

Topoisomerase IV can functionally replace all type 1A topoisomerases in *Bacillus subtilis*

Daniel R. Reuß^{1,†}, Patrick Faßhauer^{1,†}, Philipp Joel Mroch¹, Inam Ul-Haq², Byoung-Mo Koo³, Anja Pöhlein⁴, Carol A. Gross³, Rolf Daniel⁴, Sabine Brantl² and Jörg Stülke^{1,*}

¹Department of General Microbiology, GZMB, Georg-August-University Göttingen, Göttingen, Germany,

²Matthias-Schleiden-Institut, AG Bakteriengenetik, Friedrich-Schiller-University Jena, Jena, Germany, ³Department of Microbiology and Immunology, University of California, San Francisco, San Francisco, CA 94158, USA and

⁴Department of Genomic and Applied Microbiology, GZMB, Georg-August-University Göttingen, Göttingen, Germany

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ABSTRACT

DNA topoisomerases play essential roles in chromosome organization and replication. Most bacteria possess multiple topoisomerases which have specialized functions in the control of DNA supercoiling or in DNA catenation/decatenation during recombination and chromosome segregation. DNA topoisomerase I is required for the relaxation of negatively supercoiled DNA behind the transcribing RNA polymerase. Conflicting results have been reported on the essentiality of the *topA* gene encoding topoisomerase I in the model bacterium *Bacillus subtilis*. In this work, we have studied the requirement for topoisomerase I in *B. subtilis*. All stable *topA* mutants carried different chromosomal amplifications of the genomic region encompassing the *parEC* operon encoding topoisomerase IV. Using a fluorescent amplification reporter system we observed that each individual *topA* mutant had acquired such an amplification. Eventually, the amplifications were replaced by a point mutation in the *parEC* promoter region which resulted in a fivefold increase of *parEC* expression. In this strain both type I topoisomerases, encoded by *topA* and *topB*, were dispensable. Our results demonstrate that topoisomerase IV at increased expression is necessary and sufficient to take over the function of type 1A topoisomerases.

INTRODUCTION

The thorough control of DNA topology is an essential function in every living cell. In bacteria, DNA is usually present as circular covalently closed molecule that is compacted via the introduction of negative supercoils. Moreover, DNA replication and transcription involve the unwinding of the

DNA double helix, and result in the introduction of positive supercoils ahead of the polymerizing complexes. In addition, transcription generates negative supercoils behind RNA polymerase (1). To ensure the faithful operation of genetic information, these alterations of the DNA topology state must be relaxed to put the chromosome to the *status quo ante*. DNA topology is not only important for packaging of the DNA, but also for gene expression, as DNA topology affects the accessibility of specific chromosomal regions for transcription (2).

All organisms possess DNA topoisomerases to either introduce or relax supercoils to maintain the topological state. In addition to the control of DNA supercoiling, the DNA topoisomerases are also involved in the resolution of DNA catenanes that are formed at the chromosomal terminus region during DNA replication and in the resolution of Holliday junctions during DNA recombination (3). To perform all these activities most bacteria, like the model organisms *Escherichia coli* and *Bacillus subtilis*, possess four topoisomerases that are members of two different families (see Table 1) (4). The type I topoisomerases are monomeric proteins that carry out an ATP-independent reaction that cleaves one DNA strand of the double helix, passes the second strand through, and finally reseals the DNA molecule (5). The paralogous type I topoisomerases are encoded by *topA* and *topB* and called topoisomerase (topo) I and III, respectively. Both proteins belong to the type 1A topoisomerases. The type II topoisomerases are ATP-dependent heterotetramers that cleave both strands of the DNA molecule, pass one region of the DNA through another, and rejoin the molecules by re-ligating both strands (5). The type II topoisomerases are encoded by *gyrAB* and *parEC* and are referred to as DNA gyrase and topo IV, respectively.

DNA gyrase is required for introducing negative supercoils into the bacterial chromosomes, and thus essential for efficient DNA packaging. In addition, gyrase relaxes the

*To whom correspondence should be addressed. Tel: +49 551 39 33781; Fax: +49 551 39 33808; Email: jstuelk@gwdg.de

†The authors wish it to be known that, in their opinion, the first two authors should be regarded as Joint First Authors.

Table 1. DNA topoisomerases in *B. subtilis*

Name	Gene(s)	Essential	Activity
Type I topoisomerases			
Topo I	<i>topA</i>	Yes	Relaxation of negatively supercoiled DNA behind RNA polymerase
Topo III	<i>topB</i>	No	Decatenation of DNA, resolution of Holliday junctions
Type II topoisomerases			
DNA gyrase	<i>gyrAB</i>	Yes	Introduction of negative supercoils, relaxation of positive supercoils ahead of DNA and RNA polymerases
Topo IV	<i>parEC</i>	Yes	Decatenation/ segregation of replicating chromosomes

positive supercoils that form ahead of the replication and transcription complexes (6). The relaxation of negatively supercoiled DNA behind the RNA polymerase is catalyzed by topo I. Topo III and topo IV are required for the resolution of Holliday junctions during recombination and for the segregation of replicating chromosomes, respectively (3). As expected from their critical functions in maintaining the proper DNA structure and allowing DNA replication, the two type II enzymes, gyrase and topo IV, are essential both in *E. coli* and in *B. subtilis* (7,8). Topo III, encoded by *topB*, is dispensable both in *E. coli* and *B. subtilis* reflecting the specialized function of this enzyme in DNA recombination. Some laboratory strains of *B. subtilis* (JH642 and its derivatives) even lack a chromosomal region of 18 kb that encompasses the *topB* gene (9). For topo I encoded by the *topA* gene, contradicting results have been reported: The gene is essential in *E. coli*, and two studies reported it to be essential in *B. subtilis* (7,10,11). In contrast, in a recent genome-wide essentiality study, the *topA* gene could be deleted; however only by replacing the gene with a kanamycin cassette whereas replacements with other resistance cassettes were not successful (8). Thus, no clear conclusion about the essentiality of topo I is possible.

Interestingly, the essentiality of the DNA topoisomerases is also reflected by their presence in bacteria. Most bacteria possess the four enzymes described above. However, *Mycobacterium tuberculosis*, *Helicobacter pylori* and *Campylobacter jejuni* only encode topoisomerase I and gyrase, and thus seem to lack decatenation activity. In these bacteria, decatenation at the end of replication can possibly be performed by gyrase (12,13). Moreover, a few obligate bacterial endosymbionts that have undergone extreme genome reduction and therefore are fully dependent on their insect host, such as *Carsonella ruddii*, *Hodkinia cicadicola* and *Trembleya princeps*, have lost all their genes encoding DNA topoisomerases. Most likely, these bacteria acquire the enzymes from their hosts, as it is also the case in eukaryotic organelles (14). With genomes smaller than 160 kb, these bacteria belong to the organisms with the smallest genomes. Similarly, other host-adapted symbionts or pathogens have partially lost their topoisomerase complement. The endosymbionts *Buchnera aphidicola*, *Wigglesworthia glossinidia* and *Sulcia muelleri* have lost both type I topoisomerases, but do possess the essential enzymes gyrase and topo IV. In the bacteria of the genus *Mycoplasma* that are capable of living independently of host cells with the smallest known genomes, topo I, DNA gyrase and topo IV are present. These three enzymes and the encoding genes are also es-

essential for *Mycoplasma pneumoniae* and *Mycoplasma genitalium* (15,16).

