenas et al., 2012) or *in vitro* (Cox, 2007a, Galletto and Kowalczykowski, 2007). Finally, the dynamic RecA-ssDNA filaments (threads) search for a region on the resident chromosome that is homologous to incoming ssDNA. Thus, the specifically localized DNA uptake machinery extends into the cytosol, so that a complex competence machinery provides a defined vectorial path for DNA from one cell pole to an homologous region on the recipient chromosome, showing that bacterial transformation is a highly organized and ordered process (Figure 2A).

RecU (a 206-residue polypeptide chain, ~ 23 kDa), which physically interacts with RecA (Cañas et al., 2008), has two activities: to modulate RecA-ssDNA filament extension and to catalyze HJ resolution (Ayora et al., 2004, Cañas et al., 2008, Carrasco et al., 2005, Carrasco et al., 2009). The role of RecU in transformation is believed to be the regulation of RecA-ssDNA filament formation, and not the resolution of the recombination intermediate, because *in vitro* the protein is unable to cleave D-loops (Carrasco et al., 2005), and separation of function mutants (*recU56* and *recU71*) that are impaired in plasmid transformation, can promote resolution of HJs but do not promote RecA modulation (Cañas et al., 2008, Cañas et al., 2011). RecU disassembles from the cell pole concomitantly with the formation of RecA threads (Kidane et al., 2009), suggesting that when RecA is engaged

in homology search it becomes refractory to RecU action. A second set of modulators (e.g., RecX, RecF, Sms) might have an active role in RecA-ssDNA filament formation or in the modulation of the dynamics of the RecA threads during homology search (Table 2). The role of RecF (a 370-residue polypeptide chain, ~ 42 kDa), RecX (a 264-residue polypeptide chain, ~ 30 kDa) and Sms (a 458-residue polypeptide chain, ~ 49 kDa) modulators in chromosomal transformation, however, is poorly understood. RecX_{Ngo} binds ssDNA and inhibits RecA loading onto SsbA_{Ngo}-coated ssDNA (Gruenig et al., 2010). Another protein(s) might counteract the effect of RecX (e.g., RecF) and create an effectual RecA-ssDNA filament (see Baitin et al., 2008, Lusetti et al., 2006). Indeed, in the presence of RecF_{Eco} the inhibitory effect of RecX_{Eco} is reversed (Lusetti et al., 2006). However, mechanistically RecX_{Ngo} might differ from RecX_{Eco} (Stohl and Seifert, 2001, Stohl et al., 2003), because RecX_{Ngo} does not cap the growing ends of RecA_{Ngo} filaments, as it has been reported for RecX_{Eco}, but it directly facilitates a more rapid RecA_{Ngo} filament disassembly (see Drees et al., 2004a, Gruenig et al., 2010, Ragone et al., 2008, Stohl et al., 2003). RecF, which contains a consensus nucleotide-binding fold, binds ssDNA and dsDNA (Ayora and Alonso, 1997). Cytological analyses of the DSB repair response suggest that RecF might work after RecA nucleation (Kidane et al., 2004). In contrast, a role for RecF_{Eco} prior nucleation has been proposed: RecF_{Eco} inhibits RecOR_{Eco}-mediated loading of RecA_{Eco} onto SSB_{Eco}-coated ssDNA, but facilitates RecA_{Eco} nucleation to the dsDNA/ssDNA boundary (Umezue et al., 1993, Hegde et al., 1996, Morimatsu and Kowalczykowski, 2003, Cox, 2007b). In some bacterial species, RecA mediation and modulation is catalyzed by the same protein (e.g., DprA_{Spn}) (Mortier-Barriere et al., 2007), whereas in other species both activities might be carried out by different proteins (e.g., RecO and RecF, see Table 2). In *B. subtilis* DprA is a RecA mediator that does not seem to contribute to the dynamic formation of RecA threads from the cell pole to midcell (RecA-ssDNA filaments) (Tadesse and Graumann, 2007), suggesting that RecF and RecX might modulate dynamic RecA-ssDNA filament extension (Ayora et al., 2011, Cox, 2007b).

The function of CoiA (a 373-residue polypeptide chain, ~ 43 kDa) is poorly understood. CoiA_{Spn} seems to function at a later stage than that the guardian proteins, and it was suggested to be involved in promoting recombination during genetic transformation (Desai and Morrison, 2007). The biochemical activities associated with CoiA are unknown.

When the RecA-ssDNA filament finds its homologous counterpart it catalyzes DNA strand invasion with the formation of a D-loop intermediate (Figure 2A). Then, an unknown “resolver(s)” should process the RecA-mediated recombination intermediate, and the ends should be sealed by a DNA ligase (Figure 3A). The role of the essential YrrK or the non-essential DprB_{Hpy} in the resolution of the D-loops remains to be unraveled (see above).

If any recombination intermediate is improperly processed during GR a one-ended DSB might be generated, and a different set of proteins must repair this DSB. End-processing and RecA accessory factors involved in the repair of the one-ended DSB during competence are termed rescuers (Table 2). Since these proteins, which should act after the physical integration of the donor DNA, carry out repair that is necessary for the viability of the transformed cell, they should be considered as a genuine part of the transformation machinery (Fernández et al., 2000, Kickstein et al., 2007). As an example, the cytosolic ExoV enzyme (also termed AddAB [RexAB], RecBCD or AdnAB) (Niu et al., 2009), which is not expected to act during ssDNA assimilation into a recipient duplex DNA, interacts with dsDNA ends to rescue those improperly processed integration events. In *B. subtilis* the absence of one of the end-processing (AddAB) avenues or of a modulator (RecF) decreases transformation less than 3-fold, but the absence of both AddAB and RecF blocks chromosomal transformation (Table 3) (Alonso et al., 1993, Fernández et al., 2000). The involvement of the rescuer proteins in GR, however, is not generally conserved. *H. pylori*

competent cells are either not affected or marginally compromised in chromosomal transformation in the absence of AddAB and RecOR proteins (Marsin et al., 2010, Wang et al., 2011) whereas *N. gonorrhoeae* mutations in *recBCD* and *recF* decrease chromosomal transformation ~ 40-fold (Mehr and Seifert, 1998). These results suggest the existence of other uncharacterized rescuer proteins in these bacteria or that the GR process is less prone to errors in competent cells of the Proteobacteria than those of the Firmicutes Phylum.

