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Evaluation of the plasmid copy number in *B. cereus* spores, during germination, bacterial growth and sporulation using real-time PCR

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ABSTRACT

Only a small number of studies have measured the plasmid copy number (PCN) variation during bacterial growth. Besides, information about the PCN in spores is still rare. In this work, we utilized a real-time PCR assay to evaluate the PCN of four different plasmids in *Bacillus cereus*. The PCN was measured in spores as well as during germination, active bacterial growth, and sporulation. Plasmid stability was also evaluated to ensure that plasmid loss does not affect the accuracy of the PCN measurement. We demonstrated that the PCN of low and high copy number plasmids varies with growth phase as well as culture media over *B. cereus* life cycle. The PCN was minimum during the germination and maximum during the stationary growth phase for all plasmids tested. We also demonstrated that the use of antibiotic in the culture media is not enough to ensure stable inheritance in spores of plasmids carrying antibiotic resistance genes. Moreover, we revealed that the PCN in spores is related to the PCN during endospores formation. Therefore, the plasmid partitioning during sporulation is not influenced by the unequal-size of the forespores and the mother cells, even for a plasmid distributed randomly.

1. Introduction

The plasmid copy number (PCN)[1] is usually defined as the number of copies of a plasmid present per chromosome in a cell. The PCN is an important feature of plasmids and it depends on many factors, such as plasmid replication mechanism and copy number regulation as well as host factors (del Solar and Espinosa, 2000; del Solar et al., 1998). The PCN and partitioning mechanism of many plasmids are well studied in vegetative cells, but information about partitioning during sporulation and PCN in spores is still rare. It is reported in literature that less than 10% of the total plasmid content in the cell is transferred into the forespores (Mason et al., 1988; Mason and Setlow, 1987; Nicholson and Setlow, 1990). It is also suggested that the PCN might influence the number of spores with plasmids (Mason et al., 1988). Unfortunately, data available about the PCN in spores were not complemented with plasmid stability measurement. Furthermore, the PCN during germination and sporulation has not been evaluated yet.

The knowledge of plasmid stability and copy number is important in several fields. In the development of shuttle vector, these parameters must be assessed for every expected host to ensure stable inheritance and expression of the desire characteristic. Particularly for spore-forming bacteria, it is important to make sure that plasmids are transferred to the offspring during bacterial growth as well as into the forespores. Moreover, the number of copies has a huge impact on *in situ* detection techniques of

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[1] *Abbreviations used:* PCN, plasmid copy number; TSB, trypticase soy broth; TSA, trypticase soy agar; PBS, phosphate buffer saline; OD, optical density.

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plasmid-encoded genes, like fluorescence *in situ* hybridization and *in situ* PCR (Zwirglmaier, 2005).

Most available methods to evaluate the PCN, like densitometry and Southern hybridization, are time-consuming, laborious or give poorly reproducible results. In the recent years, quantitative PCR assays were developed to estimate the PCN (Carapuca et al., 2007; Lee et al., 2006a,b; Perez-Arellano et al., 2001; Pickett et al., 2005; Providenti et al., 2006; Smajs et al., 2008; Tao et al., 2005). This method is sensitive, fast, simple, and the results are highly reproducible.

In this work, we used the real-time PCR method described by Lee and collaborators to determine the PCN over the life cycle of *Bacillus cereus* (Lee et al., 2006a). We used a selection of plasmids with various copy numbers and replication mechanisms. We evaluated the stability of the selected plasmids in growth and sporulation media to ensure that the plasmid loss does not affect the accuracy of the PCN measurement. Then, we were able to evaluate the variations of the PCN during different stages of growth of *B. cereus*.