We are interested in the identification of the minimal set of genes that allows independent bacterial life. The identification of such a set of genes will be an important first step in our understanding of the functions of these genes and will undoubtedly facilitate the analysis of functional and physical interactions between the encoded proteins. Of course, there is not a single minimal gene set, but the solution to this problem depends on the lifestyle and cellular biology of the bacteria. So far, most advance in constructing minimal cells has been made with naturally genome-reduced bacteria of the genus *Mycoplasma*. With only 473 genes, the artificial organism *Mycoplasma mycoides* JCVI syn3.0 is the smallest independently viable bacterium. Among these 473 genes are the five genes encoding DNA gyrase (*gyrA*, *gyrB*), topo I (*topA*) and topo IV (*parE*, *parC*) (17). We approach genome reduction by a top-down approach using the model bacterium *B. subtilis* as the starting point. An analysis of a potential minimal genome of a *B. subtilis*-derived *Mini-Bacillus* revealed the requirement for the same set of topoisomerases (18,19). So far, we have been able to delete >36% of the genome, making *B. subtilis* the organism with the most advanced top-down genome reduction project (20).

Precise knowledge on essential genes and functions is the key to any successful minimal genome project. Essential genes by definition cannot be individually deleted in a wild type genetic background under standard growth conditions (18). In this study, we have addressed the issue of topo I essentiality in *B. subtilis*. While the *topA* gene could indeed be deleted, this was accompanied by the immediate acquisition of suppressor mutations that result in overexpression of the topo IV-encoding *parEC* operon. We demonstrate how these suppressor mutants eventually evolve from amplification of the *parEC* chromosomal region by acquiring a more stable point mutation in the *parEC* promoter. Our results suggest that topo IV, when overexpressed, can functionally replace topo I.

MATERIALS AND METHODS

Bacterial strains, growth conditions and phenotypic characterization

All *B. subtilis* strains used in this study are listed in Table 2. All strains are derived from the laboratory strain 168 (*trpC2*). *B. subtilis* was grown in Lysogeny Broth (LB medium) (21). LB plates were prepared by addition of 17 g Bacto agar/1 (Difco) to LB and SP (sporulation medium),

respectively (21,22). Quantitative studies of *lacZ* expression in *B. subtilis* were performed as described previously (22). One unit of β -galactosidase is defined as the amount of enzyme which produces 1 nmol of *o*-nitrophenol per min at 28°C.

DNA manipulation and genome sequencing

B. subtilis was transformed with plasmids, genomic DNA or PCR products according to the two-step protocol (21,22). Transformants were selected on SP or LB plates containing erythromycin (2 μ g/ml) plus lincomycin (25 μ g/ml), chloramphenicol (5 μ g/ml), kanamycin (15 μ g/ml), or spectinomycin (150 μ g/ml). Phusion DNA polymerase (Thermo Fisher Scientific, Dreieich, Germany) was used as recommended by the manufacturer. DNA fragments were purified using the peqGOLD Cycle-Pure Kit (Peqlab, Erlangen, Germany). DNA sequences were determined by the dideoxy chain termination method (21). Chromosomal DNA from *B. subtilis* was isolated using the peqGOLD Bacterial DNA Kit (Peqlab, Erlangen, Germany). To identify the mutations in the suppressor mutant strains GP1948, GP1952, GP1958, GP1963, GP1964, GP1974, GP1975, GP1979, GP1983, GP1985, GP1987, GP2546, GP2547 and GP2549 (see Table 2), the genomic DNA was subjected to whole-genome sequencing. The reads were mapped on the reference genome of *B. subtilis* 168 (GenBank accession number: NC_000964) (23). Mapping of the reads was performed using the Geneious software package (Biomatters Ltd., New Zealand) (24). The resulting genome sequences were compared to that of our in-house wild type strain. Single nucleotide polymorphisms were considered as significant when the total coverage depth exceeded 25 reads with a variant frequency of $\geq 90\%$. All identified mutations were verified by PCR amplification and Sanger sequencing. Copy numbers of amplified genomic regions were determined by dividing the mean coverage of the amplified regions by the mean coverage of the remaining genome (25). The raw fastq reads of strains GP1948, GP1979 and GP1985 have been deposited in the NCBI-SRA database (<https://www.ncbi.nlm.nih.gov/sra>) and are accessible under the accession numbers SRR7967955, SRR7967956, and SRR7967957, respectively.

Construction of an amplification reporter system

To assay the amplification of the *parEC* genomic region, we replaced the adjacent *ynfC* gene by integrating a cassette composed of the *gfp* and *ermC* genes encoding the green fluorescent protein and erythromycin resistance determinant, respectively. In this construct, *gfp* expression is controlled by a weakly active promoter (p_a) (26) and the ribosome binding site of the *gapA* gene. To fuse the *gfp* gene to the expression signals, we amplified the *gfp* gene using the oligonucleotides KG427 and KG429 (for oligonucleotide sequences, see Supplementary Table S1) and plasmid pBP43 (27) as the template. KG427 attaches the promoter and the ribosome binding site. The amplicon was inserted between the EcoRI and Sall sites of the expression vector pGP3101 resulting in plasmid pGP3102. pGP3101 in turn was obtained by cloning a DNA sequence specifying the immunodetectable FLAG-tag (obtained by PCR

with primers ML05/ML06 and plasmid pGP1331 (28) as the template) into the HindIII site of the expression plasmid pBQ200 (29). To fuse the *gfp* gene with its expression signals to the *ermC* resistance gene and chromosomal regions up- and downstream of the anticipated insertion site (the *ynfC* gene), these four fragments were amplified by PCR (*gfp* with DR642/DR643 from pGP3102; *ermC* with mls-fwd(kan)/mls-rev(kan) from pDG647 (30); up- and downstream fragments with DR640/DR641 and DR644/DR645, respectively, from *B. subtilis* chromosomal DNA). The PCR products were purified, fused in another round of PCR using the oligonucleotides DR640 and DR645, and the 4.1 kb fusion PCR product was used to transform *B. subtilis* 168. The resulting strain was GP1966.

Construction of *parE-lacZ* fusions

Plasmid pAC5 (31) was used to construct a translational fusion of the *parE* promoter region with the *lacZ* gene. For the construction of plasmid pGP3111 containing a *parE-lacZ* fusion, the region upstream of *parE* was amplified using the oligonucleotides PF25/PF26. The PCR product was digested with EcoRI and BamHI, and cloned into pAC5 linearized with the same enzymes. Plasmid pGP3112 for the mutant upstream region was constructed accordingly using fusion chromosomal DNA of GP1985 as the template. The fusion constructs were integrated into the *amyE* site of the *B. subtilis* chromosome by transformation of *B. subtilis* 168 with linearized plasmids. The resulting strains were GP2550 and GP1967 for the wild type and mutant promoter, respectively.

Construction of a *recA* mutant

Deletion of the *recA* gene was achieved by transformation with PCR products constructed using oligonucleotides (see Supplementary Table S1) to amplify DNA fragments flanking the target genes and intervening antibiotic resistance cassettes as described previously (30,32).

Overexpression of *E. coli topB*

To express the *E. coli topB* gene in *B. subtilis*, we amplified the gene using the oligonucleotides PF79 and PF80, and cloned it between the XbaI and BamHI sites of the expression vector pHT01 (33). *B. subtilis* GP1966 was transformed with the plasmid, and recombinant cells were selected for chloramphenicol resistance. Expression of *topB* was induced by the addition of IPTG (1 mM) to the culture.