Previously, it was shown that *B. subtilis* polynucleotide phosphorylase (PNPase) is necessary for natural transformation (Luttinger et al., 1996). PNPase, which is a multifunctional enzyme, is responsible for Mg^{2+} -dependent $3' \rightarrow 5'$ processive phosphorolytic degradation of RNA and template-independent polymerization of rNDPs into RNA (Condon, 2003, Grunberg-Manago, 1999), and also for Mn^{2+} -dependent $3' \rightarrow 5'$ processive phosphorolytic degradation of ssDNA and template-independent polymerization of dNDPs into ssDNA (Cardenas et al., 2009, Cardenas et al., 2011). Previous research showed that PNPase (also termed ComR) is necessary for the normal expression of *comG-lacZ* and *srfA-lacZ* (including *comS*) fusions, but such effect was bypassed by the absence of the MecA protein, which is an adaptor protein that drives ComK degradation by ClpCP protease (Luttinger et al., 1996). However, an attempt to bypass the need for PNPase as a post-transcriptional regulator was unsuccessful by overexpressing ComS, which competes with ComK for MecA binding and protects ComK from degradation (Luttinger et al., 1996, Prepiak and Dubnau, 2007). Recently, it was shown that PNPase, which co-purifies with RecA in *E. coli* or with RecN in *B. subtilis*, was able to degrade ssDNA with a $3' \rightarrow 5'$ polarity in the presence of Mn^{2+} and low inorganic phosphate concentration, and such activity was enhanced by the presence of RecA and inhibited by SsbA (Cardenas et al., 2009, Cardenas et al., 2011). PNPase can also extend a $3'$ -OH end in the presence of dNDP- Mn^{2+} , and RecN-dATP bound to ssDNA stimulates such activity (Cardenas et al., 2009, Cardenas et al., 2011). The role of PNPase in chromosomal and plasmid transformation needs to be revisited.

Chromosomal transformation

Chromosomal DNA transformation does not seem to require extensive DNA replication because less than 300-nt of *de novo* synthesis is sufficient for integration of the donor markers (Dubnau, 1999, Lorenz and Wackernagel, 1994). The three-stranded HR reaction, which takes place during natural transformation by recombination of the linear taken up ssDNA with recipient dsDNA is one of the most efficient ways for cells to acquire new genetic traits, because ~ 40% of internalized ssDNA becomes a recombinant product (Dubnau, 1999, Fernández et al., 2000). The size of the internalized ssDNA in competent cells varies, but it seems to average 12-kb in *B. subtilis* (Dubnau, 1999). During chromosomal transformation the SsbB, SsbA, RecN or SbcE guardian proteins protect the internalized ssDNA or the $3'$ -OH end of it (see above, Table 2, Figure 3A). It was hypothesized that the internalized ssDNA remains intact as long as RecA is searching for homology (chromosomal transformation) or an internal homology in a self-replicating molecule is found (plasmid transformation), but it is degraded when none of these conditions are fulfilled (Dubnau et al., 1973, Lacks, 1988). The homology search could be divided into three steps: i) alignment of the RecA-ssDNA filament with a given tract along duplex DNA, ii) unwinding of the duplex DNA and strand invasion of the $3'$ -ssDNA into the intact homologous recipient duplex, and iii) extension of the pairing with the complementary strand resulting in the formation of a three stranded synaptic intermediate (D-loop) (Figure 2A and 3A) (Claverys and Lacks, 1986, Sanchez et al., 2007b). It is likely that a similar series of events takes place in Proteobacteria (*e.g.*, *N. gonorrhoeae*, *H. pylori*) (Figure 3B and 3C). Due to homology, the invaded region can be extended by branch migration, but the function(s) that promote(s) branch migration of a D-loop is not or poorly

characterized. Once a stable D-loop is formed, branch migration seems to proceed relatively easily through regions of high divergence. This could explain why gene disruption, by a heterologous (antibiotic resistance marker) DNA segment surrounded by homologous DNA segments, is a relatively efficient process (Sanchez et al., 2007b). An uncharacterized D-loop resolvase(s) (resolver[s]), (Table 2), rather than the RecU or SKIN-encoded RusA-like HJ resolvase (Table 3), may resolve the D-loop intermediate. The role of the essential YrrK function in the processing of D-loop intermediates need to be revisited (see above). Then, a DNA ligase seals the DNA ends without extensive DNA replication (Sanchez et al., 2007b). The displaced ssDNA should be then degraded. Alternatively, both ends of the taken up ssDNA are used by RecA to catalyze two independent strand invasion events into the recipient DNA, and these events should occur nearly simultaneously, then, spontaneous branch migration from the invaded ends through the entire molecule forms the three-strand intermediate (Majewski and Cohan, 1999). The processing of this putative three-strand intermediate is unknown.

The replisome, as judged by the localization of YFP-DnaX, remains at or near midcell not only during exponential growth (Lemon and Grossman, 1998), but also during competence development and transformation with chromosomal DNA (Figure 2), suggesting that the stationary replisome is more static than previously thought (our unpublished results). Upon replication fork reactivation and one round of DNA replication, the strand where the donor DNA was integrated will be segregated from the unreactive recipient strand, resulting in one transformed and one parental genome. In contrast, competent cells with more than one chromosome (e.g., *D. radiodurans*) need to segregate first the recombinant strand and in a second step recombination between the chromosomes and segregation will render a genotypic transformant (homogenate).

If a plasmid molecule contains a segment of homology with the chromosome or with another plasmid already present in the recipient, the plasmid, even as a monomer, is transformed and established via RecA-dependent intermolecular recombination (plasmid facilitation) (Figure 3B). Plasmid facilitation, as well as chromosomal DNA, follows a first order kinetics with respect to DNA concentration indicating that a competent cell interacts with a single plasmid molecule to render a plasmid transformant (Contente and Dubnau, 1979, Canosi et al., 1981, López et al., 1982, Gromkova and Goodgal, 1981). Mechanistically, plasmid facilitation resembles chromosomal transformation, but the internalized ssDNA does not necessarily integrate into the recipient molecule (Sanchez et al., 2007b). It is likely that after DNA synthesis primed by the invasive end the D-loop is disrupted by the action of an uncharacterized DNA helicase, and ligation of the partially duplex molecule reconstitutes a circular plasmid molecule. Plasmid facilitation mechanistically resembles synthesis-dependent strand annealing (Ayora et al., 2011).

Plasmid transformation

The DNA uptake machinery does not distinguish between linear chromosomal from supercoiled plasmid DNA (Dubnau, 1999, Stewart and Carlson, 1986), and they compete for receptor sites at the cell surface (Gromkova and Goodgal, 1981). In the absence of homology with recipient DNA (< 30-nt) (Alonso et al., 1986, Majewski and Cohan, 1999), an oligomeric plasmid molecule is transformed with ~100-fold lower efficiency than chromosomal DNA (Canosi et al., 1978, Dubnau et al., 1980). In *B. subtilis* monomeric plasmid DNA is inactive for transformation, but its artificial oligomerization markedly increases plasmid transformation efficiency, suggesting that monomeric plasmid DNA is not the proper substrate for naturally transforming bacteria (see below) (Notani et al., 1981, Canosi and Trautner, 1979, Eisenstein et al., 1977). The host-range of the plasmid

replication machinery is the only limiting factor for plasmid transformation between competent cells of different species.

