Multiple links connect central carbon metabolism to DNA replication initiation and elongation in *Bacillus subtilis*

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**Abstract**

DNA replication is coupled to growth by an unknown mechanism. Here, we investigated this coupling by analyzing growth and replication in 15 mutants of central carbon metabolism (CCM) cultivated in three rich media. In about one-fourth of the condition tested, defects in replication resulting from changes in initiation or elongation were detected. This uncovered 11 CCM genes important for replication and showed that some of these genes have an effect in one, two or three media. Additional results presented here and elsewhere (Jannièrè, L., Canceill, D., Suski, C., et al. (2007), PLoS One, 2, e447.) showed that, in the LB medium, the...
CCM genes important for DNA elongation \((gapA\) and \(ackA\)) are genetically linked to the lagging strand polymerase DnaE while those important for initiation \((pgk\) and \(pykA\)) are genetically linked to the replication enzymes DnaC (helicase), DnaG (primase) and DnaE. Our work thus shows that the coupling between growth and replication involves multiple, medium-dependent links between CCM and replication. They also suggest that changes in CCM may affect initiation by altering the functional recruitment of DnaC, DnaG and DnaE at the chromosomal origin, and may affect elongation by altering the activity of DnaE at the replication fork. The underlying mechanism is discussed.

**Key words:** cell cycle, DNA replication, metabolism, signalling, integrative biology

1. Introduction

DNA replication must be coordinated with the cell cycle and cell division to ensure that chromosomes are faithfully replicated once and only once per cell cycle. This is carried out by overlapping mechanisms that control the activity of replication origins and replication enzymes for initiating DNA strand opening and recruitment of the replication machinery at origins every cell cycle.1–3

It has been known for several decades that replication is also subject to a metabolic control. In steady-state bacteria, this control couples the rate of replication to the rate of growth afforded by nutrients by modulating the initiation frequency and/or the speed of replication forks.4–7 The net result is a precise and reproducible timing of DNA synthesis in the cell cycle across a wide range of nutritional conditions. Intriguingly, this timing primarily depends on the energy and precursors extracted from nutrients rather than on the actual nature of the carbon and nitrogen sources, as *Escherichia coli* cells growing at the same rate in different media exhibit similar temporal replication patterns.7 The temporal control of replication in the cell cycle defines two major periods: the C period, which corresponds to the time taken to replicate the chromosome, and the D period, which corresponds to the time between termination of DNA replication and cell septation. At the growth rates analysed in this study (1–3 doublings/h (db/h)), the C and D periods are rather constant in *E. coli*8,9 while they substantially vary in *Bacillus subtilis* (see below). In chemostat cultures of *Saccharomyces cerevisiae* and possibly higher eukaryotes, the metabolic control confines DNA synthesis to the reduction phase of a redox metabolic cycle that is repeated several times per cell cycle.8–11

Despite a long history of investigation, the exact nature of the determinants involved in the metabolic control of replication remains elusive. Equally elusive is the mechanism at play and the way it acts in concert with classical control functions of replication initiation. The long-standing hypothesis is that the metabolic control of replication depends on the concentration of the active form of the replication initiator protein DnaA, linking CCM to initiation.32–34

Central carbon metabolism (CCM) extracts the precursors and energy needed for macromolecular synthesis and biomass production from nutrients. This breakdown process involves about 30 key reactions that are highly conserved across the phyla. CCM is fed at various positions by different metabolites and the metabolite entry point determines the polarity of the carbon flux travelling through it, either glycolytic or gluconeogenic. The CCM catabolic reactions are grouped in pathways of which glycolysis, gluconeogenesis, the pentose phosphate pathway and the tricarboxylic acid (TCA) cycle form the main routes for breaking down nutrients (see the schematic representation in Supplementary Fig. S1). By directly sensing the supply and the demand in biosynthetic reactions, CCM is in a strategic position for producing signals that cells may use for adapting the main cellular activities to nutrient richness as recently demonstrated for cell division in *E. coli* and *B. subtilis*.26–28

A role for CCM in replication was first suggested in the model organisms *B. subtilis*, *E. coli* and *S. cerevisiae* by a series of related genetic studies that analysed the viability of thermosensitive replication mutants at high temperature in cells compromised in CCM genes. In *B. subtilis*, this analysis uncovered strong genetic links between the 3-carbon part of the glycolysis/gluconeogenesis pathway (i.e. gapA, pgk, pgn, eno and pykA see Supplementary Fig. S1) and three replication enzymes, namely the DnaC helicase, DnaG primase and DnaE polymerase.29 Significantly, these three replication enzymes are loaded early at oriC during the initiation of replication and form a ternary complex in the replisome to carry out DNA melting and lagging strand synthesis.30–31 This would make these enzymes good target candidates for modulating replication initiation and elongation in response to changes in CCM activity. In *E. coli*, studies have uncovered strong genetic links between *pta*, *ackA* and genes encoding pyruvate dehydrogenase subunits (*fdA* and *aceE*) (Supplementary Fig. S1) and the replication initiator protein DnaA, linking CCM to initiation.32–34

