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Complex polar machinery required for proper chromosome segregation in vegetative and sporulating cells of *Bacillus subtilis*

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Summary

Chromosome segregation is an essential process of cell multiplication. In prokaryotes, segregation starts with the newly replicated sister origins of replication, oriCs, which move apart to defined positions in the cell. We have developed a genetic screen to identify mutants defective in placement of oriC during spore development in the Gram-positive bacterium *Bacillus subtilis*. In addition to the previously identified proteins Soj and DivIVA, our screen identified several new factors involved in polar recruitment of oriC: a reported regulator of competence ComN, and the regulators of division site selection MinD and MinJ. Previous work implicated Soj as an important regulator of oriC positioning in the cell. Our results suggest a model in which the DivIVA-interacting proteins ComN and MinJ recruit MinD to the cell pole, and that these proteins work upstream of Soj to enable oriC placement. We show that these proteins form a polar complex, which acts in parallel with but distinct from the sporulation-specific RacA pathway of oriC placement, and also functions during vegetative growth. Our study further shows that MinD has two distinct cell cycle roles, in cell division and chromosome segregation, and highlights that cell probably use multiple parallel mechanisms to ensure accurate chromosome segregation.

Introduction

Chromosome segregation is one of the most fundamental processes in biology. However, details of the mechanisms responsible for accurately configuring and segregating bacterial chromosomes remain poorly resolved. Spore formation in the Gram-positive bacterium *Bacillus subtilis* offers a particularly tractable system for studying chromosome organization and segregation (Errington, 2010; Possoz et al., 2012). Typical of the bacteria, *B. subtilis* has a single circular chromosome and a single fixed origin of replication (oriC). Bidirectional replication forks emanate from oriC and meet at the terminus, terC. The cells are haploid and replication is regulated so as to occur once per cell cycle, in parallel with cell growth (Toro and Shapiro, 2010).

Endospore formation (hereafter, ‘sporulation’) is a complex adaptive response to starvation that is characteristic of the Firmicutes group of bacteria, largely those of the genera *Bacillus* and *Clostridium* (Al-Hinai et al., 2015). During sporulation, the cell cycle is modified such that a cell with two and only two completely replicated chromosomes is generated prior to septation (Burkholder et al., 2001; Veening et al., 2009). Morphologically, it begins with a conformational change of the nucleoid to form an elongated structure called the axial filament (Hilbert and Piggot, 2004), followed by an asymmetric cell division, which divides the sporulating cell into two unequally sized cell compartments: the small prespore or forespore, which develops into a mature, dormant endospore and the larger mother cell, which lyses after contributing its resources to the development of the spore (Errington, 2010; Tan and Ramamurthi, 2014). The axial filament extends from pole to pole, with the two oriC regions of the sister chromosomes associated with opposite cell poles (Ben-Yehuda et al., 2003; Wu and Errington, 2003) and the two terC regions located close to each other.
at about mid cell (Fig. 1A). Chromosomal loci between these sites are arranged in a linear order between poles and mid cell, reflecting their relative positions on the chromosome ‘left’ and ‘right’ arms (Webb et al., 1997; Teleman et al., 1998; Wu and Errington, 1998; Wang et al., 2014). The sporulation division septum, which is located very close to one of the cell poles, closes around the axial filament in such a way that initially only about 30% of the chromosome destined for the spore is located inside the small prespore compartment, while the mother cell houses the remaining 70% of this chromosome, together with the second chromosome that belongs to the mother cell (Fig. 1A) (Wu and Errington, 1994; Wu et al., 1995; Wu and Errington, 1997). A widely conserved protein, SpoIIIE (FtsK) (Sherratt et al., 2010), then translocates the remaining segment of the chromosome into the prespore compartment to complete the segregation process (Wu and Errington, 1994; Bath et al., 2000). The prespore and the mother cell subsequently undergo a complex differentiation process that is initiated by activation of a specialized RNA polymerase sigma subunit, σF, in the prespore compartment (Fig. 1A) (Haldenwang, 1995; Londoño-Vallejo et al., 1997; Iber et al., 2006). While the prespore and the mother cell ultimately each receive one intact chromosome, the transient genetic asymmetry that exists prior to the completion of chromosome translocation is crucial for the activation of σF in the prespore and the establishment of compartment-specific programmes of transcription (Fransden et al., 1999).

The DNA translocase SpoIIIE is located at the leading edge of the asymmetric septum (Wu and Errington, 1997; Fleming et al., 2010; Fiche et al., 2013) (Fig. 1A). Certain mutations in the spoIIIE gene abolish DNA translocation activity but enable assembly of a stable complex with the DNA enclosed by the constricting septum (Wu and Errington, 1997). Although in these cells the chromosomes are ‘frozen’ in an asymmetric state, with 70% of the prespore chromosome stuck in the mother cell, σF is correctly activated in the small prespore compartment and it can turn on σF-dependent genes if those genes are on the segment of DNA that locates inside the prespore (Wu and Errington, 1994; Wu et al., 1995; Wu and Errington, 1997). A ‘spoIIIE trapping assay’ based on σF-dependent reporters in a transfer-inactive spoIIIE mutant has been used extensively to probe the segment of DNA initially trapped in the prespore compartment, as well as the factors required for chromosome orientation and

![Image of compartmentalization during sporulation](https://example.com/compartmentalization.png)

**Fig. 1.** Organization of the oriC region and design of the genetic screen for mutants with an ‘ORI zone out’ phenotype.

A. Schematic illustration of the compartmentalization that occurs during the early stages of sporulation, where asymmetric division leads to the generation of a small prespore containing only one third of a chromosome, and a larger mother cell. σF drives expression of the spoIIQ (PspoIIQ) gene exclusively in the prespore compartment. Expression of the lacZ reporter is induced by σF but this is overruled by repression by LacI via its operator lacO, when expressed in the prespore compartment from the PspoIIQ. SpoIIIE(frozen) (spoIIIE36) indicates the mutant form of SpoIIIE that does not allow DNA translocation into the prespore compartment.