Successful transformation requires reconstitution of the plasmid in the new host cell (plasmid transformation or establishment), and unlike chromosomal transformation, this process is independent of RecA. Dimeric or higher order oligomeric plasmid DNA follows a first order kinetics with respect to DNA concentration indicating that a competent cell interacts with a single plasmid molecule to render a plasmid transformant via a RecO- or DprA-dependent avenue (Figure 3C) (Stewart and Carlson, 1986, Lacks, 1988, Sanchez et al., 2007b, Yadav et al., 2012). The molecular bases of plasmid establishment are poorly understood, and two models (1 and 2 that are represented in Figures 4A and 4B, respectively) are still under evaluation. Model 1 predicts that only a linear ssDNA multimer (*e.g.*, a dimer) in a head-to-tail configuration is internalized and DNA synthesis, as shown for conjugative transfer, generates the strand with the opposite polarity (Lacks, 1988, Saunders and Guild, 1981) (Figure 3C and 4A). Model 2 predicts that the DNA uptake machinery after internalizing one strand (Watson strand) of the plasmid molecule longer than the monomer length with low efficiency might shift strands and now the complementary strand (Crick strand) is internalized with subsequent degradation of the Watson strand, suggesting that the transforming DNA substrate should be at least a trimer, and that the uptake machinery provides the strands of opposite polarity (Canosi and Trautner, 1979, Dubnau et al., 1980, de Vos et al., 1981).

In both cases, the internalized ssDNA should be protected by the guardian proteins (RecN, SbcE, SsbB and perhaps SsbA) localized at the cell pole, as described previously (Table 2, Figure 4) (Kidane and Graumann, 2005, Kidane et al., 2009, Krishnamurthy et al., 2010, Tadesse and Graumann, 2007). The RecA mediators displace the guardians and recruit RecA onto the internalized plasmid ssDNA coated by SsbA or SsbB and RecA polymerizes to form a dynamic RecA-ssDNA filament (thread). If RecA fails to find a homologous region, it is disassembled from the internalized plasmid ssDNA by the action of RecU (Carrasco et al., 2005, Cañas et al., 2008) or any other RecA modulator (*e.g.*, perhaps RecX in concert with RecF, Table 2). This is consistent with the fact that RecU inhibits RecA-ssDNA filament dynamic extension in the absence of a recombination intermediate (Ayora et al., 2004, McGregor et al., 2005, Carrasco et al., 2005, Cañas et al., 2011). It was proposed that the RecA-ssDNA filaments exert an inhibitory effect in plasmid transformation and that the role of RecU is to reverse such inhibition (Cañas et al., 2008, Cañas et al., 2011). Indeed, the absence of RecA suppresses the RecU requirement in plasmid transformation, and RecU disassembles from the pole concomitantly with RecO accumulation (Cañas et al., 2008, Kidane et al., 2009).

In model 1, SsbA bound to the incoming lagging strand might recruit DnaE or PriA onto the primosome assembly site (*pas* site), then the replisome is recruited. The replisome primes DNA synthesis until the end of the template DNA, generating a tailed linear duplex with redundancy at both DNA ends (reviewed by Lacks, 1988). Then, SsbA-bound to the ssDNA tails recruits RecO onto SsbA- or SsbA and SsbB-coated ssDNA and such interaction overcomes the negative exerted by SsbB on RecO-mediated strand annealing reaction (Kidane et al., 2009, Manfredi et al., 2010, Yadav et al., 2012). RecO alone or in concert with a DNA helicase circularizes the duplex molecule by invasion and annealing of the terminal redundant ends (Figure 4A) (Lacks, 1988, Manfredi et al., Yadav et al., 2012). Unlike DprA_{Spn} (Mortier-Barriere et al., 2007), it was documented that DprA fails to catalyze strand annealing between two naked homologous DNA strands (Claverys et al., 2009), but our genetic data suggested that DprA is required for plasmid transformation (see Table 3). Since it is unknown whether DprA could promote annealing of complementary strands coated by SsbA or SsbB, we will only discuss the activity of RecO-mediated SSA in

plasmid transformation. In model 2, RecO catalyzes intramolecular annealing of the internalized complementary oligomeric molecules (Canosi and Trautner, 1979, Dubnau et al., 1980, de Vos et al., 1981) (Figure 4B), coated by SsbA and/or both SsbA and SsbB to render a linear tailed molecule, with complementary single strand tails and RecO-mediated strand annealing generates a nicked circular plasmid molecule (Manfredi et al., 2010, Yadav et al., 2012). Unlike chromosomal transformation where a D-loop resolvase will generate ligatable ends, if the DNA ends of the linear plasmid oligomer cannot be ligated they are potentially dependent of the NHEJ pathway as the ultimate rescuer to seal the DNA ends upon processing by Ku and LigD (Shuman and Glickman, 2007, Kidane et al., 2009). The circular plasmid molecule will be then replicated. Unlike in chromosomal transformation where the replisome is stationary and at mid cell, localization studies indicated that the plasmid replication machineries had extra foci of the replisome, and they often appeared to be clusters of plasmids located predominantly at or near a cell pole (Figure 4) (Wang et al., 2004). In a final step, the oligomeric plasmid molecules that replicate by the theta mode are converted into a monomer by site-specific recombinases (Ceglowski et al., 1993), and those plasmids that replicate by the rolling circle mode are converted into a monomer by the action of the initiator/terminator protein during DNA replication (Alonso et al., 1986, Novick et al., 1986).

The requirement of multimeric forms of plasmid DNA in transformation appears to reflect the mode of DNA processing in transformation of *B. subtilis* (Kidane et al., 2009). Indeed, when a donor plasmid molecule contains a directly repeated sequence, the pseudo-monomeric forms can be sufficient to produce plasmid transformants in *B. subtilis* competent cells (monomeric activation) (Figure 3D) (Michel et al., 1982). This is also a first order process and the number of transformants increases with the square of the length of the repeated region, but its transformation frequency decreases in the *recA* context (Michel et al., 1982). Most of the established plasmids, however, consist of a portion of the original plasmid, with only ~ 10% of the transformed cells bearing the initial donor plasmid DNA (Figure 3D) (Michel et al., 1982).