In the budding yeast, three glycolytic enzymes (HXK2, ENO2 and *pykA*) are genetically linked to the lagging strand synthesis.30–31 This would make these enzymes good target candidates for modulating replication initiation and elongation in response to changes in CCM activity. In *E. coli*, studies have uncovered strong genetic links between *pta*, *ackA* and genes encoding pyruvate dehydrogenase subunits (*fdA* and *aceE*) (Supplementary Fig. S1) and the replication initiator protein DnaA, linking CCM to initiation.32–34

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2. Materials and methods

2.1. Strains and plasmids

Strains and plasmids are listed in Supplementary Table S1. B. subtilis cells were grown at 37°C in LB supplement or not with malate 0.2% or in minimal medium (KH2PO4: 80 mM; KH2PO3: 44 mM; (NH4)2SO4: 15 mM; CaCl2·2H2O: 3.4 mM; MgSO4: 2 mM; FeIII citrate: 11 μg/ml; MnCl2: 10 μM; FeSO4: 1 μM; FeIII: 4 μg/ml; Trp 50 μg/ml) supplemented with glucose 0.4%, casein hydrolysate 0.2%, malate 0.4%, glutamine 0.4%, proline 0.4% and/or succinate 0.4%, as listed in Supplementary Table S2. Unless stated otherwise, antibiotics were used at the following concentrations: spectinomycin (Sp, 60 μg/ml); kanamycin (Km, 5 μg/ml); erythromycin (Em, 0.6 μg/ml); chloramphenicol (Cm, 5 μg/ml); phleomycin (Pm, 10 μg/ml). Liquid cultures were performed in a water bath under strong shaking (200–230 rpm). The LB medium is a complex rich medium that supports steady-state growth at OD600 nm < 0.3 and a gradual decrease of the growth rate up to saturation (OD600 nm > 3).41 The regimen is glycolytic in the first part of the growth curve as shown by direct measurement of the activity of promoters sensitive to the glycolytic (gapA promoter) and neoglucogenic (pckA and gapB promoters) carbon flux.29 The E. coli plasmid pMUTIN2-Pm is a derivative of pMUTIN2 (EmR) in which the erm gene was replaced by a Pm (pomr) marker. The plasmid pLJH88 and pLJH89 were constructed by replacing the small HindIII-Nhel fragment of the E. coli plasmid pDR111 (SpcR)45 by PCR HindIII-Nhel fragments produced from pALS1046 containing the relA or relA ori/ter ORFs previously ribosomed-binding site, respectively. The relA constructions were then inserted at the amyE locus of the B. subtilis chromosome by double crossover event to permit RelA and RelAi expression from the IPTG-dependent Papank promoter. The plasmid pLJH90 is a CmR derivative of pLJH88. It was constructed by replacing the spc-containing HindIII-SacII fragment of pLJH88 by the cat-containing HindIII-SacII fragment of pDG1661.47 The plasmids pLJH92 and pLJH93 are derivatives of pMTLBs7 containing the cat gene. This was done by inserting the marker frequency analysis (see Supplementary Table S3 for details) were used at 200 μM in 12 μl of the 1× SYBR qPCR Premix Ex Taq (Tli RNaseH Plus) (Ozyme, Saint Quentin en Yvelines, France) and DNA amplification was carried out as follows: denaturation: 95°C, 30 s; amplification: (33 cycles) 95°C, 5 s and 60°C, 1 min. For every qPCR reaction, the primer efficiency (97 to 103%) was controlled. The oriter ratio of the non-replicating sporelets was 0.585 ± 0.019, a value consistent with the size of the PCR products (Supplementary Table S3). The amplifications were carried out on a Mastercycler® ep realplex (Eppendorf, Hamburg, Germany) and the ACq were determined automatically from the cycle threshold.

The pgmIP and enoLP metabolic mutants (DGRM335 and DGRM334, respectively) are thermosensitive (Supplementary Fig. S2). To determine the effect of these mutations on cell cycle parameters, the following procedure was followed. The strains were precultured overnight at 30°C in triplicates, diluted 1,000-fold for growth at restrictive temperature (47°C for pgmIP and 45°C for enoLP) and cell cycle parameters (growth, oriter ratio and the C period) were determined as above. Results (Supplementary Table S4) were then used to calculate the corresponding parameters at 37°C. For this, we first analysed the effect of temperature on growth, the oriter ratio and C period of the wild-type (TF8A), Δysf (DGRM330) and ΔytoI (DGRM360) strains (these mutations are present in the pgmIP and enoLP strains, Supplementary Table S1). Results showed that temperature has nearly no effect on the oriter ratio while it changes the growth rate and the C period in a similar way in the three strains (Supplementary Fig. S3), as expected from previous studies.11,13 They in addition showed that the Δysf and ΔytoI mutations have no effect on the growth-oriter ratio coupling (compare data...
Supplementary Fig. S3 37°C with Figs 1 and 2). The equation of the slopes Supplementary Fig. S3 were then used to derive the cell cycle parameters of the thermosensitive CCM mutants at 37°C (Supplementary Table S5).