2. Materials and methods

2.1. Bacterial strains, plasmids, and media

Bacterial strains and plasmids used in this study are listed in Table 1. The plasmid pMTL500Eres use a theta replication mechanism of group D (pAMβ1-pIP501) of Osborn replicon classification (Osborn, 1999). The plasmids pT181, pLS1, and pC194 utilize rolling circle replication mechanisms of groups I, II, and III, respectively, according to the same classification (Osborn, 1999). Plasmids were introduced into *B. cereus* ATCC14579 strain using the previously described electroporation protocol (Turgeon et al., 2006). All *Bacillus* strains were grown at 30 °C, 225 rpm in trypticase soy broth (TSB) (Difco Laboratories, Detroit, MI) or on trypticase soy agar (TSA) (Difco Laboratories). *Escherichia coli* strains were grown at 37 °C, 225 rpm in LB (Difco Laboratories). *Staphylococcus aureus* strain was grown in TSB at 37 °C, 225 rpm. When required, 100 μg/ml ampicillin or 20 μg/ml chloramphenicol or tetracycline were added for *E. coli,* and 20 μg/ml of chloramphenicol, tetracycline or erythromycin were added for *B. cereus*, *Bacillus subtilis*, and *S. aureus*. *E. coli*, *B. subtilis*, and *S. aureus* strains were used as plasmid source only. All antibiotics and chemical reagents were supplied by Sigma–Aldrich (Oakville, Ontario, Canada).

2.2. Spores preparation

Spores were obtained from a 7 days culture in chemically defined YLHG sporulation medium (de Vries et al., 2005), 30 °C, 225 rpm. One liter of YLHG medium was prepared as follow: 500 ml of miliQ water, 10 ml of 100X oligoelements solution (250 μM $CuCl_2$, 1.25 mM $ZnCl_2$, 100 mM $MgCl_2$, 250 μM Na_2MoO_4, 250 μM $CoCl_2$, 100 mM $Ca(NO_3)_2$, 6.6 mM $MnSO_4$) and 5 ml 1 M $(NH_4)_2SO_4$ were mixed and sterilized by autoclave. Then 100 ml 1 M phosphate buffer, pH 7.2, 20 ml 1 M glutamic acid, 50 ml of 20X amino acids solution (120 mM L-leucine, 50 mM L-valine, 28 mM L-threonine, 10 mM L-methionine, 6 mM L-histidine), 5 ml 1 M lactic acid, 1 ml 1 M acetic acid and 10 ml 5 mM $FeCl_3$ were sterilely added and the volume was completed to 1 L with sterile miliQ water. Spores were harvested by centrifugation at 9000*g*, for 2 min and washed several times with phosphate buffer saline (PBS) to removed cellular debris. Spores were kept at 4 °C. Before their utilization, spores were incubated 30 min at 37 °C in the following buffer to eliminate remaining vegetative cells and chromosomal DNA: 40 mM Tris–HCl, pH 8, 3 mM $MgCl_2$, 3 mM $MgSO_4$, 200 U/ml DNase I (Roche Diagnostics, Laval, Québec, Canada), 5 mg/ml lysozyme (Sigma–Aldrich) and 5 U/ml mutanolysin (Sigma–Aldrich). The enzymes were heat inactivated (80 °C, 10 min). Spores were utilized immediately. Plate counts were performed to ensure that this treatment does not affect the culturability of the spores.

2.3. Plasmids stability

To evaluate the plasmid stability in *B. cereus* vegetative cells, cells were grown in TSB, 30 °C, 225 rpm, during 100 generations without antibiotic. To obtain a continuous culture during 100 generations, serial 10-fold dilutions of the cultures were made and 10^2–10^3 cells were inoculated in fresh medium twice a day. Cell growth was monitored using optical density (O.D.) at 600 nm with a GeneQuant pro UV/Vis spectrophotometer (Biochrom Ltd, Cambridge, UK). At each passage, serial 10-fold dilutions were made and plate counts were performed on TSA to measure the number of generations reach by the culture. At each step, 100 isolated colonies were transferred on TSA and TSA supplied with antibiotics and incubated overnight at 37 °C. The number of cells with plasmids was calculated as the ratio of the antibiotic resistant colonies to all obtained colonies.

To evaluate the stability of plasmids during spore formation, vegetative cells were incubated in YLHG medium at 30 °C, 225 rpm. Samples were taken at every 24 h until free spores were observed by phase contrast microscopy. The samples were plated on TSA to evaluate the number of generation reached by the culture and the percentage of cells with plasmids as described for vegetative cells.