In certain bacterial species (e.g., *S. pneumoniae*) the monomeric plasmid molecules are active in transforming competent cells, but this process is mechanistically different from plasmid transformation (Stewart and Carlson, 1986, Lacks, 1988). Monomeric plasmid transformation resembles bacteriophage transformation (also termed viral transfection). Both process show a second order dependence on DNA concentration. Viral transfection requires both RecA- and RecO-dependent recombination avenues and the viral-encoded SSA protein for viral establishment and amplification (Ayora et al., 2002, Lo Piano et al., 2011, Kidane et al., 2009). The separate entry of at least two complementary linear monomeric plasmid molecules is necessary to transform *S. pneumoniae* competent cells (Saunders and Guild, 1981, Lacks, 1988). The host requirement is known because in the absence of RecA or DprA the incoming ssDNA is degraded (Berge et al., 2003).

In *S. pneumoniae* competent cells, transformation with native plasmid DNA (unfractionated) should follow two different pathways: at low DNA concentration plasmid transformation follows a first order kinetics (oligomeric ssDNA) and it is marginally affected by the absence of SsbB_{Spn}, whereas at high DNA concentration plasmid transformation follows a second order kinetics (monomeric ssDNA) and its transformation frequency is markedly enhanced in the Δ ssbB_{Spn} context (Attaiech et al., 2011), suggesting that SsbB_{Spn} preferentially antagonizes plasmid transformation.

Unlike in *S. pneumoniae*, various lines of evidence have indicated that the inability of plasmid monomers to transform otherwise wt *B. subtilis* competent cells. Since a DNA uptake machinery may assemble at each pole, albeit at relatively low frequency (see Hahn et

al., 2005), and there is no bias for strand assimilation, monomeric plasmid DNA should be active in transformation if both assembly apparatus are active to recombine the incoming ssDNA. Alternatively, the failure of plasmid monomers to transform *B. subtilis* competent cells might be a consequence of saturation of the uptake machinery (see above) rather than of a deficiency of the recombination machinery (Canosi et al., 1978, de Vos et al., 1981, Kidane et al., 2009).

Genetic recombination shares some steps with recombinational repair

In bacteria, the primary functions of HR are template-dependent recombinational DNA repair and programmed GR. Recombination-dependent DNA repair facilitates genomic surveillance and removal of harmful DNA damage in one (daughter strand gap repair) or both strands (DSB repair) in order to promote correct chromosomal segregation (Kowalczykowski et al., 1994, Kuzminov, 1999). In contrast, programmed GR, which is a unidirectional three-strand recombination reaction, provides genetic variation by integration of horizontally acquired ssDNA into a recipient genome (Thomas and Nielsen, 2005, Sanchez et al., 2007b). Part of the machinery required for GR (*e.g.*, DNA uptake) exclusively evolves for the taken up and processing of internalized ssDNA, but functions involved in recombinational repair play an essential role in natural transformation (Figure 5).

Vegetative growing *B. subtilis* cells contain several specific DNA repair pathways and the ultimate and general recombinational repair mechanism, that facilitates genetic material exchange between the damaged DNA and its homologous partner (Ayora et al., 2011). The presynaptic stage of DNA DSB repair consists of three basic steps (damage recognition, end processing and RecA-ssDNA filament formation) (Kidane et al., 2004). Among the first responders to a DSB is the RecN protein that recognizes and stabilizes the DNA ends within the first 15 – 20 min of DNA damage in cells grown in minimal medium and at 25° C (Figure 5) (Kidane et al., 2004, Ayora et al., 2011). After basal end processing, RecN tethers the 3'-ends perhaps by mediation of PNPase (Cardenas et al., 2009, Cardenas et al., 2011). The 5'-ends at the DSBs are resected by two different pathways (Figure 5) (Sanchez et al., 2006). In the first pathway, end processing and RecA loading might be coupled: the AddAB complex in concert with SsbA, upon interaction with the *chi* site, might facilitate loading of RecA onto naked ssDNA (Kowalczykowski et al., 1994, Yeeles et al., 2011). The RecBCD_{Eco} complex actively loads RecA_{Eco} onto the exposed naked ssDNA (Anderson et al., 1999, Dillingham and Kowalczykowski, 2008, Lucarelli et al., 2009), but the AddAB-dependent RecA loading avenue remains to be documented (Yeeles and Dillingham, 2010). In the second pathway, end processing and RecA loading are uncoupled. Here the RecJ ssDNA exonuclease in concert with a RecQ-like DNA helicase (RecQ or RecS) and SsbA may leave a duplex with an SsbA-coated 3'-tailed end (Figure 5) (Sanchez et al., 2006). A mediator (*e.g.*, RecOR) upon interaction with SsbA delivers RecA onto 3'-tailed ssDNA end coated by SsbA (Carrasco et al., 2008, Manfredi et al., 2008, Manfredi et al., 2010). Cytological data have shown that *B. subtilis* RecO, RecR, RecA co-localize with a damage-induced RecN focus 30 – 40 min upon DNA damage (Figure 5) (Kidane et al., 2004, Sanchez et al., 2006). Concomitantly with RecF, RecU and RecX assembly (60 min after damage), the RecA foci are converted to highly dynamic filamentous structures (threads) that cross the nucleoids (Figure 5) (Kidane et al., 2004, Kidane and Graumann, 2005, Sanchez et al., 2006, Cardenas et al., 2012). After RecA nucleation by any of the above pathways, RecA polymerizes on 3' ssDNA with concomitant displacement of SsbA from ssDNA (Figure 5) (Carrasco et al., 2008, Manfredi et al., 2008).

In both recombinational repair and GR, RecA-mediated DNA strand exchange involves the detection of the homologous sequence within a huge amount of heterologous sequences, and

extensive homologies are required for the reaction to be efficient (Lovett et al., 2002). Once a region of homology is located, RecA catalyzes DNA strand invasion to ensue that ssDNA forms base-pairs with its homologous strand within the duplex. During recombinational repair, the 3'-OH end of the invading strand is extended by the action of the replicase by copying the sequence information from the sister chromatid onto the broken DNA end. If this D-loop is disrupted after DNA synthesis from the first processed DNA end, by the action of an uncharacterized DNA helicase, the free end can anneal with the other resected end of the break, leading to non-cross-over (NCO) events and synthesis-dependent strand annealing (Figure 5). This process during GR should result in abortive chromosomal transformation, but the displaced ends can reconstitute a circular plasmid as described above for plasmid facilitation (Figure 3B).