2.2.2. Measurement of the C period

Cell growth, genomic DNAs extraction and amplification reactions were as above. Primer pairs, their positions on the chromosome map and the size of the PCR products are given Supplementary Table S3. Results were reported to the terC signal and corrected for the size of the PCR products. The C period was extracted from the slope of the straight line of the plot (log2(Nm)) against m where N is the relative copy number of a sequence N at position m (with 0 < m < 1 along the ori-ter axis) and t the generation time in minutes. Control experiments showed that the marker frequency of the different loci in the non-replicating DNA was close to 1 (1.011 ± 0.045) as expected. Similar data were obtained with primers on the right and left arm of the chromosome.

2.3. Flow cytometry analysis

2.3.1. Measurement of the number of origins/cell

Strains were grown as indicated in the previous section and at OD650 nm = 0.1-0.15, chloramphenicol (200 µg/mL final) was added to the cultures to impede replication initiation, cell division and allow completion of ongoing rounds of replication. After 4-6 hours of drug treatment, 10⁸ cells were fixed in filtered ethanol 70% and stored at 4°C. Stored cells were then washed in filtered 0.1 M phosphate buffer (PB) pH 9, resuspended in 1 mL of Tris-buffered saline buffer (TBS 150, filtered) (20 mM Tris-HCl pH 7.5, 150 mM NaCl) and stained with Hoechst 33258 (1.5 µg/mL) for at least 30 min as described elsewhere. Flow cytometry analysis was performed using a MoFlow Astrios cell sorter (Beckman Coulter, Life Sciences) equipped with a 355 nm krypton laser and a 448/59 nm bandpass filter used to collect Hoechst 33258 fluorescence. Data were analysed with the Kaluza software (Beckman Coulter, Life Sciences). We counted 50,000 to 100,000 events. In most of the tested samples, DNA histograms showed 1-2 main peaks with a 2ⁿ distribution. The number of origins/cell was obtained from the proportion of cells with 1, 2, 4, 8 and/or 16 chromosomes.

2.3.2. Measurement of the D period

The D period was calculated from the equation D = [ln(ori/cell)]/τ - ln(2) – C where τ is the generation time and C the C period.

2.4. Suppression assay

Three independently constructed mutants containing a dna(Ts) and the ackA deletion were grown overnight in LB at 30°C. Appropriate dilutions were then spread on solid LB medium and incubated at permissive (30°C) or restrictive temperature as indicated previously.

2.5. Microscopy analysis

For analysing the production of filament in dna(ts) mutants, cells were grown overnight at 30°C in LB and diluted 500-fold in the same medium for further growth at permissive or restrictive temperature. After 2 hours of incubation, cells were collected and the nucleoid was stained with 4',6'-diamidino-2-phenylindole (DAPI, 10 µg/mL) (Sigma). For analysing the effect of (p)ppGpp production on cell size, relevant strains were grown at 44°C in LB in the presence of different concentrations of IPTG. After about 10 generations of growth and at OD₆₅₀ nm = 0.4, cells were collected and the membrane was stained by FM5-95 (Biotium Inc, Fremont, CA). Stained cells were then mounted on glass slides (VWR) covered with 1.2% agarose (low melting point, Sigma) in minimal medium supplemented with malate 0.2% and cas 0.2% and a 0.17 mm glass coverslip (VWR) was placed on top. The microscopy was performed on an epifluorescence microscope (Zeiss, Axio Observer.Z1) with a ×100 magnification oil-immersion objective (Plan-APOCHROMAT Ph3) and a CMOS camera (Orca Flash 4.0 LT Hamamatsu). Digital images were acquired and analysed using Zen 2012 software. All experiments were independently performed at least three times and representative pictures are shown.