B. Diagram of the oriC region of the chromosome (horizontal bar) that is trapped in the prespore during the initial stage of spore formation, divided into the left and right ARM zones and the ORI zone according to the results of our prespore chromosome trapping assays. Regions enriched in RacA binding sites (ram, green) and Spo0J binding sites (parS, purple) are indicated above. Colour intensity roughly corresponds to the distribution with which the respective motifs occur in the DNA. Vertical arrows indicate the positions of the various genetic constructs used in the screen and for the prespore-specific fluorescent reporter assay.
configuration in the early stages of sporulation (Wu and Errington, 1998; Wu and Errington, 2002). It is now known that the DNA segment that is already in the prespore compartment when the asymmetric septum forms centres slightly to the left of oriC (Fig. 1B) (Wu and Errington, 1998; Wu and Errington, 2002), and that many factors are involved in ensuring correct chromosome configuration and establishing the interaction between the DNA segment and the cell pole, including sporulation-specific RacA (Ben-Yehuda et al., 2003; Wu and Errington, 2003), and vegetatively expressed DivIVA (Thomaides et al., 2001; Perry and Edwards, 2004). Spo0J (ParB) (Sharpe and Errington, 1996; Wu and Errington, 2002) and Soj (ParA) (Wu and Errington, 2002; Sullivan et al., 2009).

DivIVA is a conserved protein that is involved in a wide range of processes associated with cell pole development in Gram-positive bacteria (Edwards and Errington, 1997; Lenarcic et al., 2009). In B. subtilis, DivIVA is the topological determinant of the Min system involved in division site placement during vegetative growth (Edwards and Errington, 1997; Marston et al., 1998), and in sporulation it acts as an important factor in polar chromosome attachment (Thomaides et al., 2001; Perry and Edwards, 2004; Perry and Edwards, 2006). The two functions are separable (Thomaides et al., 2001) and, in a divIVA mutant that specifically affects chromosome segregation, the prespore chromosome is trapped in an unusual configuration in which the oriC region ('ORI zone'; perhaps 200 kbp or so of the oriC-proximal DNA, Fig. 1A and B) lies outside the prespore compartment, while flanking regions either side of the ORI zone (left and right ARM zones; Fig. 1B) are correctly placed (Wu and Errington, 2003). Exclusion of the ORI zone from the prespore has also been reported for the soj null mutant, though the defect is much milder (Sullivan et al., 2009). Finally, RacA was identified as a sporulation-specific factor required for accurate prespore chromosome segregation (Ben-Yehuda et al., 2003; Wu and Errington, 2003; Ben-Yehuda et al., 2005). It is recruited to the cell poles by DivIVA and it binds to multiple RacA-binding (ram) sites that are located mainly in a 250 kbp region to the left of oriC (Fig. 1B). Surprisingly, although loss of RacA also resulted in a defect in chromosome segregation, the phenotype was different from that of the divIVA mutant: about half of the prespores failed to capture any DNA, and the other half of the cells had the correct oriC segment of the chromosome (Ben-Yehuda et al., 2003; Wu and Errington, 2003).

The soj (parA) and spo0J (parB) genes encode well known, widely conserved factors originally identified through their roles in the stable maintenance of low copy number plasmids (Møller-Jensen et al., 2000; Surtees and Funnell, 2003; Mierzejewska and Jagura-Burdzyn, 2012). They are now known to be present in most bacterial genomes (with the curious exception of E. coli and close relatives), and thought likewise to be involved in chromosome segregation. Mutations in soj and/or spo0J affect proper capturing of the prespore chromosome in the spoIIIE trapping assay (Sharpe and Errington, 1996; Wu and Errington, 2003; Sullivan et al., 2009). During vegetative growth, these proteins not only are required for proper chromosome segregation (Lee et al., 2003; Lee and Grossman, 2006; Gruber and Errington, 2009), but also contribute to regulation of the initiation of DNA replication and, indirectly, to the initiation of sporulation (Murray and Errington, 2008; Veening et al., 2009). Spo0J is a site-specific DNA binding protein that binds to several parS sites, located mainly around the oriC region (Fig. 1B) (Breier and Grossman, 2007). It spreads from primary binding sites by a mechanism that probably involves direct lateral protein–protein interactions as well as bridging or looping (Murray et al., 2006; Graham et al., 2014). ParB-parS complexes can recruit bacterial condensin (ScpAB-SMC), which is important for chromosome organisation and segregation, at least in vegetative cells (Gruber and Errington, 2009; Sullivan et al., 2009; Gruber et al., 2014). Soj is a dimeric ATPase that in some organisms has been shown to participate in active movement of parS-oriC complexes (Ptacin et al., 2010; Schofield et al., 2010; Lim et al., 2014). Its ATPase activity can be stimulated by a short N-terminal peptide of Spo0J, and different forms (ADP or ATP-bound, monomeric or dimeric) of B. subtilis Soj can interact with Spo0J-DNA (Schofield et al., 2011) and the DNA replication initiation protein DnaA (Murray and Errington, 2008). Perturbation of this latter interaction can lead to a defect in the initiation of sporulation, which resulted in the original naming of spo0J (Ireton et al., 1994; Veening et al., 2009).

To dissect further the mechanism of prespore chromosome segregation, we developed a screen for mutations that generate a divIVA-like ‘ORI zone out’ phenotype (9). Characterization of a large collection of transposon mutants with the required phenotype identified two genes, minD and comN, that have other, unrelated functions in vegetative growth, and defined a multicomponent polarly-located machinery required for correct placement of the oriC region in B. subtilis during spore development as well as in vegetative cells.

**Results**

A screen for mutations affecting oriC to pole recruitment during sporulation

The basis of the transposon screen is illustrated in Fig. 1A. We built two strains, one carrying a lacZ reporter
gene located in the right ARM zone (amyE, +327 kbp clockwise from oriC on the 4218 kbp chromosome) and the other in the left (spolIID, −438 kbp; see also Fig. 1B). The lacZ reporter was driven by a prespore-specific $\sigma^F$-dependent promoter, $P_{spolIO}$ (Londono-Vallejo et al., 1997), which was modified to be regulated negatively by LacI. The lacI gene was expressed from an unmodified $P_{spolIO}$ promoter and was located in the ORI zone (−192 kbp). In an otherwise wild-type [but spolIIEfrozen] background, the lacZ reporter gene would not be expressed significantly, because it occupies the same compartment as the lacI gene, giving white colonies on X-Gal plates unless IPTG was included in the medium (Supporting Information Fig. S1). Mutants giving an ORI zone out phenotype would give blue colonies because null mutants are both defective in comN and minD which were also found in a gene called comN (yrzD). ComN is reported to be involved in post-translational regulation of the comE competence operon (Ogura and Tanaka, 2009), but had not previously been implicated in chromosome segregation. Interestingly, localization of ComN is also influenced by DivIVA (dos Santos et al., 2012). Of note, multiple hits in soj, comN and minD were obtained independent of whether the lacZ gene was located in the right or the left ARM region.