The capture of the second processed end of the DNA break may occur via RecO and leads to formation of a double HJ (Ayora et al., 2011). These recombination intermediates are resolved by RecU in concert with the RuvAB translocase, which generates preferentially NCO products (Ayora et al., 2004), or might be dissolved by Topo III in concert with a RecQ-like helicase, ultimately to result in a faithfully restored chromosome and NCO products (Sanchez et al., 2007b). Unlike recombinational repair, single or double HJs, produced by second end capture, are not formed during GR and the D-loops are resolved by an uncharacterized enzyme(s).

Why do bacteria take up DNA from microenvironments?

The possible answer for this question lies in four hypotheses (Engelmoer and Rozen, 2011, Michod et al., 2008, Vos, 2009):

(i) Nutrient source

Bacteria inhabit a wide variety of niches including mammalian hosts (Matthews et al., 1963) and within many of these environments the extracellular DNA concentration is estimated to be in the range of 0.2 to 44 µg/liter (Lorenz and Wackernagel, 1994). With such an abundance of DNA available in the environment, it is quite reasonable to think that bacteria would take advantage of this rich nutrient source, and that the dsDNA uptake machinery has been developed as a means of acquisition of nutrients (Redfield, 1993, Stewart and Carlson, 1986). In addition, some studies have presented evidence that mutant bacteria that were unable to consume DNA as a sole source of carbon and energy suffered a significant loss of fitness during stationary-phase competition (Finkel and Kolter, 2001, Palchevskiy and Finkel, 2006). If so, do bacteria have a selective mechanism to make the decision to use DNA as nutrient source or for gaining genetic information? Since the taken up ssDNA is dependent on the USS or DUS sequence in some species, and bacteria of the Firmicutes Phylum activates transcription of > 150 genes with only ~ 13 % of those encode proteins whose products are required for the DNA uptake machinery that only internalize ssDNA, we do not favor the idea that this expensive uptake system is to provide DNA as source of carbon and energy.

(ii) DNA uptake for acquisition of a DNA repair template

Early reports have presented the view that GR, and also sexual reproduction originated as a recombinational repair process, and the preferred reason for DNA uptake in competent bacteria is to acquire templates for DNA repair rather than the generation of evolutionary novelty (Maynard Smith, 1978, Bernstein et al., 1987, Hoelzer and Michod, 1991). It was proposed that *B. subtilis* competent cells exposed to UV-light show increased cellular survival through transformation-mediated recombination (Michod et al., 1988). However, non-competent growth-arrested cells were also better protected from DNA damaging agents

than their exponentially growing siblings (Carrasco et al., 2004). The small fraction of taken up genomic DNA (< 0.4%) should be unable to replace the damaged ssDNA and by this way to increase survival of cells during UV-exposure competence cells. Furthermore, if transformation has been adopted to repair DNA, it should be expected that UV-light or mitomycin C induces transformation. This was not observed in either *H. influenzae* or *B. subtilis*. However, in *S. pneumoniae* and *H. pylori* cells competence development is induced by DNA damage or by inhibition of protein synthesis (see above). It is likely that in these bacteria DNA uptake might correlate with DNA repair (Redfield, 2001, Claverys et al., 2006, Engelmoer and Rozen, 2011).

(iii) DNA uptake for evolution

GR between species is of major importance for prokaryotic evolution (Fraser et al., 2007, Donati et al., 2010, Didelot and Maiden, 2010). The main evolutionary advantage and disadvantage of acquiring DNA lies on the genetic variation generated by recombination of a given gene with the host genome (Fraser et al., 2007, Donati et al., 2010, Didelot and Maiden, 2010). When bacteria are subjected to an environmental change that challenges their fitness, there are three survival strategies: i) decreasing the efficiency of error-free repair for the acquisition of novel genetic traits (mutations), ii) modulation of phenotype in response to specific environmental cues and, iii) stochastic phenotype switching by noisy molecular fluctuations (Ackermann et al., 2008, Moxon et al., 1994). In the latter two scenarios, DNA released by non-competent sibling cells could be used to increase genomic plasticity and metabolic flexibility in order to increase the adaptive capability of a microbial community leading to species with non-clonal population structures (Feil et al., 1999, Fraser et al., 2007, Donati et al., 2010, Mell et al., 2011). For many bacterial species it is clear that the acquired genes play a prominent role in adaptive evolution as a source of genetic variation, eventually leading to habitat-and-niche expansion of up to ~ 8% of the genome (Thomas and Nielsen, 2005, Johnsen et al., 2009, Croucher et al., 2011, Donati et al., 2010, Hiller et al., 2010). Many microorganisms become competent to survive the selection pressure and to increase fitness. For example, under certain environmental conditions, 10 – 20% of a *B. subtilis* population becomes competent (Dubnau, 1999). If the acquired genes cause deleterious effects to the recipient bacterium, then these bacteria will lose fitness and will be lost from the population over time. However, if the acquired DNA confers a selective advantage to the host (*e.g.*, antibiotic resistance), this lineage has the potential to spread rapidly within a bacterial population.

(iv) Competence as stress management

The master competence transcription factor activates its own transcription as well as the transcription of > 150 genes, however only a fraction of those genes (~ 30 genes) encode proteins whose products are required for GR (Berka et al., 2002, Hamoen et al., 2002, Ogura et al., 2002, Peterson et al., 2004). Differentiation into a certain physiological state (*e.g.*, competence) depends on the input of multiple genetic pathways and extracellular signal molecules affecting the bacterial heterogeneity, suggesting that competence is a broader process required to relieve stress that can be separated from GR (Prudhomme et al., 2006, Engelmoer and Rozen, 2011, Shank and Kolter, 2011).

Conclusions

Based on the recent progress on the knowledge of bacterial genetics specifically in DNA transformation and DNA repair, a successful transformation event requires several steps: (i) competence development, (ii) DNA uptake and processing that includes DNA binding, translocation and processing, so that ssDNA is taken up into the cytosol, and (iii) the GR process, which integrates the incoming ssDNA into a recipient genome (chromosomal

transformation) or establishes an autonomous replicating element (plasmid and viral transformation). A set of competence (SsbA, SsbB) and DNA repair (RecN, SbcE) guardian proteins protect the incoming ssDNA. Competence (DprA) and DNA repair (RecO, perhaps in concert with RecN and RecR) specific mediators can displace the SsbA and SsbB guardians and recruit the RecA onto SsbA- or SsbB-coated ssDNA (visualized as foci). RecA polymerization (threads) onto ssDNA might be modulated by RecU, RecF, and perhaps RecX. The RecA-ssDNA filament (threads) recognizes its target and catalyzes recombination with the homologous host genome. An uncharacterized resolver cleave(s) the D-loop and a DNA ligase seals the DNA ends. If the incoming oligomeric ssDNA molecule does not share homology with the recipient, but it contains an autonomous replicating region it can be established as an episome (plasmid transformation). Here the guardians protect the internalized oligomeric ssDNA and recruits the mediators. RecO or DprA might favor RecA-mediated recombination, but in the absence of homology in a recipient supercoiled molecule (e.g. the chromosome) the modulators may promote RecA disassembly and SsbA and/or SsbB may re-coat the ssDNA. In a second try, RecO and/or DprA promote strand annealing of linear molecules and re-circularization of the tailed duplex molecule using its terminal redundancy. If both chromosomal or plasmid transformation fails, a set of rescuer proteins (SbcCD, AddAB, RecJ in concert with RecQ [RecS or RecQ], RecF or RecH) will fix any putative DNA damage or correct the ends if the internalized ssDNA is improperly processed.