2.6. (p)ppGpp analysis

The (p)ppGpp production in WT cells and (p)ppGpp mutants was analysed before and after induction of the stringent response by serine hydroxamate for E. coli and arginine hydroxamate (250 µM) for B. subtilis as previously described.
Figure 2. Identification of CCM mutations important for the growth-replication coupling in rich media. Top panels: Growth-ori/ter ratio and growth-C period coupling in CCM mutants grown in rich media (LB, LB + malate and malate + casa) (see Supplementary Table S5 for details). Closed dots and dotted line: results from wild-type cells (Fig. 1); Green and red symbols: CCM mutants with parameters that deviate from the typifying curve by less or more than 20%, respectively. Bars in the growth-ori/ter ratio relationship stand for mean and standard deviation obtained from at least three independent biological repeats (obtained generally from independent constructs) and two technical repeats (SD/mean < 10%). Bottom panels: Green symbols: CCM genes dispensable for both the growth-ori/ter ratio and growth-C period relationships (class I mutants). Red: CCM genes important for both relationships (class II mutants). Blue: CCM genes important for at least one relationship (class III mutants). zwf: glucose 6-phosphate dehydrogenase; ptsG: phosphoenolpyruvate: carbohydrate phosphotransferase enzymeIICBA; pgi: glucose 6-phosphate isomerase; gapA: glyceraldehyde-3-phosphate dehydrogenase, NAD-dependent; gapB: glyceraldehyde-3-phosphate dehydrogenase, NADP-dependent; pgk: phosphoglycerate kinase; pgm: phosphoglycerate mutase; eno: enolase; pykA: pyruvate kinase; pdhB: pyruvate dehydrogenase (E1 beta subunit); pdhC: pyruvate dehydrogenase (dihydrolipoamide acetyltransferase E2 subunit); pta: phosphotransacetylase; ackA: acetate kinase; citC: isocitrate dehydrogenase; mdh: malate dehydrogenase; ytsJ: malic enzyme (NADH-producing); pckA: phosphoenolpyruvate carboxykinase; pycA: pyruvate carboxylase; pps: phosphoenolpyruvate synthase.
3. Results

3.1. Growth-replication coupling in wild-type cells

A hallmark of growing populations of *E. coli* and *B. subtilis* cells is the concurrent increase in the rate of growth and DNA synthesis. This coupling implies that (i) bacteria growing slowly in poor carbon sources host a chromosome that either is not being replicated or is undergoing a single round of replication while (ii) fast-growing bacteria in rich media contain multi-forked chromosomes undergoing up to three overlapping replication cycles. To characterize in more detail the growth-replication relationship in *B. subtilis*, we quantified cell cycle parameters of a wild-type strain growing at rates ranging from 0.5 to 3 doublings per hour (db/h) in different media. This strain (TF8A) and the derivatives used below, are free of prophages to avoid any interference of phage induction on chromosomal replication. Growth was assessed by spectrophotometry, and DNA replication by marker frequency using qPCR. In the latter analysis, the amount of replication terminus (ter) sequences was compared with the amount of 18 other loci regularly scattered along both arms of the chromosome and including the replication origin (*ori*). This gave us two major replication parameters: the relative copy number of *ori* versus *ter* sequences (the *ori/ter* ratio) and the *C* period. This latter parameter was extracted from the slope of the straight line of the plot \( \log(N_m) \) against \( m \) where \( N_m \) is the relative copy number of a sequence \( N \) at position \( m \) (with \( 0 < m < 1 \) along the *ori-ter* axis) and \( \tau \) the generation time in minutes. The results show a strong positive correlation between growth and the *ori/ter* ratio, as expected (Fig. 1, left panel, Supplementary Table S2). They also show a significant negative correlation between growth and the *C* period: the time taken to replicate the chromosome increases from about 40 to 50 min when the growth rate decreases from 3 to 0.5 db/h. Calculation of the *C* period (C) from the *ori/ter* ratio and \( \tau \) using the formula \( \text{C} = \frac{2N_0}{\ln(2) \cdot \tau} \) confirms this negative correlation (Supplementary Table S2).

To get further insights into the growth-replication relationship, the number of origins/cell and the *D* period of the wild-type cells grown in various media were measured by flow cytometry. To that goal, we first analyzed the size of events detected by the flow cytometer in order to discriminate single cells from the chains of cells that often accumulate in fast growing *B. subtilis* cultures. This entailed using density plots of area versus width of fluorescence signals generated by cultures stained with a DNA specific dye (Hoechst 33258). Representative results are presented Supplementary Fig. S4 (left panels). The width axis corresponds to the flight time of particles through the laser beam spot. It reveals small (S) and large (L) events and that the proportion of large events strongly increases (from 1 to up to 60%) with the medium richness and growth rate. The area axis gives the integral of fluorescent signals. It shows that the intensity of fluorescence signals of small events varies while that of large events is rather constant and strong. Taken together, these results show that small events correspond to single cells while large events correspond to cell-chains and/or to clump of cells.

The samples used to measure the number of origins/cell and the *D* period corresponds to exponentially growing cultures treated for 4–5 hours with Cm. This treatment allows completion of ongoing replication cycles but prevents re-initiation of new rounds of replication and cell division. Hence, at the end of the treatment, cells contain an integral number of fully replicated chromosomes that corresponds to the number of origins at the time of drug addition (replication run-out assay). It is of interest to note here that the proportion of cell-chains in Cm-treated cultures is greatly reduced (to 1–5%) as shown by density plots in Supplementary Fig. S4, right panel. DNA histograms of Cm-treated, single cells (Supplementary Fig. S5) show a smooth increase (from 2 to 6) of the number of origins/cell at growth rates ranging from 0.5 to 2 db/h followed by a stronger increase and a plateau to 9 origins/cell for cells growing at rates >2 db/h (Fig. 1, right panel and Supplementary Table S2). The *D* period, calculated from the equation \( D = \log(\text{ori/ter}) + \text{C} \), varies with the growth rate in *B. subtilis* as expected (Fig. 1 and Supplementary Table S2).