Staining and microscopy

For microscopy, cells were grown at 37°C in liquid LB medium overnight. Cells were harvested and re-suspended in PBS buffer (pH 7.4). DAPI and Nile Red were added to a final concentration of 20 μ g/ μ l and staining occurred for 15 min in the dark. The cells were washed three times in PBS buffer (pH 7.4). Images were acquired using an Axioskop 40 FL fluorescence microscope, equipped with digital camera AxioCam MRm and AxioVision Rel (version 4.8) software for image processing (Carl Zeiss, Göttingen,

Table 2. *B. subtilis* strains used in this study

Strain	Genotype	Source or reference
168	<i>trpC2</i>	Laboratory collection
BKE04260	<i>trpC2 ΔtopB::ermC</i>	8
BKK16120	<i>trpC2 ΔtopA::aphA3</i>	8
GP1948 ^a	<i>trpC2 ΔtopA::aphA3 (yneP-xynC)₁₉</i>	This study
GP1952 ^a	<i>trpC2 ΔtopA::aphA3 (yneE-alsT)₁₇</i>	This study
GP1958 ^a	<i>trpC2 ΔtopA::aphA3 (yneP-xynC)₁₇</i>	This study
GP1963 ^a	<i>trpC2 ΔtopA::aphA3 (citB-alsT)₁₆</i>	This study
GP1964 ^a	<i>trpC2 ΔtopA::aphA3 (yneP-xynC)₂₈ ΔtopB::ermC</i>	BKE04260 → GP1963
GP1966	<i>trpC2 ΔynfC::(p_{alf4}-gfp ermC)</i>	This study
GP1967	<i>trpC2 amyE::(p_{parE}*-lacZ cat)^b</i>	This study
GP1974 ^a	<i>trpC2 ΔtopA::aphA3 (citB-alsT)₁₅</i>	This study
GP1975 ^a	<i>trpC2 ΔtopA::aphA3 (citB-alsT)₁₅</i>	This study
GP1979 ^a	<i>trpC2 ΔtopA::aphA3 (yneP... parEC... ΔynfC::(p_{alf4}-gfp ermC)... xynC)₁₅</i>	This study
GP1983 ^a	<i>trpC2 ΔtopA::aphA3 (yneP... parEC... ΔynfC::(p_{alf4}-gfp ermC)... xynC)_{4codY}</i> (S129L) <i>rocG</i> (S61R)	This study
GP1985 ^{a,b}	<i>trpC2 ΔtopA::aphA3 ΔynfC::(p_{alf4}-gfp ermC) codY</i> (S129L) <i>rocG</i> (S61R) <i>gltA</i> (V1181A) S672 (C5T)	This study
GP1896	<i>trpC2 ΔtopB::cat</i>	11
GP1987 ^a	<i>trpC2 ΔtopA::aphA3 (yneP... parEC... ΔynfC::(p_{alf4}-gfp ermC)... xynC)_{4codY}</i> (S129L) <i>rocG</i> (S61R)	This study
GP2542	<i>trpC2 ΔrecA::spc</i>	This study
GP2543	<i>trpC2 ΔtopA::aphA3 (... parEC... ΔynfC::(p_{alf4}-gfp ermC)...)_n</i>	This study
GP2544	<i>trpC2 ΔtopA::aphA3 (... parEC... ΔynfC::(p_{alf4}-gfp ermC)...)_n</i>	This study
GP2545	<i>trpC2 ΔtopA::aphA3 (... parEC... ΔynfC::(p_{alf4}-gfp ermC)...)_n</i>	This study
GP2546 ^a	<i>trpC2 ΔtopA::aphA3 (... parEC... ΔynfC::(p_{alf4}-gfp ermC)...)₃</i>	This study
GP2547 ^a	<i>trpC2 ΔtopA::aphA3 ΔynfC::(p_{alf4}-gfp ermC) S672 (C5T)</i>	This study
GP2548	<i>trpC2 ΔtopA::aphA3 ΔynfC::(p_{alf4}-gfp ermC) S672 (C5T) ΔtopB::cat</i>	GP1896 → GP2547
GP2549 ^a	<i>trpC2 ΔtopA::aphA3 ΔynfC::(p_{alf4}-gfp ermC) S672 (C5T)</i>	This study
GP2550	<i>trpC2 amyE::(p_{parE}-lacZ cat)</i>	pGP3111 → 168

^aThe genomic DNA of these strains was analyzed by whole genome sequencing.

^bThe *parE* promoter region of this strain carries the C → T substitution in the -10 region of the promoter (see Figure 4).

Germany) and Neofluar series objective at ×100 primary magnification. We used the Filter set 49 (G 365, FT 395, BP 445/50; Carl Zeiss) for DAPI detection and Filter set 43 (BP 545/25, FT 570, BP 605/70) for Nile Red detection. Overlays of stained images were created with ImageJ 1.44p (National Institutes of Health, Bethesda, USA).

Primer extension

Total RNA was isolated from *B. subtilis* strains 168 (wild-type) and GP1985 grown in complex TY medium by the hot-phenol method (34) and treated with RNase-free DNase I (NEB) for 1 h at 37°C. 10 μg total RNA were hybridized to 5'-labeled-oligonucleotide SB3204 by incubation at 65°C for 5 min followed by quick cooling on ice. Reverse transcription was performed with Superscript IV RT (Invitrogen) in three subsequent 20 min steps at 50°C, 55°C and 65°C. Products were heat-denatured with formamide loading dye at 95°C for 5 min and separated on a 6% denaturing PAA gel along a Sanger sequencing reaction of plasmid pGP3111 with oligonucleotide SB3204 (Supplementary Table S1).

RESULTS

Inactivation of the *topA* gene results in the amplification of the *parEC* chromosomal region

Contradictory results have been reported about the essentiality of the *topA* gene encoding topo I in *B. subtilis*. While *topA* was found to be essential in two studies (10,11), the

gene could be deleted in other genome-wide analyses (8,35). However, Koo *et al.* (8), were able to replace the *topA* gene by a kanamycin resistance gene, but not by other resistance cassettes, highlighting the importance of the gene. In an attempt to verify the possibility of *topA* deletion, the *ΔtopA::aphA3* cassette from the mutant strain BKK16120 was amplified by PCR and used to transform our wild type strain *B. subtilis* 168. This transformation yielded two distinct types of colonies: the smaller colonies lysed rapidly whereas the larger colonies were stable. To test whether the deletion of the *topA* gene had occurred without further mutations, the chromosomal DNAs of two of the large transformants were subjected to whole genome sequencing. As expected, the *topA* gene was deleted and replaced by the *aphA3* resistance gene in the two transformants. For both strains, the genome sequence revealed the amplification of a genomic region (see Figure 1A). In case of strain GP1948, the 12 kb *yneP-xynC* chromosomal region was amplified, whereas the 18 kb *sirA-alsT* region was amplified in the second strain, GP1952. The amplified regions of both strains overlap. The *yneQ* and *yneR* genes encoding proteins of unknown function, the essential *plsY* gene encoding the acylphosphate:glycerol-phosphate acyltransferase required for phospholipid biosynthesis, the *yneT* gene encoding a predicted coenzyme A-binding protein, the *parEC* operon encoding the two subunits of topo IV, and the *ynfC* gene of unknown function form the common core of the amplified region. Strikingly, the deletion of the *topA* gene generates a selective pressure to maintain the amplification of