During the last few years remarkable progresses have been made in our understanding of the molecular mechanism of DNA uptake during bacterial competence. Nevertheless, many questions about the molecular mechanism of GR remain unanswered. Does the bacterial DNA uptake process create a signaling pathway to recruit appropriate DNA repair mediator proteins to the uptake site based on the type of DNA? Is there any checkpoint activation to coordinate the DNA uptake and GR proteins at the cell pole? The future will unravel this mysterious puzzle in bacterial genetics.

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Figure 1. Protein-protein interaction network of DNA repair and competence-induced proteins in *B. subtilis*

he proteins that participate in recombinational repair (light gray/yellow background) and genetic recombination (dark gray/light-green background) and that are required for transformation are shown. The proteins that participate in both processes are represented at the interphase between them. ComGA and ComFA, which reside in the inner face of the uptake apparatus and interact with cytosolic competence-induced proteins (RecA, DprA, SsbB, and CoiA), are used as representatives of the DNA uptake machinery. The solid arrows show the interaction between two proteins proven either by biochemical or biophysical experiments. The dashed arrows show the suggested biochemical interaction.

Figure 2. Model of coordinated action of DNA uptake and DNA repair proteins assembled at the cell pole during competence when homology with the chromosome is present

At the competent state, specialized proteins assemble into a DNA uptake and recombination complexes at the cell pole, RecN (or SbcE) oscillates from pole to pole and whereas DNA PolC localizes at the nucleoid. The model for DNA uptake, chromosomal transformation and DNA assimilation into recipient duplex in Firmicutes (e.g., *B. subtilis*, [A]) and in Proteobacteria (e.g., *N. gonorrhoeae* [B] and *H. pylori* [C]) can be divided in discrete steps: The first two steps did not apply to Firmicutes competent cells. Step 3, dsDNA binds to the cell surface and becomes processed to ssDNA by an endonuclease, the ssDNA is internalized into the cytosol via ComEC channel using a proton motive force, driven by the ComFA ATPase, and RecN loses its dynamic movement. Step 4, the guardians RecN, SbcE, SsbB (and perhaps SsbA) protect the incoming DNA from nuclease attack. Step 5, DprA (RecO) and RecA contribute to the formation of an active RecA-ssDNA filament. The RecA-ssDNA filament (thread), perhaps modulated by RecU, RecX and RecF, promotes homology search to recombine the incoming ssDNA with bacterial genome. In (B), a model for chromosomal transformation in Proteobacteria that are required DNA uptake sequence (DUS) (e.g., *N. gonorrhoeae*). In (C), a model for chromosomal transformation in Proteobacteria (e.g., *H. pylori*) is presented. In Both B and C models DNA uptake and DNA assimilation into recipient duplex can be divided in five different steps. Step 1, specialized proteins, which assemble into a DNA transport complex on cell surface, bind dsDNA bearing a DUS site (B) or dsDNA of any source (C). Step 2, dsDNA is taken up to the periplasmic region. Step 3, dsDNA binds to the inner membrane surface and becomes processed to ssDNA by an endonuclease and enters through a ComEC-like channel in the cytoplasmic membrane to reach the cytosol. The role of RecN is unknown in these bacteria. Step 4, guardians proteins protect the incoming DNA from nuclease attack ssDNA. Step 5, RecA mediators might contribute to RecA-ssDNA filament formation and accessory proteins contribute to RecA-mediated homology search to recombine the incoming ssDNA with bacterial genome.

Figure 3. DNA substrates processed by different avenues during natural transformation

(A) Chromosomal transformation. A linear ssDNA of bacterial or plasmid origin that displaces the homologous strand in the duplex of chromosomal or recipient plasmid (gray/red line) DNA pairs with the complementary strand (black line). The formed D-loop structure is resolved as indicated by white triangles, and the displaced DNA strand degraded. The ends are sealed and one of the daughter cell inherits the donor DNA. (B) Plasmid facilitation. The ends of a linear ssDNA of plasmid origin show homology to the duplex resident and pair with the complementary strand. The invaded strand then primes DNA synthesis using the intact homologous chromosome as a template, the ends are sealed and an unidentified DNA helicase might disassemble the paired molecule. DNA synthesis of the complementary strand, initiated at the primosome assemble site (*pas*, dark-gray/light blue), renders a circular duplex molecule. (C) Plasmid transformation. An oligomeric ssDNA plasmid molecule (dotted line), two - three times the unit-length plasmid genome (indicated by vertical bars), either is used as template to synthesize the complementary strand, (DNA synthesis initiated at the *pas* site, model 1, see below) or anneals to the also imported complementary and oligomeric strand (model 2). Then, the linear duplex by a RecA-independent recombination event, anneals at the redundant complementary regions to generate a circular oligomeric intermediate that is then converted into a unit-length monomer. (D) Monomeric activation. A plasmid molecule carrying direct internal repeats (1.5 – 2-kb long) by a poorly understood mechanism produce, by recombination between repeats (partial RecA-dependency), circular viable plasmids, either with similar to the input DNA or deleted plasmid variants.