Overall, the cell cycle parameters determined here show that the coupling between growth and replication in *B. subtilis* cells growing at rates >0.5 db/h depends on a concerted modulation of the initiation and elongation phases of replication. In agreement with *E. coli* studies, they also support the hypothesis that this coupling depends on the precursors and energy extracted from nutrients rather than on the nature of available carbon sources.

3.2. Identification of CCM genes important for the growth-replication coupling

To look for a possible involvement of CCM in the growth-replication couplings, we analyzed cell cycle parameters of CCM mutants grown in rich media and compared the results with data obtained for the wild-type strain grown in different carbon sources. We assumed that this approach is relevant as coupling depends on the energy and precursors extracted from nutrients and not on the amount and nature of nutrients available in the environment (see above). This assumption is further supported here by a coupling analysis in *B. subtilis* cells extracting energy and precursors from a rich medium (LB) at different rates because of inhibition of ribosome production. Such an assay was developed in *E. coli* using cells expressing a truncated form of the *E. coli* (p)ppGpp synthetase RelA from an inducible promoter. This truncated protein (termed RelAt) synthesizes constitutively the inhibitor of ribosome synthesis (p)ppGpp without the need for metabolic stress and binding to idling ribosomes. This genetic system was proved to modulate *E. coli* growth rate and cell size in LB in an inducer-dependent manner. To develop a similar approach in *B. subtilis*, the *E. coli* relA gene was inserted in the *B. subtilis* chromosome and put under the control of an IPTG inducible promoter. A related strain expressing an inactive form of *E. coli* RelA (RelAt) was constructed as a control. As in *E. coli*, an inverse correlation between (p)ppGpp production, growth rate and cell size was found in *B. subtilis* (see Supplementary data and Supplementary Fig. S6). Measurement of the *ori/ter* ratio shows a wild-type coupling over the large range of growth rates generated by degrees of (p)ppGpp production in LB (Supplementary Fig. S7). Hence, *B. subtilis* sets the replication rate according to the growth rate determined by its metabolism and not by the richness of the growth medium. It is thus reasonable to compare data from wild-type cells grown in various media to data from CCM mutants grown in rich media. This underpins our hypothesis that CCM mutants growing at various rates in rich media will have a wild-type growth-replication relationship unless the metabolic mutation alters cell cycle parameters.

We used a library of 25 CCM mutants and three rich media to analyze the growth-replication coupling in 53 metabolic conditions.
The mutations correspond to deletions (16), point mutations (5) or inducible transcriptional fusions (4). They decrease or abolish the activity of 19 genes (i.e. two-thirds of the key CCM genes; see Supplementary Table S1 for details on the strain library) that map in glycolysis (ptsG, pgi, gapA, pgk, pgm, eno and pykA), gluconeogenesis (pckA and gapB), the pentose phosphate pathway (xylE), the TCA cycle (cirC and mdhB), the overflow pathway (pta and ackA) and in the pyruvate metabolism (pdhB, pdhC, ytsJ, pycA, ppc and pps) (see the CCM map Supplementary Fig. S1 for details). The rich media (LB; LB + malate and the minimal medium + malate + casamino acids) feed both the upper and lower parts of CCM and support the growth of mutants at rates ranging from about 0.5 to 3.5 dB/h (see Materials and Methods and Supplementary Table S5 for details) (note that the ΔpdhB and ΔpdhC strains did not grow in malate + casamino acids and that the Δpgi mutant was not analysed in LB and LB + malate as cells had abnormal shapes in these media – not shown).

The growth rate, oriter ratio and C period in the 53 metabolic conditions were measured and compared with curves typifying the growth-replication coupling in wild-type cells (Fig. 2, top panels and see Supplementary Table S5 for details). Based on the SD/mean of LBþ growth-replication coupling in wild-type cells (Fig. 2, top panels and media (Fig. 2, bottom panels). These genes are located in the main routes of carbon source degradation: the 6- and 3-carbon part of glycolysis/gluconeogenesis, the pentose phosphate pathway and the overflow pathway. Interestingly, the sets of genes important for replication differ from one medium to another with some genes being involved in just one medium (zef, pgi, gapA, gapB, pta and mdhB), and others being involved in two (pgk, pykA and ackA) or in three (eno and pgm) media (Fig. 2, bottom panels). It can thus be inferred that CCM is connected to DNA replication through multiple links that are committed in replication in a medium manner.