2.4. Plasmid DNA purification for real-time PCR standard curves

Plasmid DNA of pMTL500Eres, pLS1, and pNT2 was purified from *E. coli* with a Qiagen plasmid maxi kit (Qiagen, Chatsworth, CA). For purification of pC194 and pT181 from *B. subtilis* and *S. aureus* respectively, cells were treated 30 min at 37 °C with 10 mg/ml lysozyme (*B. subtilis*) or 15 min at 37 °C with 25 μg/ml lysostaphin (*S. aureus*) before performing plasmid purification with Qiagen plasmid maxi kit. O.D. at 260 nm was used to evaluate the DNA concentration. Purity was checked by 260/280 nm O.D. measurement. Plasmid quality was verified by agarose gel electrophoresis. The number of plasmid molecules per μl was calculated as follow, where "[DNA]" is DNA concentration in μg/μl and "DNA size" is the number of nucleotides pairs of dsDNA.

Table 1
Bacterial strains and plasmids used in this study

Bacteria and plasmids	Relevant characteristics	Source
Bacteria		
B. cereus ATCC14579		Ivanova et al. (2003)
B. cereus CD30	ATCC14579 + pLS1	This study
B. cereus CD52	ATCC14579 + pC194	This study
B. cereus CD53	ATCC14579 + pMTL500Eres	This study
B. cereus CD51	ATCC14579 + pT181	This study
Plasmids		
pNT2	pBlueScript KS^+ (Stratagene) with 799-1044 *B. cereus* ATCC14579 16S fragment, 4115 bp	This study
pLS1	pMV158 Δ*mob* derivative, from *Streptococcus agalactiae*, Tet^r, 4408 bp	Lacks et al. (1986)
pC194	From *Staphylococcus aureus,* Cm^r, 2910 bp	Gros et al. (1987)
pMTL500Eres	pAMβ1 derivative, from *Enterococcus faecalis*, Erm^r, Ap^r, 7127 bp	Swinfield et al. (1991)
pT181	From *S. aureus*, Tet^r, 4439 bp	Khan and Novick (1983)

Ap^r, ampicillin resistance; Cm^r, choramphenicol resistance; Erm^r, erythromycin resistance; Tet^r, tetracycline resistance.

$$[\mathrm{DNA}]\frac{\mu g}{\mu l}\times 10^{6}\frac{pg}{\mu g}\times\frac{1\ \mathrm{pmol}}{660\ \mathrm{pg}\times \mathrm{DNA\ size}}\times 6.022\times 10^{23}\frac{\mathrm{molecules}}{\mathrm{mol}}\times 10^{-12}\frac{\mathrm{mol}}{\mathrm{pmol}}$$

2.5. Total DNA preparation for PCN evaluation

The method used for PCN measurement by real-time PCR was previously described (Lee et al., 2006a; Smajs et al., 2008). The total DNA purification method with QIAamp DNA mini kit (Qiagen) from this assay was adapted for *B. cereus* vegetative cells and spores. Spores were incubated 15 min at 56 °C in 1 ml buffer A (20 mg/ml SDS, 100 μM DTT, 8 M Urea, for 50 mM Tris–HCl, pH 10), washed twice in PBS, and incubated 30 min at 37 °C in 180 μl buffer B (100 mM Tris–HCl, pH 8, 5 mM EDTA pH 8, 70 mg/ml lysozyme, 7 U/ml mutanolysin). Afterward, 20 μl of proteinase K (Qiagen) was added followed by an incubation of 30 min at 56 °C. Subsequently, 200 μl of buffer AL (Qiagen) was added followed by incubation at 95 °C for 15 min. After that, 200 μl of ethanol 95% was added before the adsorption and purification of the DNA on a silica-gel column (Qiagen) in accordance with the manufacturer instructions. For the purification of total DNA from *B. cereus* vegetative cells the same protocol was applied except that the incubation in buffer A and the subsequent washes in PBS were omitted.

2.6. Quantitative PCR for PCN measurement

As described previously (Lee et al., 2006a; Smajs et al., 2008), PCN was measured by comparing the number of plasmids with the number of genomes. Modifications were applied to allow the use of dual-labeled probes (Taqman®) instead of SYBR Green I technology. Primers and dual-labeled probes, supplied by Integrated DNA technology (Coralville, IA), are listed in Table 2. Probes were labeled with FAM or HEX fluorochromes in 5′ and with Iowa Black as quencher in 3′. The assay used to quantify the number of genome targets the 16S DNA region with 16S specific primers and probe adapted from a eubacteria universal assay (Bach et al., 2002). The 16S standard curves were built with pNT2 plasmid DNA, which contain the targeted *B. cereus* 16S DNA fragment. Probes and oligonucleotides for pLS1, pT181, pC194 and pMTL500Eres plasmids were designed with Beacon designer 4.0 software (Premier Biosoft International, Palo Alto, CA) within the *tetL*, *tetK*, *cat*, and *ErmR* genes, respectively.