comN and minD mutants are both defective in oriC trapping during sporulation

To verify our screening results, we constructed an in-frame marker-free deletion mutant of comN and confirmed its phenotype of oriC exclusion from the prespore. The defect could be complemented with $P_{comN-}$, which is active only in the prespore. As expected, 3 h after the induction of sporulation, prespores of the wild-type contained mostly signals from both reporters: the majority of cells (>80%; green bar in Fig. 2B), showing that both the ORI and the ARM zones were inside the prespore. In line with the genetic screen, the comN deletion mutant displayed a strong specific defect in trapping of the ORI zone marker in the prespore, with only about 1/4 of the sporulating cells showing signals from both reporters: the majority of cells (~60%) had only the signal from the reporter in the ARM zone (blue bar in Fig. 2B). A similar pattern was seen when the ARM zone marker was on the right (amyE, +327 kbp) (Supporting Information Fig. S3).

Interestingly, although the minD mutant was more difficult to work with because of its high frequency of mini-cell production, it seemed to behave indistinguishably from the comN mutant, with ~80% of the prespores showing the signal for the ARM zone (green and blue
Fig. 2. Use of a \( P_{spoIIQ} \)-based fluorescent reporter assay to quantitate trapping of ORI and ARM zones in the prespore compartment of the mutant strains indicated in the graphs.

A. Principle of the assay as developed previously by (Sullivan et al., 2009). The prespore compartment contains either both the ORI and ARM zones (green ‘BOTH IN’), only the ORI zone (yellow ‘ARM zone OUT’), only the ARM zone (blue ‘ORI zone OUT’), or neither of these (black ‘BOTH OUT’). The ORI zone is represented by a \( P_{spoIIQ}-yfp \) construct inserted at \( yycR \) (~79 kbp; yellow spot) and the ARM zone is represented by a \( P_{spoIIQ}-cfp \) construct inserted at \( ywJ \) (~418 kbp; blue spot). The microscopic image below (comN mutant and spoIIIE(frozen) mutant background) illustrates prespores of all 4 classes indicated by arrows with corresponding colours, that is, green = both reporters in the prespore, yellow = only the ORI zone (YFP) reporter in the prespore, blue = only the ARM zone reporter, white = no reporter in the prespore (CFP). Cell membranes were stained with the FM5-95 dye.

B. ORI zone out phenotypes of \( \text{comN}, \text{minD} \) and \( \text{soj} \) mutants quantitated by the fluorescent reporter assay as described in A.

C, D. Chromosome trapping phenotypes of the \( \text{minC}, \text{minJ} \) (C) and \( \text{racA} \) (D) mutants as assessed by the fluorescent reporter assay described in A.

Cells were allowed to sporulate for 3 h before microscopic imaging. At least 300 cells were counted for each strain in each experiment. The values are averages of at least three independent experiments. Error bars represent standard deviations. For the wild-type, data were averaged among all experiments and therefore, the same numbers are shown in panels B–D.
bars in Fig. 2B) but most of these (over 50%) did not have the ORI zone signal (blue bar in Fig. 2B).

To probe the positioning of the ORI zone directly, we used the tetO-TetR fluorescent repressor operator system (FROS) with a tetO array inserted close to oriC (yycR, −79 kbp) that can be visualized by binding of TetR-GFP expressed from amyE (−327 kbp) (Wagner et al., 2009) (Supporting Information Fig. S4A). In the comN mutant, approximately 60% of the cells had both oriC foci in the mother cell compartment, whereas almost all (>98%) wild-type cells had one oriC in the prespore and one in the mother cell (Supporting Information Fig. S4B and C). This result lent support to the idea that MinD plays a role in fixing the ORI zone in the mother cell, whereas, in the prespore, it is not fixed yet, as seen in the comN mutant. The mean trapping of the ORI in both wild-type and comN mutant cells is similar, as expected.

oriC trapping is perturbed by mutations in comN, minD, soj, racA, and yycR

Notably, in a spoIIE+, mother cell (Supporting Information Fig. S4B). Notably, in a spoIIE+ background, which did not lead to the ‘frozen’ state of the prespore chromosome, the comN mutant still mislocalized 35% of the ORI zone versus <2% for the wild-type (Supporting Information Fig. S4C). Again, the minD mutant had a very similar phenotype to that of the comN mutant (Supporting Information Fig. S4B and C). This result lent support to the PspoilQ-cfp/yfp-based reporter assays and confirmed that in the absence of ComN or MinD the ORI zone is excluded from the prespore in a large portion of the sporulating cells.

minJ but not minC is also required for correct positioning of oriC in the prespore

Both MinD and DivIVA are members of the Min system that spatially regulates division septum placement, in which the cell division inhibitor MinC forms a complex with MinD to inhibit FtsZ assembly near cell poles and nascent septa (Marston et al., 1998; Marston and Errington, 1999b; Gregory et al., 2008). The complex is recruited to DivIVA through a fourth protein, MinJ, an integral membrane protein that bridges DivIVA and MinD (Bramkamp et al., 2008; Patrick and Kearns, 2008). To see whether MinC and MinJ were also required for oriC positioning at the cell pole during sporulation, we examined trapping of the ORI and ARM markers as described above in mutants of minC and minJ. We tested a single, non-polar minC mutant and were surprised to obtain essentially wild-type level of chromosome trapping (Fig. 2C and Supporting Information Fig. S4C), indicating that perturbation of cell division through loss of Min function is not the reason for the observed trapping defect of the minD mutants, and therefore that this is a separate additional function of MinD.

Mutants of minJ have a strong MinCD-dependent cell division defect (Bramkamp et al., 2008; Patrick and Kearns, 2008). To be able to test the involvement of MinJ in oriC placement we introduced a minC mutation into the minJ mutant, and compared this with a minC single mutant. As shown in Fig. 2C, the minJ mutation, similarly to the comN and minD mutations, also severely affected trapping of the ORI but not the ARM zone in the prespore. Therefore, MinJ is also required for oriC positioning, potentially through its role in MinD recruitment. We assume that minJ mutations were not picked up in the screen because of the growth and division defect in minJ single mutants, just as for divIVA.