3.3. CCM mutations affect the initiation or elongation phase of replication

To determine which replication step is defective in CCM mutants, we constructed a series of strains replicating their genome from a plasmid replicon rather than from the chromosomal origin oriC. The replicon used (termed oriN thereafter) originates from the low copy-number plasmid pLS32. It encodes an initiator protein RepN that binds the plasmid origin embedded in the repN gene to initiate replication.61,62 In addition to RepN, initiation at oriN depends on the host-encoded factors DnaD, DnaB (two helicase loaders), DnaC (the helicase) and DnaN (the DNA polymerase clamp). It is however independent of the initiator DnaA and the oriC sequence.63 Once assembled at oriN, the initiation proteins (RepN, DnaB, DnaC and DnaD) recruit the rest of the chromosomal replication machinery to carry out bidirectional replication. In the series of strains constructed here, the plasmid replicon was inserted 2 kb to the left of the chromosomal origin and normal initiation of chromosomal replication was inactivated by introducing three ochre mutations into the DnaA-encoding gene or by deleting oriC sequences.63,64

The plasmid replicon used responds poorly to differences in carbon sources13 (see below). This means that if uncoupling between growth and replication in CCM mutants were to result from changes in initiation (i.e. the productive interaction of DnaA with oriC), cells replicating their genome from the plasmid replicon would not suffer from the metabolic mutation and would thus exhibit the coupling of oriN-dependent cells, wild-type for metabolic functions. In contrast, if uncoupling were to result from changes in DNA elongation (or in an initiation event downstream of the formation of an active DnaA/ oriC complex), plasmid replicon-dependent cells would still suffer from the metabolic mutation and would thus not exhibit the coupling typical of the CCM+ oriN-dependent cells (i.e. growth and replication would be uncoupled).

We first analysed the growth-oriter coupling in dnaAΔtyp or ΔoriC cells (which replicate the chromosome from oriN) growing in different media. As a control, an isogenic strain containing oriN and a functional chromosomal origin (DnaA+ OriC+) was analysed. The results show that this control strain (which contains oriN but does not depend on it) had the strong, positive relationship between growth and the oriter ratio characteristic of B. subtilis (Supplementary Fig. S9, top Table and panel) whereas oriN-dependent cells had a much weaker positive relationship (Supplementary Fig. S9, bottom Table and panel). This weaker response is probably because the plasmid replicon (i) is relatively insensitive to differences in the richness of media and (ii) cannot support high growth rates since it is unable to provide the high frequency of DNA initiation events required in rich media.

To determine which replication step is defective in CCM mutants, the growth-oriter ratio coupling in oriN-dependent derivatives was analysed as above. This analysis was focused on class II metabolic conditions where both the oriter ratio and the C period are affected at 37°C (i.e. the class II metabolic conditions affecting replication at high temperature and involving the pgmIP and enoLP mutations were not tested here, as the replication activity of the oriN replicon at high temperature is not known). Out of the nine tested metabolic conditions, four give a coupling typical of oriN-dependent cells while five exhibit a perturbed oriN-type coupling (Fig. 3 and
As reasoned above, the loss of the uncoupling phenotype in oriN-dependent CCM mutants shows that the corresponding CCM mutations affect initiation (i.e., the formation of an active DnaA/oriC complex) at the chromosomal origin in oriC-dependent strains, while the persistence of this phenotype in oriN-dependent cells shows that the CCM mutations affect DNA elongation (or in a step downstream of the formation of an active DnaA/oriC complex) in the parental oriC-dependent strains. This interpretation is strengthened by control experiments showing that the assay is not sensitive to the way initiation at the chromosomal origin is abrogated (by the dnaAochre or DoriC mutation, Supplementary Fig. S10).

Collectively, our results reveal that CCM genes are connected to the initiation (i.e., the formation of an active DnaA/oriC complex) or elongation (or an initiation step downstream of the formation of an active DnaA/oriC complex) phase of replication in a medium-dependent manner (Fig. 3). Although we investigated a limited number of class II conditions, links between CCM genes and different key replication phases were found in two out of the three tested media. This suggested that the mechanism of the growth-replication coupling in B. subtilis, which depends on a concerted modulation of the initiation frequency and elongation velocities (Fig. 1), may involve the combined effect of groups of CCM genes on the initiation and elongation phase of replication. This hypothesis is supported by the results presented below.