Figure 4. Differential protein localization at the cell pole during plasmid transformation
 Models 1 (A) and 2 (B) of plasmid DNA transformation in *B. subtilis*. Chromosomal and plasmid transformations share the first steps of DNA uptake (see Figure 2), but in the presence of DNA with self-annealing potential RecO also localizes at the cell pole. The guardians (SsbA, SsbB, RecN and SbcE) protect the incoming oligomeric ssDNA from nuclease attack, the mediator (RecO or DprA) loads RecA onto ssDNA, the modulator (RecU, and perhaps RecX and RecF) inactivates RecA-ssDNA filament formation, and SsbA and/or SsbB re-coated the ssDNA (steps common for both models). In (A), only one strand enters and replication, which is initiated at the *pas* site (see Figure 3), generates a partial duplex DNA with ssDNA tails. RecO (or DprA) catalyzes annealing of the terminal redundant ends generating a gapped circular plasmid molecule. In (B), first enters the Watson strand and then the Crick strand of the same oligomeric plasmid particle. RecO (or DprA) catalyzes annealing of the complementary strands, generating a tailed duplex DNA with terminal redundancy. Then, RecO (or DprA) catalyzes a second annealing reaction of the complementary ssDNA tails coated by SsbA or SsbA and SsbB so that the plasmid is circularized. In both cases DNA replication reconstitutes the molecule and allows plasmid establishment. However, in the second model no extensive DNA replication is required.

Figure 5. Temporal order of DNA repairs proteins recruitment during DNA double strand break

Three of the structural maintenance of chromosomes family of proteins (e.g., SMC, SbcC and SbcE) might contribute to chromosome cohesion and/or condensation during the entire cell cycle, whereas the fourth, RecN, binds and protects the tails of the broken DNA ends upon DNA damage (Sanchez and Alonso, 2005, Sanchez et al., 2008). After basal resection by PNPase (among others) (Cardenas et al., 2009, Cardenas et al., 2011) long-range end processing takes place (Sanchez et al., 2007a, Sanchez et al., 2006). The 5' ends are resected either by the nuclease-helicase (AddAB/RecBCD/AdnAB) complex or by the RecJ ssDNA exonuclease, in concert with a RecQ-like (RecQ and/or RecS) in association with SsbA/SSB to generate the 3' tailed DNA that would help for the polymerization of RecA (Sanchez et al., 2006). After basal and long-range end resection RecN tethers the DNA ends leading to a discrete RC (15 – 20 min). RecO, RecR and RecA co-localize with RecN 15 to 30 min later (30 – 50 min) (Kidane et al., 2004, Sanchez et al., 2006). At a later stage, RecF (min 60) is recruited to the RecN-mediated repair centre (Kidane et al., 2004). RecX foci start to accumulate min 60 after DNA damage (Cardenas et al., 2012). The recruitment of RecU to a recombination intermediate, in the presence of RuvAB, is visualized at min 100 – 110 after DNA damage (Sanchez et al., 2005). Growth resumption in minimal medium at 25 °C takes place ~ 180 min upon DNA damage in wt cell (Mascarenhas et al., 2006).

Table 1

Common DNA repair and recombination genes that participate in bacterial competence.

Gene Name	Function and phenotype ^a	ComK regulated ^b	Bacterial species ^c	References
<i>ssbA</i> ^d	Up regulated (~ 8-times), binds ssDNA. Essential gene.	Yes	<i>Bsu, Spn</i>	(Berge et al., 2003, Peterson et al., 2000)
<i>ssbB</i>	Up regulated (~ 14-times), binds ssDNA. Decreased DNA transformation.	Yes	<i>Bsu, Spn</i>	(Berka et al., 2002, Ogura et al., 2002, Peterson et al., 2000)
<i>dprA</i>	Up regulated (~ 12-times), binds ssDNA. Decreased DNA transformation.	Yes	<i>Bsu, Spn, Hin, Ngo</i>	(Berge et al., 2003, Ogura et al., 2002, Tadesse and Graumann, 2007)
<i>topA</i>	Up regulated (~ 2-times). Essential gene, operon with <i>dprA</i> . DNA Topoisomerase I.	Yes	<i>Bsu, Hin</i>	(Chandler and Smith, 1996, Tadesse and Graumann, 2006)
<i>coiA</i>	Up regulated (~ 6-times). Decreased DNA transformation. Activity unknown.	Yes	<i>Bsu, Spn Ngo, Pst</i>	(Berka et al., 2002, Ogura et al., 2002, Peterson et al., 2000)
<i>recA</i>	Up regulated (~ 6-times), recombinase. Blocked in chromosomal transformation.	Yes	<i>Bsu, Spn</i>	(Kidane and Graumann, 2005, Kidane et al., 2004)
<i>radC</i>	Up regulated (~ 10-times). No defect in chromosomal transformation. Activity unknown.	Yes	<i>Bsu, Spn, Sgo, Hin</i>	(Berka et al., 2002, Ogura et al., 2002, Peterson et al., 2000, Redfield et al., 2005, Attaiech et al., 2008)
<i>Sms (radA)</i>	Induced in <i>Spn</i> . defective in DNA repair and chromosomal transformation. Activity unknown.	No	<i>Bsu, Spn</i>	(Carrasco et al., 2002, Peterson et al., 2000)
<i>cinA</i> ^d	Induced in <i>Spn</i> . CinA is a nucleoid-associated protein that has a minor role during DNA transformation.	No	<i>Bsu, Spn</i>	(Kaimer and Graumann, 2010, Peterson et al., 2000, Masure et al., 1998)
<i>addAB</i> (β) ^e	Indirectly ComA-induced. Helicase-nuclease, DSB repair and recombination.	No	<i>Bsu, Ngo</i>	(Amundsen et al., 2008, Sanchez et al., 2006)
<i>sbcE</i> (κ)	Indirectly ComA-induced. Affected in chromosomal transformation. Sensitive to MMC.	No	<i>Bsu</i>	(Krishnamurthy et al., 2010)
<i>recO</i> (α)	Essential for transformation in $\Delta dprA$ cells. Very deficient in DNA repair. RecA mediator and DNA annealing.	No	<i>Bsu</i>	(Kidane et al., 2009)
<i>recF</i> (α)	Essential for transformation in the absence of RecH. Very deficient in DNA repair. Binds ss and dsDNA.	No	<i>Bsu</i>	(Kidane et al., 2009, Kidane et al., 2004)
<i>recH</i> (γ)	Impairs DNA transformation. Enforces sexual isolation. Moderately deficient in DNA repair.	No	<i>Bsu</i>	(Alonso et al., 1993, Zawadzki et al., 1995)
<i>recN</i> (δ)	Sensitive to DSBs, binds ssDNA. Decreased DNA transformation in the absence of SbcE.	No	<i>Bsu, Hin</i>	(Kidane et al., 2004, Skaar et al., 2002, Wang and Maier, 2008)
<i>recU</i> (ϵ)	Impairs plasmid transformation. Deficient in DNA repair. HJ resolvase and RecA modulator.	No	<i>Bsu</i>	(Kidane et al., 2009)
<i>recG</i> (η)	Branch migration translocase, DSB repair. Chromosomal segregation defect. Very deficient in DNA repair.	No	<i>Bsu, Spn</i>	(Sanchez et al., 2007c, Martin et al., 1996)
<i>recX</i> (θ)	Moderate sensitivity to DNA damaging agents. Impairs chromosomal transformation. RecA modulator.	No	<i>Bsu, Ngo</i>	(Stohl and Seifert, 2001 our unpublished results)
<i>mutS</i>	Mismatch repair recognition, deficient in GR	No	<i>Pst</i>	(Meier and Wackernagel, 2005)

Gene Name	Function and phenotype ^a	ComK regulated ^b	Bacterial species ^c	References
<i>ku</i>	Impairs plasmid transformation and DNA repair in the absence of transient diploidy. Binds DNA ends.	No	<i>Bsu</i>	(Kidane et al., 2009)
<i>priA</i>	loader of the replicative helicase	No	<i>Ngo</i>	(Kline and Seifert, 2005)

^aFunction and/or biochemical activity associated with a given gene product.