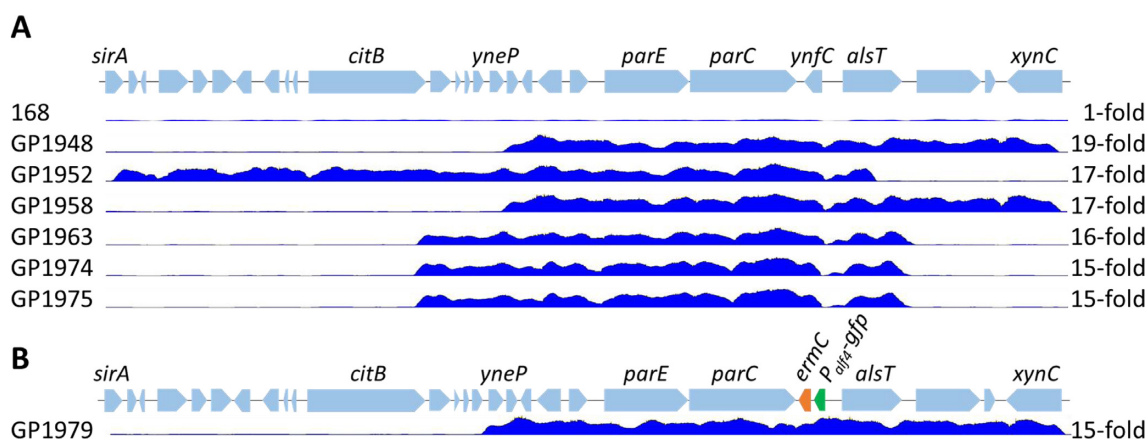


Figure 1. Gene amplifications in $\Delta topA$ suppressor strains. (A) Scheme of the *parEC* region of the *B. subtilis* wild type chromosome and of the amplifications in the suppressor mutants. (B) Scheme of the *parEC* region of the amplification reporter strain GP1966. The *gfp* gene replaces the *ynfC* gene. The suppressor strain GP1979 was isolated after deletion of *topA* in GP1966. The blue curves indicate the genome coverage of the corresponding genomic regions. Copy numbers are indicated on the right. Pictures of genome coverage were created using the Geneious Software version 10.0 (Biomatters Ltd., New Zealand).

a topoisomerase-encoding operon. In contrast, the paralogous *topB* gene, encoding the second type I topoisomerase (topo III), was not affected by any mutation. In addition, we found several point mutations. Both strains carried a point mutation in the *mreB* gene, and an insertion of an A in the terminator region of the *yfhJ* gene. Mutations in *mreB* are frequently encountered in suppressor screens (11), and the mutation in the *yfhJ* terminator is not expected to have a phenotypic effect. Therefore, these mutations can be regarded as genetic hitchhikers. Moreover, strain GP1952 had a mutation in the *panC* gene resulting in an amino acid substitution (V128G) in the pantothenate synthase. The relevance of this mutation is unknown.

The suppressor strains grew well on LB plates, however, the colonies lysed after prolonged incubation. To observe the further evolution of one of the *topA* mutants, we passaged strain GP1948 several times on LB plates. This resulted in the appearance of papilla-like colonies. One of them (GP1958) was selected, and subjected to further rounds of passaging on LB plates. This again gave rise to papillae that survived longer periods of incubation on plates (GP1963). The latter strain was subsequently passaged several times in liquid medium, and single colonies were isolated after eight passages. Two of these colonies (GP1974 and GP1975) were chosen for further analysis. The genomes of the four suppressor strains were sequenced. Interestingly, all these strains carried the amplification around the *parEC* operon, however, both the boundaries and the copy numbers of the amplified regions differed from strain to strain indicating ongoing dynamic evolution (see Figure 1A). In the first evolved strain (GP1958), the amplified region was identical to that of the parent strain. The next round of evolution exemplified by GP1963 resulted in a shift of the amplified region to the 12 kb *citB-alsT* region with about 16 copies of the amplified region per genome. Further cultivation had no effect on the boundaries and copy number of the amplified region (see Figure 1A). This suggests that this state of the amplification provided sufficient genome stability for the bacteria.

Topoisomerase I is essential for *B. subtilis*

The results described above suggest that the deletion of the *topA* gene results in selection for amplification of the *parEC* genomic region. To get further evidence for this conclusion, we decided to construct a screening system that allows the rapid detection of the *parEC* amplification. For this purpose, we inserted a cassette encoding the green fluorescent protein and an erythromycin resistance determinant (*gfp-ermC*) under the control of a weak constitutive promoter immediately downstream of the *parEC* operon (see Materials and Methods for the details of strain construction). The resulting reporter strain, GP1966, exhibited a faint fluorescence (see Figure 2). We then deleted the *topA* gene, and tested fluorescence of the emerging colonies. Upon *topA* deletion from GP1966, all resulting colonies immediately showed an intense fluorescence suggestive of an amplification of the *parEC* chromosomal region. Indeed, whole genome sequencing of one of the resulting mutants (GP1979) confirmed the amplification of *parEC* chromosomal region (see Figures 1B and 2). In this strain, the 12 kb *yneP-xynC* region was amplified 15-fold. The observation that no single stable transformant with the deletion of *topA* showed a faint fluorescence, i.e. had the *parEC gfp* region in the single copy state, indicates that the *topA* mutant is not viable unless this genomic region has been amplified. Thus, our results support the conclusion that the *topA* gene is essential and suggest that topo IV encoded by *parEC* might be able to substitute for topo I.

The amplification of chromosomal regions depends on the functional recombination protein RecA. It has been shown previously that the lack of the RecA protein results in a drastic reduction of the amplification frequency in response to a selective pressure (25). In turn, the absence of RecA facilitates the detection of rare point mutations that may also suppress the original mutation. To test this idea for the suppression of the *topA* deletion, we intended to construct a *recA topA* double mutant to isolate new suppressor mutants. However, all attempts to construct such a strain failed, supporting the idea that *topA* suppression can only occur by amplification of the *parEC* region.

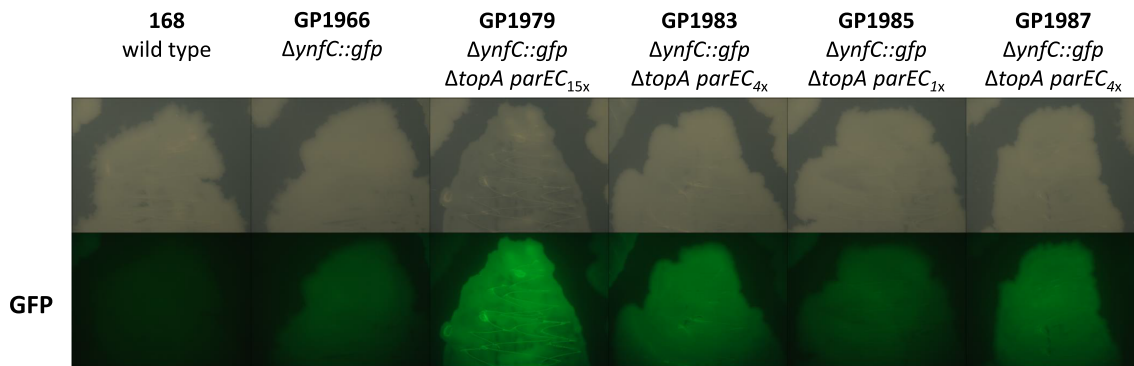


Figure 2. Visualization of the *parEC* copy number using the amplification reporter system. Colony images (upper panel) and GFP intensities (lower panel) of the different strains are shown. The indexed numbers in the genotypes indicate the copy number of the *parEC* chromosomal region. Please note that the single copy *parEC* operon present in the suppressor strain GP1985 carries a promoter-up mutation.