### 3.4. Genetic links between CCM and replication genes in LB

The cell cycle studies of oriN-dependent cells presented above show that the genes encoding catalysts of the terminal reactions of glycolysis (gapA, pgk, pgm, eno, and pykA) and of downstream reactions (pta and ackA) are important for replication initiation (i.e., the formation of an active DnaA/oriC complex) or elongation (or a step downstream of the formation of an active DnaA/oriC complex) in the LB medium as summarized Fig. 4A, right part of the panel. Intriguingly, a previous study carried out in the same medium showed that gapA, pgk, pgm, eno and pykA are also genetically linked to the replication genes dnaC, dnaG and dnaE, while gapA is genetically linked to dnaE30 (Fig. 4A, left part of the panel). Interestingly, the enzymes encoded by these replication genes (the helicase DnaC, the primase DnaG and the lagging strand polymerase DnaE) were recently shown to be required for the initiation and elongation phase of replication.30,31,65–69 Indeed, during initiation of DNA replication, the conserved replication initiator DnaA protein, aided by the initiation factors DnaB, DnaD and Dnai (a helicase loader), melts the origin, oriC, to mediate anti-parallel loading of two hexameric rings of the DnaC helicase, one on each melted strand.30 The primase, DnaG, and the lagging strand polymerase, DnaE, are then recruited to synthesize RNA primers and start DNA polymerization.30,31 The remaining replisomal components, including the clamp, DnaN,
Figure 4. Suppression of dnaE(Ts) mutations in the ΔackA context. (A): Functional (dark lines) and genetic (dotted lines) links between (i) CCM genes, (ii) the initiation and elongation phase of replication and (iii) the DnaC, DnaG and DnaE replication enzymes. (B): Viability and microscopy analysis of a ΔackA dnaE(Ts) double mutant. Wild-type cells and strains carrying the thermosensitive dnaE2.6 mutations combined or not with the deletion of the ackA gene were grown in LB at permissive temperature. They were then streaked on LB plates or inoculated in liquid LB broth and then incubated at permissive or restrictive temperature. Left panels: colony formation at permissive (30°C) and restrictive (42°C) temperatures. Right panels: Microscopic analysis of cells grown at permissive or after 2 hours of incubation at restrictive temperature. Merge images of phase contrast and DAPI stained nucleoid (colored in blue). White arrows: anucleated minicells.

4. Discussion

We report here on a rather large study aimed at better understanding the important and likely universal relationship between central carbon metabolism (CCM) and DNA replication. To achieve this, we characterized the cell cycle parameters in three rich media of a collection of 25 CCM B. subtilis mutants. The results reveal defects in the initiation and/or elongation phase of replication in about one-third of the metabolic conditions tested. These defects are rather strong, causing a significant increase in the concentration of the origin sequences per cell and an up to 2-fold decrease in the mean velocity of replication forks. We infer from these results that CCM is important for replication and, in particular, for the coupling between growth and replication that positions the replication cycle within the cell cycle. These roles for CCM in replication are even more surprising as the replication phenotypes reported here were found in cells that are fully proficient in replication enzymes and replication control functions.

Overall, 11 genes mapping in different CCM pathways (the pentose-phosphate, glycolysis/neoglucogenesis, TCA and overflow pathways) appear to be important for replication in rich media (Fig. 2). Interestingly, their commitment in replication is medium dependent, forming partly overlapping subgroups with some genes only being needed for a single medium and others being needed for two or three media (Fig. 2). Moreover, some CCM genes are important for initiation while others are important for elongation or for both replication steps (pgk) (Fig. 3). Taken together, our results suggest that cells evolved a toolbox of intermingled CCM-replication links that it partially activates to cope with requirements imposed by the environment.

The results reported here show that the coupling between growth and replication in wild-type cells depends both on the modulation of the frequency of initiation and the mean velocity of replication forks (Fig. 1). Studies on CCM mutants confirm this conclusion (Figs 3 and 4) and suggest that this coupling may depend on both CCM-initiation and CCM-elongation links. It is thus tempting to speculate that these links are part of the metabolic control of DNA replication in B. subtilis.

Additional results presented here and in a previously publication uncover strong genetic links between six CCM genes (gapA, pgk, pgm, eno, pykA and ackA) and three replication genes (dnaC, dnaG and dnaE) in cells growing in the rich LB medium (Fig. 4A). These links allow CCM mutations to restore the viability of thermosensitive replication mutants at restrictive temperatures. Interestingly, the links were suggested to depend on metabolic-induced conformational changes that restore the activity of the thermosensitive replication enzymes at restrictive temperature. They however may not dependent on a (i) deep change in the replication machinery that make thermosensitive replication enzymes dispensable for replication, (ii) the overexpression of dna(Ts) genes, (iii) a decay in the degradation of the thermosensitive replication protein, (iv) a reduction in ATP concentration or (v) an induction of a stress response.
Moreover, the replication enzymes responding to CCM alteration encode the helicase DnaC, the primase DnaG and the lagging strand polymerase DnaE that are loaded early at oriC during the initiation of replication and form a ternary complex in the replisome for carrying out DNA melting, RNA priming and essential polymerase functions in lagging strand synthesis. 30,31 Although the suppression assay and the CCM-replication studies are based on distinct approaches and concepts, their combination gives us for the first time a coherent picture on how cell’s metabolism and DNA replication may be coupled in LB. Indeed, results showed that at least two metabolic genes important for initiation (pykA and pykA) are genetically linked to dnaC, dnaG and dnaE while CCM genes important for elongation (gapA and ackA) are genetically linked to dnaE (Fig. 4). This suggests that, in the LB medium, CCM may modulate initiation by altering the functional recruitment of DnaC, DnaG and DnaE to oriC and may modulate DNA elongation by altering the activity of the polymerase DnaE in replication forks. This latter hypothesis is consistent with data showing that fork progression depends on lagging strand operations71–74 and that the rate of DNA melting by helicases depends on the speed of DNA polymerases.75–77 Along this line, the physical interaction between the helicase DnaC and the polymerase DnaE78 may provide a direct means for adapting the rate of duplex unwinding and the concurrent synthesis of the leading and lagging strand to the activity of the DnaE polymerase.