^bInduced gene in *B. subtilis* (*Bsu*) competence.

^cBacterial species in which the given function was characterized.

^dThe function is induced in *Bsu* and is not in *S. pneumoniae* (*Spn*) or *vice versa*.

^eThe *rec* genes, others than *recA*, are classified into seven different epistatic groups (α to κ) denoted between parenthesis. Abbreviations: *Sgo*, *S. gordonii*; *Ngo*, *N. gonorrhoeae*; *Hin*, *H. influenzae*; *Hpy*, *H. pylori*; *Pst*, *P. stutzeri*.

Table 2

DNA repair proteins function at different stage of DNA uptake.

Role	Name of proteins	Function
Guardians ^a	SsbB, SsbA RecN, SbcE, (DprA) ^b , (RecA) ^b	Bind ssDNA
Mediators ^c	RecO, DprA	Bind ssDNA and overcome SSB inhibition of RecA loading.
Recombinase ^d	RecA	Binds ssDNA and dsDNA, recombines homologous DNAs
Modulators ^e	RecF, RecU, Sms	Bind ssDNA and dsDNA and modulate RecA activities.
Resolver(s)	NN	Uncharacterized
Rescuers ^f	AddAB, RecJ-RecQ(S), RecFOR, RecH	DNA processing, DNA binding, RecA modulation
unknown	CoiA, Ku	Uncharacterized

^a (Kidane et al., 2009, Kidane and Graumann, 2005, Krishnamurthy et al., 2010, Morrison et al., 2007, Mortier-Barriere et al., 2007, Tadesse and Graumann, 2007, Kramer et al., 2007).

^b See text, DprA_{Spn} and RecA_{Spn} also bind dsDNA and are required to protect ssDNA from degradation (Berge et al., 2003, Mortier-Barriere et al., 2007).

^c (Kidane et al., 2009, Manfredi et al., 2010, Morrison et al., 2007, Mortier-Barriere et al., 2007, Tadesse and Graumann, 2007, Beernink and Morrical, 1999, Cox, 2007a).

^d (Cox, 2007b, Galletto and Kowalczykowski, 2007, Kidane and Graumann, 2005).

^e (Drees et al., 2004a, Drees et al., 2004b, Gruenig et al., 2006, Stohl et al., 2003, Cañas et al., 2008, Carrasco et al., 2005, Kidane et al., 2009).

^f (Alonso et al., 1993, Cox, 2007b, Cox, 2007a, Kidane et al., 2009, Kidane and Graumann, 2005).

Table 3

Efficiency of natural chromosomal and plasmid transformation in *B. subtilis* and *S. pneumoniae* competent cells

Relevant Genotype (epistatic group)	Chromosomal transformation		Plasmid transformation	
	<i>Bsu</i> ^a	<i>Spn</i> ^b	<i>Bsu</i> ^a	<i>Spn</i> ^b
<i>rec</i> ⁺	100	100	100	100
$\Delta ssbB^c$	10 – 20 ^d	27	ND	ND
$\Delta coiA^c$	10 – 20 ^d	1.7 – 7	ND	ND
$\Delta dprA^c$	1 – 10 ^d	< 0.01	2	< 0.01
$\Delta recA^c$	< 0.01	< 0.01	98	< 0.01
$\Delta recO(\alpha)^e$	56	ND	2.9	ND
<i>recF15</i> or $\Delta recR^f(\alpha)$	74	ND	86	ND
<i>addA5</i> (β)	58	46	74	85
$\Delta recN(\delta)$	72	98	55	ND
<i>recH342</i> (γ)	61	ND	90	ND
$\Delta recU(e)$	53	ND	3.0	ND
$\Delta recU \Delta rusA$	57	ND	2.9	ND
$\Delta recU(e) \Delta recA$	< 0.01	ND	44	ND
$\Delta ruvAB(e)$	69	ND	23	ND
$\Delta recJ(\zeta)$	77	ND	88	ND
$\Delta recG(\eta)$	47	4	ND	ND
$\Delta recX(\theta)$	0.5	ND	1.8	ND
$\Delta sbcE(\kappa)$	5	NA	ND	NA
Δsms	10	1	77	ND
Δku	56	ND	17	ND
<i>recF15 addA5</i>	< 0.1	ND	< 0.1	ND
$\Delta recN \Delta sbcE$	1.2	ND	ND	ND
$\Delta recO \Delta dprA$	< 0.1	ND	< 0.1	ND

^aThe yield of *B. subtilis* (*Bsu*) chromosomal (*met*⁺, with SB19 DNA) or plasmid (Km^R, pUB110 plasmid DNA) transformants per 0.1 μ g DNA/ml was corrected for DNA uptake and cell viability, and the values obtained were normalized relative to those of the *rec*⁺ strain, which were taken as 100 (Alonso et al., 1993, Carrasco et al., 2002, Cañas et al., 2008, Fernández et al., 2000, Kidane et al., 2009).

^bThe yield of *S. pneumoniae* (*Spn*) chromosomal or plasmid transformants derived from different groups using different experimental conditions (see Berge et al., 2003, Halpern et al., 2004, Desai and Morrison, 2006, Burghout et al., 2007, Morrison et al., 2007, Martin et al., 1996, Mortier-Barriere et al., 2007).

^cGenes induced by competence both in *Bsu* and *Spn* strains.

^dThe yields of *B. subtilis* chromosomal and plasmid transformants are derived from different groups using different experimental conditions (see Berka et al., 2002, Hamoen et al., 2002, Krishnamurthy et al., 2010, Ogura et al., 2002).

^eIn parentheses are denoted the epistatic groups of the different *rec*⁺ strains other than *recA*.

^fSimilar results were reported for *recF15* and $\Delta recR$ strains. ND, not determined; NA, not applied.