Interestingly, in *E. coli* loss of *topA* can also be suppressed by overexpression of *topB* (36). However, we failed to detect any mutants with increased *topB* expression. Therefore, we attempted to test suppression of the *B. subtilis topA* mutant by overexpression of *E. coli topB*. For this purpose, we have overexpressed plasmid-borne *E. coli* topo III (using plasmid pGP3121) in *B. subtilis*, and then attempted deletion of *topA* (see Figure 3). In this case, we observed that initially only a small part of the mutants had the *parEC* amplification (as judged by the GFP signal). In contrast, all *topA* mutants carrying the empty vector pHT01 had acquired the *parEC* amplification in the parallel control experiment. However, upon cultivation in liquid medium and re-isolation, many colonies exhibited fluorescence suggesting that they had acquired the suppressing amplification of the *parEC* region. This indicates that *E. coli topB* can indeed partially compensate for the loss of *topA* in *B. subtilis*. However, the eventual acquisition of the *parEC* amplification suggests that the suppression by *topB* is not sufficient and that the cells still experience a significant selective pressure to overexpress *parEC*.

Evolution of a strain carrying multiple *parEC* copies results in de-amplification of the *parEC* chromosomal region and the acquisition of point mutations

As shown above, it was impossible to obtain a *topA* mutant without concomitant amplification of the *parEC* locus suggesting that overexpression of topo IV compensates for the loss of topo I. However, strains with amplifications are genetically less stable, and we reasoned that our suppressor strains might become more stable with reduced copy number of the *parEC* locus. If this would be the case, one would expect a decrease in the brightness of fluorescence of the suppressor strain GP1979 upon long-term cultivation. To test this hypothesis, GP1979 (with 15 copies of *parEC*) was passaged ten times in LB medium. After each passage, a dilution series of the culture was plated on LB medium, and the colonies were screened for fluorescence intensity. After ten passages, we observed colonies with strongly reduced intensity of fluorescence, and one colony was as faint as the original strain GP1966 that contains only a single copy of the *parEC* region (see Figure 2). The strain with the strongest loss of fluorescence intensity (GP1985) as well

as two of the strains (GP1983 and GP1987) with reduced intensity were analyzed by genome sequencing. Strikingly, only one copy of the *parEC* region was present in strain GP1985 whereas the other two strains still harbored four copies of this genomic region. This finding confirms our hypothesis that the strain with the amplification undergoes evolution to reduce the copy number of the amplified region and thus to stabilize the genome. The reduction of the *parEC* chromosomal region copy number in the suppressor strains was accompanied by the appearance of novel point mutations. In GP1985, mutations resulting in amino acid substitutions in CodY (Ser129Leu), GltA (Val1181Ala) and RocG (Ser61Arg) were detected. In addition, this strain had a mutation in the upstream region of the *parEC* operon. This operon is preceded by a 315 nucleotides long leader RNA (RNA feature S672 according to 37), and the mutation changed the C at position 5 of the RNA to a T (see Figure 4A, note that primer extension experiments revealed that the mutation affected the promoter upstream of this RNA feature, see below). The mutations affecting CodY and RocG were also found in the strains with intermediate levels of amplification (GP1983 and GP1987). Interestingly, these strains were ambiguous concerning the sequence of the *parEC* upstream region. In these strains, we detected both the wild type sequence as well as the C5T variant of the S672 RNA feature. It is likely that the multiple copies of the *parEC* chromosomal region in these strains encode these different variants of the S672 RNA feature resulting in a mixed sequence at this position. Taken together, our results suggest that we have observed distinct steps in the evolution pathway of the *topA* suppressor mutants: The amplification of the *parEC* region is followed by the reduction in copy number (de-amplification) and the concomitant acquisition of point mutations that allow compensation of the *topA* deletion. In the second step, only one copy of the amplified region is retained, and the selected point mutations allow growth of the bacteria in the absence of topo I.

In the strain with the finalized de-amplification of the *parEC* region (GP1985), we found point mutations upstream of the *parEC* coding sequence as well as in genes encoding three presumably unrelated proteins (CodY, GltA, RocG). The mutation in the *parEC* leader mRNA might result in higher expression of this operon, similar to the am-

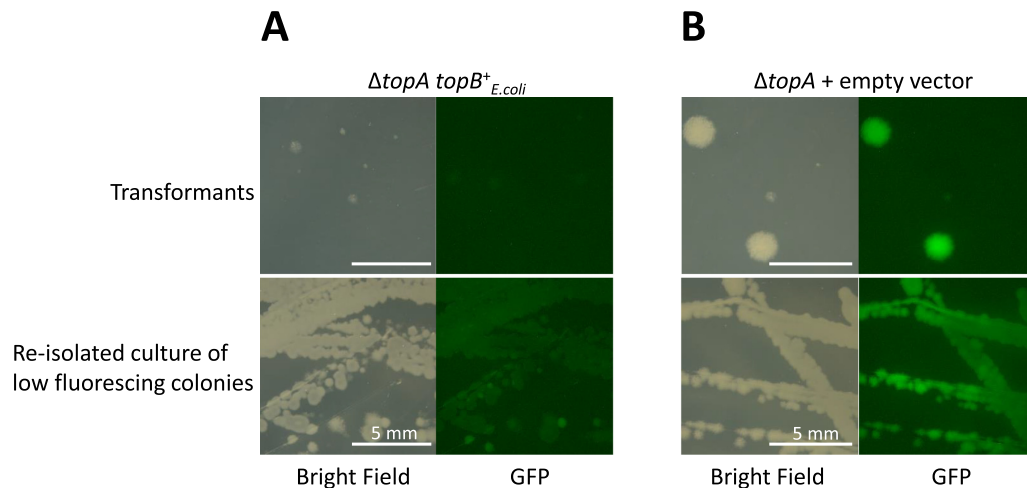


Figure 3. Suppression of a *topA* mutant by overexpression of *E. coli topB*. Strain GP1966 carrying the amplification reporter ($\Delta ynfC::gfp$) was transformed with a plasmid expressing the *E. coli topB* gene (A) or with the empty vector pHT01 (B). In these plasmid-bearing strains, the *topA* gene was deleted. The consequences for the amplification of the *parEC* chromosomal region were investigated using the *gfp* reporter gene inserted next to the *parEC* locus. Upper panel: initial transformants were photographed. Lower panel: Colonies exhibiting weak fluorescence were re-isolated, passaged in liquid LB medium, and re-analyzed for fluorescence.