ComN, MinD and Soj act in the same pathway in oriC positioning

Since deletion of comN, minD and soj all cause the oriC proximal marker specifically to be excluded from the prespore (Fig. 2B) (Sullivan et al., 2009), we reasoned that these three proteins act in a common pathway that governs oriC positioning. To test this we first combined comN and minD mutations and found that the double mutant exhibited a similar ORI zone trapping defect as the single mutants, consistent with ComN and MinD acting in the same pathway (Fig. 2B). We then examined the effects of a soj mutation, which has a slightly milder trapping defect than the comN or minD deletion (Fig. 2B). Interestingly, although double mutants of comN soj and minD soj both exhibited an ORI zone trapping defect, it was relatively mild, of similar magnitude to that of the soj single mutant in terms of reduction in cells with both markers correctly trapped (Fig. 2B). Similar results were obtained with the direct trapping assay using the tetO/TetR system (Supporting Information Fig. S4C).

ComN and MinD act in an oriC-trapping pathway separate from that of RacA

We previously showed that racA and soj mutations act synergistically and that the double mutant has a much stronger defect in oriC trapping than either single mutant (Wu and Errington, 2003). The action of RacA is quite well established and its binding (ram) sites on the chromosome mainly lie to the left of oriC (Fig. 1B) (Ben-Yehuda et al., 2005). Using the PspoilQ-based fluorescent reporter system we found that, in contrast to ComN and MinD, RacA predominantly affects trapping of the ARM zone (Fig. 2D). When the racA deletion was combined with the comN mutation, a more severe defect was also obtained and most prespores were devoid of both the CFP and YFP signals during sporulation (Fig. 2D), similar to our previously reported results with racA and soj (Wu and Errington, 2003). Consistent with the trapping assay, the sporulation efficiency of the comN racA double mutant was also greatly reduced when compared with the single mutants (Supporting
All these results support the idea that there are at least two distinct mechanisms governing the positioning of the oriC region of the chromosome during sporulation; one dependent on RacA and the other on ComN, MinD and Soj.

**The comN/minD defect affects oriC positioning before asymmetric septation**

To test when mislocalization of oriC occurs, we measured the distance between the oriC (TetR-GFP) foci and the cell pole (as a fraction of cell length) in minD, comN and soj mutants just before asymmetric septation commences. As shown in Fig. 3A, this distance was indeed significantly greater in the mutants than in wild-type cells. minD mutants, and to a lesser extent comN mutants, have an altered length distribution compared to the wild-type (Bramkamp et al., 2008) (Supporting Information Fig. S5C). To rule out an effect of cell length, oriC-pole distances in cells of the same length class (Supporting Information Fig. S5A) were measured in the comN mutant and the wild-type. This again showed a similar shift in the distribution of oriC-pole distances (Supporting Information Fig. S5B). Of note, the efficiency of prespore formation in the comN mutant over the course of 3 h after induction of sporulation was very similar to the wild-type strain, that is, at 100, 125 and 180 min after induction of sporulation the percentages of cells sporulating were 3%, 8% and 52% in the wild-type and 3%, 12% and 55% in the comN mutant, respectively. As a further control, we then examined cells of a minC mutant, which has a very similar cell length distribution as minD mutants (Bramkamp et al., 2008). As shown in Fig. 3B, the distribution of oriC-pole distance of the minC mutant was similar to that of the wild-type. Thus, the comN, minD and soj mutants are all defective in sub-polar positioning of the oriC region before the asymmetric septum forms.

**ComN and minD mutants are affected in oriC positioning in vegetative cells**

Since MinD and ComN are expressed throughout growth and sporulation, unlike RacA which is sporulation specific (Ben-Yehuda et al., 2003; Wu and Errington, 2003), we wondered whether these factors might also have a role in oriC positioning during vegetative growth. Indeed,
although neither of the mutants had an overt chromosome segregation defect, oriC-pole distances were significantly greater in minD and comN mutant cells growing vegetatively compared to wild-type cells (Fig. 3C). Curiously, no such effect was detectable for cells of a vegetatively growing soj mutant (but note that the soj mutant has a milder effect on oriC positioning in sporulating cells than comN or minD mutants; Fig. 3C). Again, the increased distances of oriC from the cell pole in vegetatively growing cells of minD and comN mutants were not a result of minicell production and/or increased cell length, because a minC mutant, which had a very similar
Fig. 4. Septal/polar localization of GFP-MinD and GFP-Soj is dependent on ComN.  
A. Localization of GFP-MinD expressed from the native locus (JAG118 from Gregory et al., 2008) in the wild-type (left panels) and the comN mutant (right panels) during vegetative growth in CH medium at 30°C (upper panels) and 37°C (lower panels).  
B. Localization of GFP-MinC expressed from the native locus (EBS499 from Gregory et al., 2008) in the wild-type (left panels) and the comN mutant (right panels) during vegetative growth in CH medium at 30°C (upper panels) and 37°C (lower panels).  
C. Localization of GFP-MinD expressed from the native locus (unpublished construct by Wu Li, Schweewess J and Errington J) in the wild-type (upper panels) and the comN mutant (lower panels) 85 min after the induction of sporulation. DNA was stained with DAPI. The right hand panels are merged images of the GFP and DNA images showing the positions of the MinD signals in relation to the chromosomes. The green colour in the cartoon in the upper right hand panel indicates the cellular positions of fluorescent signals from GFP-MinD at (asymmetric) septa and cell poles. Outlines of the cells are in white.  
D, E. Colocalisation of GFP-MinD and mCherry-ComN during sporulation (D) as well as during vegetative growth (E). For, D, the yellow colour septa and cell poles. Outlines of the cells are in white.  
For A–F, arrows point to (asymmetric) septa, and arrowheads indicate cell poles (Fig. A–E) or cytoplasmic foci (Fig. 4F). Scale bars represent 3 μm.  

minicell phenotype and cell length distribution to those of minD mutants, did not have an increased oriC-pole distance (Fig. 3D). In fact, the distances were in general slightly reduced when compared to the wild-type.

ComN is required for proper localization of MinD but not MinC

It has previously been shown that localization of ComN to division septa requires DivIVA but not MinJ or MinD (dos Santos et al., 2012). To further study the role of ComN in chromosome segregation, we constructed a ComN-GFP fusion expressed ectopically at amyE from its native promoter and checked that this was functional as judged by complementation of the oriC trapping defect of a comN null mutant (Supporting Information Fig. S2). In accordance with the previous study (dos Santos et al., 2012), we confirmed that ComN localizes to the septum and cell poles, and that this localization is dependent on DivIVA but not MinD, MinC or MinJ (Supporting Information Fig. S6).