How might CCM impact DNA replication? Long-standing hypotheses that the overall process of regulating replication depends on the concentration of the active form of the replication initiator (DnaA–ATP), on the sub-saturation of the replication machinery in precursors and/or on the concentration of (p)ppGpp have been challenged.12–14 The results presented here also argue against the sub-saturation hypothesis, as there is no obvious correlation between the impact (positive or negative) of CCM mutations on replication precursor pathways (the main source of (d)NTPs and ATP are the pentose phosphate pathways and the TCA cycle, respectively) and their coupling or uncoupling phenotype (Fig. 2). Post-translational modification of replication enzymes is an alternative mechanism for the metabolism-replication coupling system as such modifications are important parts of the eukaryotic arsenal for regulating DNA replication.76,77 Moreover, several lines of experimental evidence indicate that this level of regulation may also exist in bacteria. First, recent advances in proteomic technologies have uncovered post-translational modifications of numerous bacterial proteins including replication enzymes.80–83 Second, independent studies show that the E. coli replication initiator DnaA is acetylated and its level of acetylation may play a critical role in regulating DNA replication initiation,84 and that phosphorylation of the single-stranded DNA-binding (SSB) protein is important for replication in B. subtilis.33,35 Third, indirect data suggest that the CCM-replication links detected in B. subtilis and S. cerevisiae depend on a metabolism-dependent allosteric regulation of replication enzymes.29,35

Post-translational modifications of replication enzymes are carried out by protein kinases/phosphatases and protein acetyltransferases/deacetylases in eukaryote cells. Such enzymes are thus potential effectors of the coupling between CCM and replication, although little is known on how their activity is regulated by metabolism (see however Ref. 87). An alternative way to achieve metabolism-dependent, allosteric-regulation of replication enzymes is via CCM itself. This group of catabolite reactions is central to the supply of precursors and energy from nutrients and to the demand in biosynthetic reactions, moreover, its activity responds almost immediately to changes in carbon sources. CCM is thus at a strategic position to dynamically integrate metabolism with replication. This dynamic integration may involve underepressed activities/functions of CCM metabolites and proteins. Indeed, several CCM metabolites have emerged as important modulators of various cellular activities by modulating dynamically protein activities via post-translational modifications or allosteric binding (see as reviews).85,86 Key metabolites among others are acetyl-coenzyme A and ATP, which are the major donors of acetyl and phosphoryl groups, respectively, and acetyl phosphate which lies at the crossroads between protein phosphorylation and protein acetylation.90–93 In addition, CCM enzymes from bacteria to eukarya are often multi-functional proteins carrying out both catalysis of a canonical metabolic reaction and non-metabolic functions involved in processes as diverse as DNA replication, transcription, cell division, signalling, apoptosis and pathogenicity. These moonlighting activities, found in the cytoplasm and the nucleus, involve novel enzymatic activities (like protein kinase activity) and/or interactions with non-metabolic enzymes (see for instance Refs. 94–97). We are currently exploring the fascinating possibility that the CCM-replication links in B. subtilis involve moonlighting activities of CCM enzymes and/or signalling metabolites.

DNA double-stranded breaks are chromosomal lesions with a high mutagenic potential.85,87 Their frequency increases in response to replication control defects and the importance of this threat is highlighted by the presence of multiple mechanisms ensuring faithful genome duplication once per cell cycle, and preventing re-initiation of replication.100,101 In cells with aberrant timing of initiation, re-initiation of replication may increase the risk of double-stranded breaks by collision between forks or by forks encountering gaps in the DNA template. Such a risk may also increase in cells with a lengthened C period in consequence of a higher probability of fork breakage over time. Through their effect on replication and the timing of DNA synthesis in the cell cycle, the CCM-replication links described in this study may thus form a new determinant of genetic stability. In support of this hypothesis, it was found in E. coli that CCM mutations influence the fidelity of DNA replication102 and that mutants of the ackA-pta overflow pathway, which is genetically connected to the replication initiator DnaA,12,33 accumulate double strand breaks.101 Moreover, mutants of the budding yeast impeded in the temporal control of DNA replication may exhibit substantial increases in the spontaneous mutation rate.104 Further investigations are required to better evaluate the mechanism of CCM-replication links and its role in genetic stability and potentially in cancer as early events engaging normal human cells into the path in tumorigenesis involve major changes in CCM activity (the Warburg effect) and genetic instability.105–107

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Conflict of interest
None declared.

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Supplementary data

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