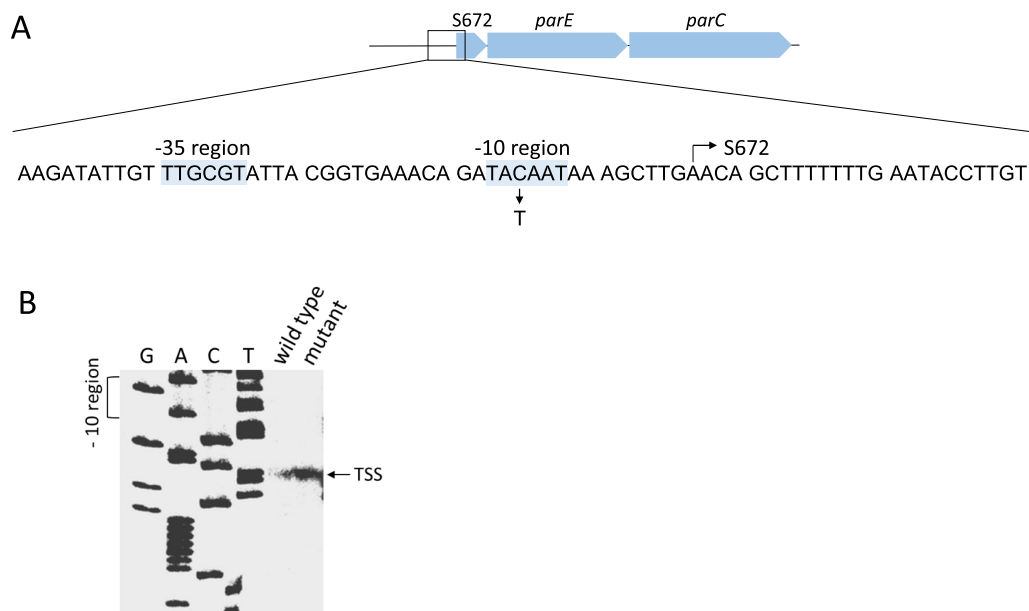


Figure 4. Genetic organization of the *parE* promoter region. (A) Structure of the *parE* promoter region. Sequence of the promoter region showing the -35 and -10 regions; as well as the transcription start site. (B) Reverse-transcriptase mapping of the transcriptional start point of the *parE* gene using the primer SB3204. Sequencing reactions were performed using the same oligonucleotide as primer.

plification of the region. This hypothesis suggests that the point mutation might be sufficient for the suppression. To test this idea, we used chromosomal DNA of the suppressor mutant GP1985 to transform the wild type strain *B. subtilis* 168. Transformants were selected for both kanamycin and erythromycin resistance, i.e. for the simultaneous transfer of both the *topA* deletion and the amplification reporter cassette downstream of the *parEC* operon. All transformants exhibited faint fluorescence excluding amplification of the *parEC* region. In 13 of these transformants, the chromosomal regions carrying point mutations in GP1985 (*codY*, *gltA*, *rocG*, S672) were sequenced. The C5T mutation up-

stream of *parEC* was present in all sequenced transformants, whereas *gltA* and *rocG* were wild type (one exception of transfer of the *rocG* mutation from GP1985). The *codY* alleles of the sequenced transformants exhibited variability: In most cases, the mutation from GP1985 was transferred with the resistance markers, and only in 25% of the transformants we observed the wild type allele of *codY*. The frequent transfer of the *codY* mutation is likely caused by the genetic linkage between *topA* (replaced by the *aphA3* resistance gene) and *codY* rather than by a selective pressure. One of the strains with only the point mutation upstream of *parEC* was selected for further experiments and designated

GP2547. This strain was subjected to whole genome sequencing. As expected, we found a single copy of the *parEC* region and the C5T point mutation upstream of *parEC*. Moreover, a SNP was present in the *yhcF* gene. Since this mutation did not result in an amino acid exchange, it is unlikely to be related to the suppressor phenotype.

To get independent confirmation for the reduction of the *parEC* copy number after the initial amplification, we passaged three further independently isolated suppressor mutants (GP2543, GP2544, GP2545) that carried the *parEC* amplification, until the intensity of the fluorescence caused by the concomitant *gfp* amplification was reduced to the levels observed for the original strain GP1966. For each of the three suppressor mutants, we tested several of the evolved strains for the presence of the *parEC* point mutation. Indeed, each independently isolated suppressor mutant gave rise to evolved strains that had acquired the same point mutation upstream of *parEC* as described above. Moreover, two of the evolved strains (one with the promoter mutation and one lacking it) were subjected to genome sequencing. In strain GP2549, we found the *parEC* point mutation accompanied by a point mutation in *rpoC* affecting the β' subunit of RNA polymerase (Thr891Ile). In the second strain, three copies of the *parEC* region (*yneP* ... *xynC*, see Figure 1) were present, and the *parEC* upstream region was identical to that of the wild type. This indicates that a triplication of the *parEC* genomic region is sufficient for the suppression of the loss of the *topA* gene.

Taken together, these results demonstrate that the original suppressor mutants reduce the copy number of the amplified region, and replace the amplification by a point mutation upstream of the *parEC* operon. This mutation is sufficient for the suppression of the loss of topo I in *B. subtilis*. This conclusion is supported by the observation of similar DNA supercoiling in the wild type strain 168 and in the suppressed *topA* mutant GP1985 (see Supplementary Figure S1).

The observed evolution of the strains upon *topA* deletion suggests that the physiological properties of the bacteria in the distinct states of evolution were different. To test this idea, we compared growth of the wild type strain *B. subtilis* 168, the wild type strain carrying the amplification reporter system GP1966, as well as of the *topA* mutant with the amplification of *parEC* or the point mutation upstream of the *parEC* operon (GP1979 and GP2547, respectively) (see Figure 5). The growth of the wild type strain and the wild type strain carrying the reporter construct was indistinguishable indicating that the loss of the *ynfC* gene and the introduction of the *gfp-ermC* reporter cassette did not compromise the physiology of the bacteria. In contrast, deletion of *topA* accompanied by the amplification of the *parEC* genomic region resulted in a severe delay of growth. Moreover, we observed a biphasic growth of the bacteria indicating a re-organisation of metabolism during growth. As expected for evolved bacteria, the strain with a single *parEC* operon copy but the point mutation in the upstream region (GP2547), grew significantly faster compared to the strain with the amplification, but slightly slower than the isogenic wild type strain (GP1966). However, this strain also exhibited diauxic growth indicative of a re-adaptation of the bacteria during cultivation (see Discussion). The growth properties of

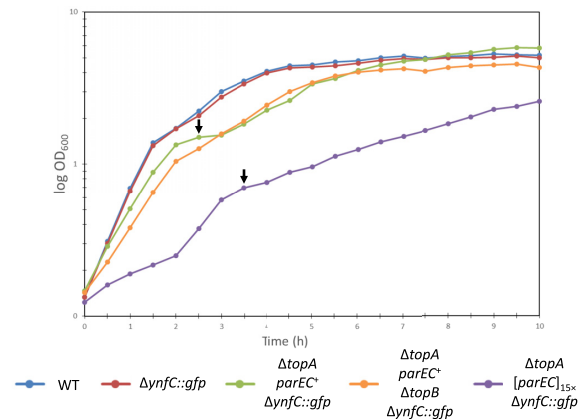


Figure 5. $\Delta topA$ suppressor strains exhibit reduced growth. Strains were grown in liquid LB medium for 10 h at 37°C and 240 rpm. The OD_{600} was measured every 30 min. Curves represent the average of three independent replicates. Arrows indicate a second lag phase indicating diauxic growth of the mutants. Genotypes: 168: Wild type. GP1966: $\Delta ynfC::P_{alf4-gfp-ermC}$. GP2547: $\Delta ynfC::P_{alf4-gfp-ermC} \Delta topA::aphA3 [parEC]_{15x} C \rightarrow T$ mutation in -10 region of *parEC* promoter. GP1979: *trpC2* $\Delta ynfC::P_{alf4-gfp-ermC} \Delta topA::aphA3 [parEC]_{15x}$. GP2548: $\Delta ynfC::P_{alf4-gfp-ermC} \Delta topA::aphA3 \Delta topB::cat [parEC]_{15x} C \rightarrow T$ mutation in -10 region of *parEC* promoter.