We then carried out the reciprocal experiments examining the effects of comN deletion on the localization of GFP fusions to MinD and MinC (Fig. 4A–E). Two different GFP-MinD constructs were examined, and in both cases substantial effects on localization were observed. In particular, the prominent fluorescent bands associated with nascent division septa (arrows in Fig. 4A), and the foci at the poles (arrowheads in Fig. 4A) were greatly reduced in intensity or abolished in vegetative cells (Fig. 4A). Western blot analysis revealed no difference in the levels of MinD between the comN mutant and the wild type strain (data not shown). Thus, recruitment of MinD to the division site and retention at the cell pole is at least partially dependent on ComN. Surprisingly, localization of a MinC-GFP fusion was not visibly affected by deletion of comN and most division sites had prominent fluorescent bands irrespective of whether or not the cells had a functional comN gene (Fig. 4B). To test how much Min function was affected by the delocalization of MinD in the absence of ComN, we examined the number of minicells produced by the mutants and found that the comN mutant indeed produced a small number of minicells (1.5% minicells (n = 1842) vs. 0.3% (n = 1450) in the wild-type), but the number was far less than that of the minD mutant (25% minicells, n = 291). It has been shown that in B. subtilis MinD is present in excess of MinC (sixfold to ninefold) (Muntel et al., 2014). It is therefore possible that the residual MinD localization in the absence of comN is sufficient to recruit MinC and restrict division to the mid cell site.

In the absence of the nucleoid occlusion protein, Noc, Min function becomes crucial for cell viability - noc min double mutants are conditionally lethal at temperatures higher than 37°C (Wu and Errington, 2004). We reasoned that if ComN affects mainly the oriC segregation but not the division function of MinD, comN noc double mutants should be viable at 48°C. Indeed, a comN noc double mutant was able to grow at 48°C, whereas a minD noc double mutant was not (data not shown), consistent with the above conclusion that MinD has separable functions and that the cell division function of MinD in vegetative growth is largely unaffected in the absence of ComN.

ComN and MinD colocalize at cell poles in sporulation

The involvement of ComN and MinD in the polar placement of the oriC region of the chromosome during spore development, and the dependence of MinD localization on ComN during growth predicted that the two proteins colocalized at cell poles during sporulation. Indeed, both MinD and ComN could be seen at the asymmetric septum and as a focus at the cell pole (arrows and arrowheads, respectively, in Fig. 4C and D). Importantly, although weak bands of GFP-MinD were still present at the asymmetric septa in the comN mutant, the polar foci of GFP-MinD were effectively absent, and instead a faint, uniform signal could sometimes be seen all over the membrane...
surrounding the prespore, probably due to its general affinity for the membrane (Fig. 4C). To test the colocalization, we constructed a strain carrying fusions expressing both GFP-MinD and mCherry-ComN. As shown in Fig. 4D and E, GFP-MinD colocalized with mCherry-ComN at septa as well as at the poles in both sporulating and vegetative cells, consistent with the idea that ComN and MinD are present in the same functional complex.

ComN, like MinD, is required for septal recruitment of Soj

Soj protein has a complex localization pattern, showing association with the nucleoid, the division septum, DnaA foci and Spo0J foci (Marston and Errington, 1999a; Quisel et al., 1999; Murray and Errington, 2008). We previously showed that septal recruitment of Soj depends on MinD (Murray and Errington, 2008), and based on the results described above it might also depend on ComN. In wild-type cells, GFP-Soj was localized to division septa (arrows in Fig. 4F) and as foci in the cytoplasm (arrowheads in Fig. 4F), as previously reported (Murray and Errington, 2008). In the comN deletion mutant, however, the septal bands of Soj were absent (Fig. 4F). Thus, ComN is, like MinD (Fig. 4F) (Murray and Errington, 2008), required for septal localization of Soj. Consistent with the hierarchy of interactions described above, GFP-Soj localization at the cell poles was also dependent on MinJ (Fig. 4F).

A network of interactions between the proteins required for ORI zone segregation during sporulation

To identify which of the putative protein-protein interactions predicted by the results described above might be direct, we carried out systematic bacterial two-hybrid analyses (Karimova et al., 2005). The results, shown in Fig. 5, confirmed the previously detected self-interactions of MinD, MinC, MinJ, DivIVA, Soj and ComN (Bramkamp et al., 2008; Scholefield et al., 2011; dos Santos et al., 2012), as well as interactions between DivIVA-MinJ, MinJ-MinD and MinD-MinC (Bramkamp et al., 2008). Our tests did reveal new interactions between ComN and MinJ, and ComN and MinD (see also Supporting Information Fig. S7), consistent with these proteins acting as a complex. Our tests did not show an interaction between ComN and DivIVA as previously detected by yeast two-hybrid (dos Santos et al., 2012), probably because the adenylate cyclase fusion constructs were not fully functional for the interaction.

We did not detect interactions between Soj and the other polar proteins even though a positive Soj self-interaction was obtained. It is possible that fusion of Soj to fragments of adenylate cyclase interfered with its interaction with some of the partners but still allowed Soj self-interaction. It has previously been reported that expression of Soj in *E. coli*, without Spo0J, is deleterious to the cells (Hester and Lutkenhaus, 2007). It will be interesting to test whether interactions with Soj could occur when Spo0J is co-expressed in the system.

Fig. 5. ComN interacts with both MinJ and MinD as assessed by bacterial two-hybrid studies. Interactions between ComN, DivIVA, MinD, MinC, MinJ and Soj proteins were tested using bacterial two-hybrid assays. Only one combination of the interactions is shown for simplicity. A plus sign (+) denotes a positive interaction. Labels on the left of the image correspond to the T18 fusions cloned into the pUT18 plasmid and labels on the top of the image correspond to the T25 fusions cloned into the pKT25 plasmid. ComN, DivIVA and Soj were N-terminal fusions to T18 on pUT18, whereas MinD, MinC and MinJ were C-terminal fusions to T18. MinC and MinJ are N-terminal fusions to T25 on pKT25, whereas ComN, DivIVA, MinD and Soj are expressed as C-terminal fusion to T25. The positive control is the two halves of a leucine zipper from GCN4 in yeast fused C-terminal to T18 and T25 in pUT18 and pKT25 and the negative control is empty pUT18 and pKT25 plasmids (Karimova et al., 1998).

Discussion

Three new proteins involved in prespore chromosome segregation

Previous work established that capture of the oriC region at the pole of the cell was crucial for successful
prespore chromosome segregation, and that this capture was probably dependent on two distinct mechanisms based on the Soj-Spo0J and RacA systems (reviewed in Errington, 2010). RacA is present only in sporulating cells and it operates via a relatively simple, well-defined mechanism involving binding to multiple specific DNA binding (ram) sites close to oriC and recruitment to the cell pole via a direct interaction with DivIVA (Ben-Yehuda et al., 2003; Wu and Errington, 2003; Ben-Yehuda et al., 2005; Lenarcic et al., 2009). The Soj-Spo0J system has proved more difficult to characterize but seems also to be dependent on DivIVA, directly or indirectly (Wu and Errington, 2003; Perry and Edwards, 2004; Perry and Edwards, 2006). In this work we have identified three additional proteins specifically involved in the Soj-Spo0J pathway.

MinJ probably works indirectly by linking MinD to DivIVA (Bramkamp et al., 2008; Patrick and Kearns, 2008; van Baarle and Bramkamp, 2010). The protein has 6 predicted transmembrane segments (van Baarle and Bramkamp, 2010) and a likely protein interaction (PDZ) domain, but its biochemical function, other than participating in complexes of proteins at the cell pole, remains unclear.

Although previous work has hinted at a role for MinD in the localization of Soj (Autret et al., 2001; Murray and Errington, 2008), the absence of MinD is not known to affect the function of Soj in regulating DnaA, and minD mutants do not have a replication over-initiation phenotype that is associated with the deletion of soj (RL and LJW, unpublished). We were therefore surprised to find that MinD (but not MinC) was clearly involved in polar oriC placement. MinD has a very well characterized role in division site selection in which it recruits and activates the division inhibitor MinC. Localization of MinC at nascent septum and cell poles prevents the FtsZ-based division machine from assembling away from its proper mid cell division site (Gregory et al., 2008; reviewed by Lutkenhaus, 2007; Rowlett and Margolin, 2015). Involvement of the MinD protein in correct placement of the oriC region could explain various old results including its influence on Soj localization (see below) and the curious observation of reverse SpolIIIE pumping (Sharp and Pogliano, 2002). SpolIIIE protein uses skewered octameric SRS sequences to orientate DNA pumping (Ptacin et al., 2008; Cattoni et al., 2013; Fiche et al., 2013). In wild-type cells in which the oriC region is correctly captured in the prespore compartment, DNA translocation is directed from mother cell to prespore. However, if the oriC region lies outside the prespore, as we now show frequently occurs in minD mutants, translocation could be misdirected away from the prespore, leading to reverse transfer of the prespore chromosome. We predict that this should also occur in the various other mutants that fail to capture oriC in the prespore compartment.

ComN was previously implicated in the regulation of competence (Ogura and Tanaka, 2009; dos Santos et al., 2012). This finding did not offer an obvious reason for localization of the protein at the cell pole via DivIVA, but its involvement in polar oriC capture now provides a functional explanation. The ComN protein is small and highly charged, and although its precise function in oriC positioning remains to be uncovered, our results suggest that ComN separates the two specific activities of MinC: ComN in complex with MinJ stabilizes and primes MinD to regulate oriC positioning in the cell, together with Soj, whereas the MinJ-MinD complex allows proper control of division site selection via MinC (Fig. 6).

A polar segregation complex targeted on Soj

Several lines of evidence point to the proteins mentioned above forming an interactive complex at the cell pole (summarized in Fig. 6), which contributes to oriC positioning at least partly by modulating the localization and activity of Soj protein. First, all of the proteins have been visualized at the cell poles in sporulating and vegetative cells by imaging methods. Second, localization dependence experiments have identified likely direct pairwise interactions within the complex, bacterial two-hybrid experiments have identified likely direct pairwise interactions between several of the proteins (Fig. 5).

Previous work on the Soj protein has revealed a complex and dynamic localization pattern involving binding to the nucleoid, Spo0J, DnaA and the division septum (Marston and Errington, 1999a; Quisel et al., 1999; Hester and Lutkenhaus, 2007; Murray and Errington, 2008). The different localization sites are thought to be dependent on different nucleotide associated states and or monomer/dimer forms (Murray and Errington, 2008). The septal localization was previously shown to depend on MinD protein but not MinC (Murray and Errington, 2008). Although we are still some way off understanding the precise role of the Soj protein in prespore chromosome capture, it now seems that the association of Soj with the septum is important for oriC capture during sporulation and that this activity is influenced by the polar segregation complex of proteins identified here. Three other lines of evidence support this idea. First,
deletions of \( \text{minJ} \), \( \text{comN} \) and \( \text{minD} \), all generate an ‘ORI zone out’ chromosome defect, similar to that of the \( \text{soj} \) mutant, if not stronger (Fig. 2) (Sullivan et al., 2009). Second, mutation of either \( \text{comN} \) or \( \text{minD} \) exacerbates the effects of a \( \text{racA} \) mutation on prespore chromosome capture, just as described for \( \text{racA} \) and \( \text{soj} \) previously (Wu and Errington, 2003). Third, \( \text{soj} \), \( \text{minD} \) and \( \text{comN} \) seem to work in the same pathway.

At present, based on analysis of the localization hierarchy and two-hybrid results, \( \text{MinD} \) is the most likely candidate for the immediate target for \( \text{Soj} \) interaction at the cell pole (Fig. 6). Intriguingly, \( \text{MinD} \) is closely related to \( \text{Soj} \) in structure and mechanism of regulation. Both proteins are deviant Walker ATPase proteins. They both undergo reversible dimerization, which is regulated by ATP binding and hydrolysis (Lutkenhaus, 2012). It is therefore possible that the two proteins interact and, either become active for DNA binding and movement (e.g., by rebalancing the \( \text{Soj} \) cycle), or are able to stimulate the activity of another protein to bring the ORI zone of the chromosome to the cell pole. It is also possible that \( \text{Soj} \) stimulates \( \text{MinD} \) to bind DNA directly (not shown). When not in complex with \( \text{ComN} \), \( \text{MinD} \) carries out its Min function (outlined in red) by recruiting and activating the division inhibitor \( \text{MinC} \), to block cell division near the poles and nascent septum (broken red line in the red cell on the right).