GP2547 are in good agreement with the observation that no further suppressor mutations could be isolated indicating that this strain has found the best possible response to the loss of topo I.

The mutation in the *parEC* upstream region affects expression of the *parEC* operon by increasing promoter strength

The results presented above suggest that the *parEC* amplification and the point mutation in the upstream region might result in increased *parEC* expression which then causes suppression of the *topA* mutation. To test this hypothesis, we compared the promoter activities of the wild type and mutant *parEC* upstream regions using fusions to a promoterless *lacZ* reporter gene encoding β -galactosidase. The reporter strains GP2550 and GP1967 carrying the wild type and mutant promoter fusions, respectively, were cultivated in LB medium and the β -galactosidase activities were determined. The wild type promoter gave rise to 85 ± 13 units mg^{-1} of protein. In contrast, the mutant promoter region exhibited a fivefold increased activity (498 ± 26 units mg^{-1} of protein). These results suggest that the expression of *parEC* is indeed increased in the mutant.

The increased promoter activity of the mutant upstream region present in the suppressor strain may result from the generation of a novel promoter or from increased activity of the original promoter. To distinguish between these possibilities, we determined the transcription start points in the wild type and the suppressor strain. Primer extension experiments with total RNA isolated from wild type strain 168 and the mutant strain GP1985 revealed that transcription started in both strains at the same position, a single A residue (see Figure 4B). Moreover, the signal intensity was strongly increased in the mutant strain indicating that the mutation results in a stronger promoter activity. Indeed, the mutation has altered the -10 box of the Sigma A-type

promoter from TACAAT to the perfect consensus –10 box TATAAT resulting in increased strength of the transcription initiation signal.

Overexpression of topo IV allows growth of *B. subtilis* in the absence of both type I topoisomerases

The *B. subtilis* genome encodes two paralogous type I DNA topoisomerases, the essential topo I (TopA) and the non-essential topo III (TopB). Our results indicate that *B. subtilis* is viable in the absence of the essential enzyme, provided that topo IV is overexpressed. However, it is not known whether *B. subtilis* is viable in the absence of both type I topoisomerases. To construct a $\Delta topA \Delta topB$ double knockout mutant, we used chromosomal DNA of a *topA* mutant to transform the *topB* mutant BKE04260. No transformants were obtained in this way. In contrast, transformation of the *topA* mutant GP1963 which exhibited amplification of the *parEC* region with chromosomal DNA of the *topB* mutant was possible and yielded the $\Delta topA \Delta topB$ double mutant GP1964 (see Discussion). Similarly, the double mutant lacking both type I topoisomerases could be generated based on the suppressor strain carrying the mutation upstream of *parEC* resulting in strain GP2548. These results suggest that overexpression of topo IV encoded by *parEC* is sufficient to allow growth of *B. subtilis* without any type I topoisomerase.

In *E. coli*, deletion of both *topA* and *topB* results in severe growth and chromosome segregation defects (38,39). To test whether this is also the case in *B. subtilis*, we determined the growth behaviour of the suppressed *topA topB* double mutant GP2548 in liquid medium and on plates. As shown in Figure 5, growth of the double mutant was very similar to the otherwise isogenic suppressed *topA* mutant GP2547. However, as the *topA* single mutant, the double mutant exhibited long filamentous cells. DAPI staining indicated that in some of these filamentous cells the chromosomes were segregated as in the wild type, whereas some cells had a segregation defect (see Figure 6). This segregation defect was not observed in the suppressed *topA* mutant (GP2547), and is therefore a result of the simultaneous loss of both type IA topoisomerases. This observation suggests that loss of topo III in addition to deletion of the *topA* gene also results in additional problems for the cell (DNA segregation), and this problem can not be solved by overexpression of topo IV.

DISCUSSION

DNA topoisomerases are the key players in the global control of chromosome conformation. Given their important functions in chromosome compaction and segregation during DNA replication, it is not surprising that these enzymes are essential for bacterial life. In the current study, we have investigated the essentiality of topo I encoded by the *topA* gene in the Gram-positive model organism *B. subtilis*. Our results demonstrate that topo I is essential unless topo IV can compensate for the loss of the *topA* gene.

In principle, all bacterial DNA topoisomerases of both types have both the relaxation and the catenation/ decatenation activity. However, each of the enzymes has different kinetic properties that finally result in the prevailing activities for controlling DNA supercoiling (topo I and gyrase)

and DNA catenation/decatenation (topo III and topo IV) (40–43, see Table 1). Topo I, topo III and topo IV are all able to relax hyper-negative supercoils behind RNA polymerase, and therefore both topo III and topo IV are candidates for the suppression of the loss of topo I. However, in our experiments we consistently observed that overexpression of topo IV may overcome the lack of topo I, whereas overexpression of topo III was never observed. A particular feature that distinguishes the two type I topoisomerases is their C-terminal domain. While topo I contains a zinc ribbon domain at the C-terminus, this domain is not present in topo III. In *E. coli*, the zinc ribbon domain interacts with the β' subunit of RNA polymerase thus bringing the relaxing activity of topo I to the site of transcription where its activity is needed (44). Interestingly, an interaction between topo I and RNA polymerase has also been observed in *B. subtilis* (45). Moreover, the C-terminal domain of topo I is also required for the recognition of hyper-negatively supercoiled DNA, the preferred catalytic substrate of topo I (46). The ability of multi-copy *E. coli* topo III to compensate for the loss of topo I both in *E. coli* and *B. subtilis* (36, see Figure 3) which was not observed with the *B. subtilis* protein suggests important differences between the topo III enzymes of the two bacteria.

As for *B. subtilis*, contradicting results have been obtained about the essentiality of topo I in *E. coli*. While a deletion of the *topA* gene was not possible in a genome-wide inactivation study (7), other studies reported that *topA* mutants were viable if the deletion of *topA* was accompanied by the simultaneous acquisition of suppressor mutations (47,48). Finally, one study suggested that *E. coli topA* mutants might be viable without any suppressing mutations (49). However, the genomes of the latter mutants have not been sequenced indicating that the presence of compensatory mutations cannot be excluded. In *E. coli*, *gyrA* or *gyrB* mutations resulting in reduced DNA gyrase activity and thus reduced negative DNA supercoiling support growth of the *topA* mutant (48,50). We did never observe mutations affecting DNA gyrase in our suppressor screens. Among all DNA topoisomerase genes, the *gyrAB* operon is most strongly expressed in *B. subtilis* (37) suggesting that mutations that reduce gyrase activity may not be tolerated in this organism. In addition, overexpression of either topo III encoded by *topB* or of topo IV encoded by the *parE* and *parC* genes allowed growth of the *E. coli topA* mutant (36,51,52). Similarly, overexpression of topo IV compensated for loss of topo I in *Shigella flexneri* (53). Based on our results using the amplification screen, we conclude that in *B. subtilis* the *topA* mutant is only viable if it acquires compensatory mutations, and that these compensatory mutations without any exception result in overexpression of the *parEC* operon encoding topo IV. Thus, the mutation landscape of possible *topA* suppressors is much narrower in *B. subtilis* than it is in *E. coli*. The reason for this difference is so far unknown.