**Comparison with other chromosome segregation systems**

The \( \text{parABS} \) systems have been widely studied as factors implicated in the active segregation of bacterial chromosomes and plasmids. However, several aspects of their function remain poorly understood. Given their relative simplicity – two small proteins and a recognition site – they are surprisingly diverse in apparent function. Thus, as well as active \( \text{oriC} \) segregation, they have been implicated in recruitment of SMC complexes to origins, positive and negative regulation of DNA replication, transcriptional regulation, and other diverse processes.
(Marston and Errington, 1999a; Quisel et al., 1999; Gerdes et al., 2000; Livny et al., 2007; Murray and Errington, 2008; Gruber and Errington, 2009; Sullivan et al., 2009). The role of the Caulobacter crescentus par-ABS system in chromosome segregation is now well understood. In this Gram-negative α-proteobacterium, the parABS system clearly plays a central role in chromosome segregation. Initiation of DNA replication takes place with the parent origin located at the old cell pole. The ParB-parS complex of one daughter origin then migrates rapidly to the new cell pole mediated by a ParA 'relay' mechanism (Lim et al., 2014) in which the oriC-ParB-parS complex migrates through a 'cloud' of DNA-bound ParA molecules, stimulating ATP hydrolysis and dissociation of ParA dimers in the process (Ptacin et al., 2010; Schofield et al., 2010; Shebelut et al., 2010; Lim et al., 2014). Ultimately, the origin complex is captured at the new cell pole through interaction with two polarly localized proteins, PopZ (Bowman et al., 2008) and TipN (Ptacin et al., 2010; Schofield et al., 2010).

The relatively fast and directed movement of ParB(-Spo0J)-parS complexes has not been seen in vegetative growth of B. subtilis, neither has any role for the cell poles in vegetative chromosome segregation. Nevertheless, it is interesting that both comN and minD, but not minC mutants, have an increased oriC-pole distance. We have previously shown that absence of MinD does not only affect the septal recruitment of Soj but also causes Spo0J foci to be less condensed compared to the MinC^+ cells. These observations raise the possibility that the MinJ/ComN/MinD polar complex also plays a role in chromosome segregation of vegetative B. subtilis cells.

In contrast to vegetative cells, the sporulation specific movement of oriC regions to the extreme cell poles may be equivalent to that of C. crescentus. Indeed, the sporulation segregation process now seems also to involve a series of polarly located proteins in B. subtilis, although there does not seem to be any clear primary sequence similarity between the polar proteins of the two organisms. In the light of the recent speculations about the possible evolutionary origins of the Gram-negative bacteria from an ancestral endospore forming bacterium (Tocheva et al., 2011; Errington, 2013) the apparent similarity between α-proteobacterial and endospore chromosome segregation mechanisms is highly intriguing.

**Experimental procedures**

**General methods**

Strains and primers used in this study are listed in Supporting Information Tables S1 and S2, respectively. General DNA manipulation in *E. coli* was done as described (Sambrook et al., 1989). Nutrient agar (Oxoid) was routinely used as a solid medium for growth of *E. coli* and *B. subtilis*. Liquid media used were Luria–Bertani (LB) or casein hydrolysate (CH) medium (See (Harwood and Cutting, 1990). Competence for genetic transformation was induced in *B. subtilis* as described previously (Hamoen et al., 2002). *B. subtilis* was induced to sporulate by the resuspension method [90], as modified by Partridge and Errington (Sterlini and Mandelstam, 1969). For blue/white selection 0.008% of X-gal was used. Supplements were added as required: 100 μg/ml Ampicillin, 20 μg/ml tryptophan, 5 μg/ml chloramphenicol, 2 μg/ml (for all spoIII ΔE36-kan strains, and for all alleles of the spoIIG Δ spoIII Δ spoIIE36-kan gene) or 5 μg/ml kanamycin (all other strains containing a kan marker, notably mutants obtained with the random mutagenesis screens using pMarB), 50 μg/ml spectinomycin, 5 μg/ml zeocin, 1 μg/ml phleomycin, 1 μg/ml erythromycin and 25 μg/ml lincomycin. Random transposon mutagenesis was performed using pMarB as described (Le Breton et al., 2006). Colonies were grown for up to 36 h to select blue ones by visual inspection.

**Construction of strains and plasmids**

To construct a lacZ driven by the spoIIG promoter (P_spoIIG) containing a lacO operator for artificial repression by LacI, a DNA fragment containing the P_spoIIG was PCR amplified with primer pair oTK20/oTK21 from chromosomal DNA of *B. subtilis* and cloned as an HindIII/BamHI fragment in pMUTIN4, which was amplified with primer pair oTK22/oTK23, using plasmid pMUTIN4 (Vagner et al., 1998) as a template. In this way the spac promoter was removed and replaced by the *B. subtilis* spoIIG promoter (without its RBS), upstream of the lacO operator (lacO-oid, AATTGT GACGCGTCACAATT, see (Vagner et al., 1998). The resulting plasmid, pTK31, was used as a template to PCR amplify the P_spoIIG-lacO(oid)-lacZ, together with the terminators located upstream (rmB1 + rmB2 from the original pMUTIN4 plasmid) and erythromycin gene using primer pair oTK24/oTK25. This fragment was subsequently ligated to fragments (1.5–2.0 kb) of chromosomal DNA of *B. subtilis* flanking the desired integrations sites, PCR amplified with primer pairs oTK26/oTK27 and oTK28/oTK29 [integration at amyE, (+327 kb)], oTK34/oTK35 and oTK36/oTK37 [integration next to jag (−3 kb)] or oTK30/oTK31 + oTK22/oTK23 (integration next to spoIID, (−438 kb)]. Ligation mixtures were then transformed into *B. subtilis* wild-type strain 168CA and transformants with correct integration of the erm-terminator-P_spoIIG-lacO-lacZ construct were selected and verified by phenotypic analyses.

A construct containing the spec-P_spoIIG-lacI cassette was made by fusing a DNA fragment containing P_spoIIG, PCR amplified from chromosomal DNA of *B. subtilis* using primers oTK48/oTK49 (P_spoIIG), a PCR product amplified from plasmid pMUTIN4 using primers oTK50/oTK51 (lacI, with a lambda 0 terminator downstream to prevent inward reading), and the spectinomycin resistance gene (spec) PCR amplified from plasmid pAPNC213 (Morimoto et al., 2002) using primers oTK77/oTK78. The fused PCR product was
digested with EcoRI and HindIII and cloned into plasmid pBS-KS* (Stratagene) digested with the same restriction enzymes, yielding plasmid pTK32. Next, the entire spec-PspoIIIE36-lac cassette was amplified from plasmid pTK32, using primers oTK48/oTK47 and ligated to the DNA fragments flanking the intergenic region between yxiF and yxxG (−192 kbp), amplified with primer pairs oTK42/oTK43 and oTK44/oTK45. This ligation mixture was then directly transformed into the wild-type strain 168CA and colonies containing the correct integration of the entire spec-PspoIIIE36-lac construct were verified using PCR.

The spoIIIE36 mutation [which we here also denote as spoIIIE(frozen)] containing the G1285>A transition in the spoIIIE ORF, originally isolated by Piggot (1973; Wu and Errington, 1994) was introduced into the chromosome of B. subtilis wild-type strain 168CA by fusing PCR products of the spoIIIE gene generated from chromosomal DNA of the spoIIIE36 mutant strain 36.3 with primer pairs oTK53/oTK54, oTK55/oTK56 and oTK59/oTK61, and a PCR product containing the kanamycin resistance gene was PCR amplified using oTK57/oTK58 from plasmid pHT21 (Trieu-Cuot and Courvalin, 1983), in a single reaction by means of overlap-extension PCR. The resulting PCR mixture was directly transformed into the wild-type strain 168CA, and transformants were selected on plates containing 2 μg/ml kanamycin. Clones containing both the spoIIIE36 mutation and the kan gene downstream of spoIIIE36, were verified by phenotypic characterization (Wu et al., 1995). Insertion of a cat gene downstream of the spoIIIE36 allele, replacing the kan gene, was done by ligating PCR products generated using primer pairs oTK53/oTK66 and oTK67/oTK68 from the chromosomal DNA of the spoIIIE36-kan strain, and oTK64/oTK65 from plasmid pSG1186 (for the cat gene), which were digested with the relevant restriction enzymes. The ligation mixture was directly transformed into the wild-type strain 168CA and CamR, KanR, Spo* colonies were selected and verified by sequencing. In the same way, a zeo gene was placed downstream of spoIIIE36, amplified using primers oTK69/oTK70 from plasmid p726 [97].

A marker-less comN deletion mutant was constructed using the cre-lox system as described (Yan et al., 2008), using PCR products generated with primer pairs oTK71/oTK72, oTK73/oTK74 from chromosomal DNA of the wild-type strain 168CA, and oTK75/oTK76 from plasmid p726. A 47 bp fragment of the comN ORF and the scar of the cre-lox system were left behind encoding the following peptide: VEKHAPDPTVIAYITRPGSEALELLR.

A minD deletion mutant (marker-free) was constructed by growing strain ΔminD:spec spec-mazF two consecutive times on NA plates containing 1 mM IPTG and selecting for spectinomycin-sensitive clones.

A PcomN-comN-gfp fusion was made by cloning a PCR product encompassing PcomN-comN, which was generated from B. subtilis 168CA chromosomal DNA with primers oTK10/oTK11 into the Sphi KpnI digested pSG1154mgrp [a monomeric GFP variant made by Henrik Strahl based on pSG1154 of (Lewis and Marston, 1999)], thereby removing PxyC. This construct, pTK11, was transformed into the relevant B. subtilis strains and specR, amyE transformants were selected and used for further study.

An IPTG-inducible mcherry-comN fusion was made by cloning a PCR product, generated on chromosomal DNA of B. subtilis 168CA with primers oTK7/oTK9, into the BamHI, EcoRI sites of plasmid pAPNC(cat)-mcherry (Henrik Strahl). The resulting construct was integrated into the aprE locus of the relevant strains of B. subtilis, selecting for chloramphenicol resistant colonies.

A PcomN-comN construct for complementation of the comN deletion was made by cloning a PCR product encompassing PcomN-comN, which was generated from B. subtilis 168CA chromosomal DNA with primer pair oTK10/oTK14 into Sphi, NotI digested pSG1154mGFP, thereby removing both PxyC and gfp. This construct, pTK13, was transformed into the relevant B. subtilis strains and specR, amyE transformants were selected and used for further experiments.

Strains containing the PspoIIIE36-lac and PspoIIIE36-cfp reporters were constructed by transforming the relevant plasmids described in (Sullivan et al., 2009) into B. subtilis, selecting for the appropriate antibiotic resistance and checking for double cross-over recombination.

Microscopy

To visualize cells overnight cultures grown at 30°C in CH medium were diluted 1:100 into fresh medium. For sporulation, cells were grown at 37°C. For visualisation of protein fusions to GFP, cells were grown at 30°C. 0.7 μl cells were spotted onto thin ~1.2% agar plates (containing 10% of the growth medium). Microscopic visualisation of cells was carried out as described (Murray and Koh, 2014; Adams et al., 2015). Membranes were stained with 0.4 μg.ml−1 FM5-95 dye (Invitrogen). When necessary data were analysed using the Metamorph software (fluorescent trapping assays) and ImageJ (http://rsb.info.nih.gov/ij) containing the ObjectJ plugin (cell length and oriC-pole distance measurements). Standard deviations are from at least three experiments.

Two colour colocalisation imaging was performed using a Nikon Eclipse Ti microscope equipped with a Nikon CFP APO TIRF x100/1.49 oil objective, 488 nm and 561 nm solid-state lasers, and Andor Xion X3 EMCCD camera. All image capture was conducted using Nikon NIS elements 4.0. Cells were immobilized on 1.5% agarose slides.

Bacterial two-hybrid interaction tests

For bacterial two-hybrid assays, comN was PCR amplified from chromosomal DNA of B. subtilis wild-type strain 168CA using primer pairs oTK11/oTK15, and cloned into the KpnI, XbaI sites of plasmids pKT25 and pUT18 (Karinova et al., 2005) to create both N- and C-terminal fusions to adenylate cyclase fragments T25 and T18. Other plasmids for bacterial-two-hybrid analysis were obtained from the Daniel Strain Collection, Newcastle University. The experiment was carried out as previously described (Daniel et al., 2006). 10 μl of the cotransformation mixture was plated onto plates containing X-gal (0.004%). Plates were incubated at 30°C for 48 h, followed by room temperature for 24 h.

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**Supporting information**

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