Most bacteria contain type I and type II topoisomerases. While the type I enzyme TopA as well as both type II enzymes are individually essential in *B. subtilis*, the type I protein TopB (topo III) is not (8). It was therefore of particular interest whether the deletion of *topA* would make *topB* essential. Importantly, this was not the case if the effect of the

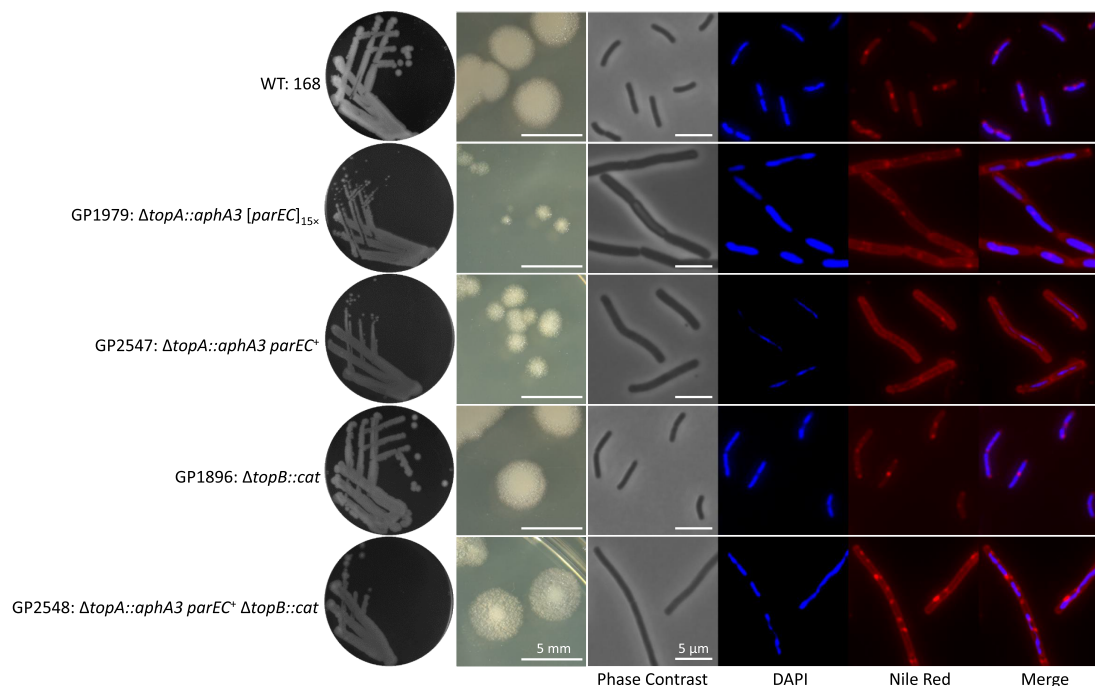


Figure 6. Phenotypic analysis of *B. subtilis* topoisomerase mutants. The wild type strain *B. subtilis* 168, the *topA* mutants GP1979 and GP2547, the *topB* mutant GP1896, and the *topA topB* double mutant GP2548 were analyzed for colony morphology (left and second left panel). Moreover, the cells were analyzed by phase contrast microscopy, for the nucleoid (DAPI stain), and for the cell membrane (Nile Red stain). The right panel shows an overlay of the DAPI and Nile Red stains.

topA deletion was suppressed by overexpression of ParEC. Similar to the *E. coli topA topB* double mutant (38), the *B. subtilis* strain lacking both type 1A topoisomerases exhibited filamentous morphology and had DNA segregation defects. As described earlier, this segregation defect may result from a redundant function of topo I and topo III during constitutive stable DNA replication to allow chromosome segregation (38).

In our work, we have observed either amplification of the *parEC* genes or a promoter-up mutation that results in about fivefold increased expression of the *parEC* operon. Moreover, DNA supercoiling was similar in the wild type and in the *topA* deletion mutant with increased *parEC* expression (see Supplementary Figure S1). Taken together, these data demonstrate that overexpression of topo IV is sufficient to compensate at least partially for the loss of topo I. This conclusion is in agreement with the results of *in vivo* studies in *E. coli* that demonstrated a five-fold reduced DNA relaxation activity of topo IV as compared to topo I (54).

One interesting result of this work is the observed diauxic growth of the suppressor mutants in complex medium (Figure 5). This observation suggests that the bacteria rapidly exhaust a particular nutrient from the medium, and that they experience difficulties in swiftly adapting their metabolism. In this context, it is interesting to note that we have observed large scale alterations in central metabolism in *B. subtilis* as a result of changes in DNA topology (11). Strikingly, glutamate biosynthesis was more efficient as a result of increased topo I activity which in turn may suggest that the expression of the *gltAB* operon encoding glutamate synthase is reduced upon loss of topo I. Interestingly,

we found point mutations in two genes involved in glutamate metabolism in the originally isolated suppressor strain that had lost the amplification of the *parEC* region. In this strain, GP1985, the GltA and RocG proteins involved in glutamate synthesis and degradation, respectively, carried mutations. While these mutations were not essential for the suppression they may contribute to improved growth. Indeed, previous work has shown that *B. subtilis* tightly controls glutamate homeostasis, by the regulation of gene expression, but also by the rapid acquisition of suppressor mutations (25,55,56). Interestingly, DNA topology also affects the synthesis of glutamine, an amino acid directly derived from glutamate in *E. coli* (57), suggesting a more general link between DNA topology and the synthesis of central amino acids.

In this work, we have established a screening system to detect chromosomal amplifications. Using this system, we were able to demonstrate that each individual stable *topA* mutant had acquired the amplification of the *parEC* region. Moreover, this system was very helpful in the analysis of the further evolution of the strains: after several passages we observed the reduction of the *parEC* copy number accompanied by the acquisition of a *parEC* promoter up mutation in some of the copies. In the final step, only one copy of the *parEC* region was present, and overexpression of *parEC* was now achieved by the stronger promoter. Based on the fact that about 10% of all cells in a bacterial population carry duplicated chromosomal segments (58), it is not surprising that strains with distinct amplifications of the *parEC* chromosomal region did readily appear. Similar results have been obtained with a *B. subtilis* strain lacking the transcriptional activator for the glutamate biosynthetic

genes. In such strains, the *gltAB* operon encoding glutamate synthase can be amplified up to 20-fold to compensate for the lack of transcription activation (25). Using our reporter system, the origin of mutations can be studied at the single cell level to the molecular details.

Taken together, our work demonstrates that the *topA* gene is essential unless the loss of topo I is compensated by the overexpression of topo IV. Increased expression of topo IV is even sufficient to replace both type IA topoisomerases, TopA and TopB. Interestingly, very similar results have very recently been reported for *E. coli*. In these bacteria, increased levels of topo IV that can relax excess negative supercoiling and prevent R-loop formation can substitute both type IA topoisomerases (59). Based on the results presented here, a set of three topoisomerases including topo I, DNA gyrase, and topo IV should be included in any minimal genome to obtain a strain that is able to grow rapidly on a complex medium.

DATA AVAILABILITY

The raw fastq reads of strains gp1948, gp1979 and gp1985 have been deposited in the NCBI-SRA database (<https://www.ncbi.nlm.nih.gov/sra>) and are accessible under the accession numbers srr7967955, srr7967956 and srr7967957, respectively.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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