The assay components per 25 μl were: 5 μl of template DNA, 12.5 pmol of each primer, 2.5 pmol of dual-labeled probes and 12.5 μl of 2X master mix QuantiTect probe PCR kit (Qiagen). The PCR run was performed as follow: hot start 94 °C for 10 min, 40 amplification cycles including denaturation at 94 °C for 20 s, annealing and elongation at 64 °C for 60 s, and fluorescence measurement. All experiments were performed using an Opticon 2 system (MJ research, Waltham, MA).

2.7. Real-time PCR data analysis

The PCN calculation was made using the absolute quantification method described previously (Lee et al., 2006a; Smajs et al., 2008). Data were analyzed with the Opticon monitor software supplied with the apparatus. For each PCR run, standard curves of 16S DNA and plasmid DNA were performed in duplicate. Serial 10-fold dilutions from 10^8 to 10^3 molecules per reaction tube were used to establish standard curves. For each sample of unknown concentration, three 10-fold dilutions made in duplicate were reported on standard curves. Two independent experiments were performed for each plasmid. The background was subtracted using global minimum function of the software. Threshold values (Ct) were determined using standard deviation method. The plotting of Ct as a function of the logarithm of DNA template gives a straight line. The slope of this graph line gives the PCR efficiency (E) according to the equation: $E = (10^{-1/\mathrm{slope}}\text{-}1)\times 100$. Results were considered as adequate when E was over 85% and error between standard points and regression curve was lower than 0.1. The number of plasmids per genomes was calculated according the following equation, where [plasmid] is the measured quantity of plasmid in the reaction tube and [16S] is the measured quantity of 16S DNA.

$$PCN = \frac{[\mathrm{plasmid}]}{[16S]/13}$$

To obtain the number of genome in the sample the number of 16S DNA measured for each sample was divided by 13, which is the number of 16S DNA operons per genome of *B. cereus*. The reported PCN are the average of all replicas.

2.8. Measurement of PCN variations during B. cereus life cycle

The *B. cereus* life cycle PCN variation was evaluated without antibiotics in culture media. Spores were inoculated in TSB and incubated at 30 °C, 225 rpm. Samples were taken during incubation and kept at −20 °C until total DNA extraction was performed. Cultures were monitored with O.D. reading. After 24 h of growth, the culture was used to inoculate YLHG sporulation medium. Incubation was prolonged at 30 °C, 225 rpm until free spores were observed by phase contrast microscopy. Total DNA extraction was performed with silica-gel column for spores and vegetative cells as described in former section. PCN was measured using real-time PCR as described previously.

Table 2
Primers and probes used in this study

Oligonucleotide	Sequences (5′–3′)	Position[a]
16SF	GGTAGTCCACGCCGTAAACG	799–818
taqman 16S	HEX-TTCGCGTTGCTTCGAATTAAACCAC-IaB[b]	951–975
16SR	GACAACCATGCACCACCTG	1063–1044
tetLF	TCAATTAGGCATCAAAAGGTTACTCC	1957–1982
taqman tetL	FAM-CCAACAAACCCAATTACCGACCCGAAACAA-IaB	2031–2002
tetLR	ATAAAACGAGCCATAATAAGTAAGGAAAAG	2069–2041
tetKF	TTTGGTAGGTTAGTACAAGGAGTAGG	1459–1484
taqman tetK	FAM-CTGCTGCATTCCCTTCACTGATTATGGTGG-IaB	1487–1516
tetKR	CCTTCACCTAAAGCTACAATTGATCC	1595–1570
catF	GTGACAAGGGTGATAAACTCAAATAC	1428–1453
taqman cat	FAM-ACCTAACTCTCCGTCGCTATTGTAACCAGT-IaB	1493–1464
catR	TGTATAAAGTGGCTCTAACTTATCCC	1523–1498
ErmF	CACTCAAGTCTCGATTCAGCAATTG	6664–6688
taqman Erm	FAM-CTGCCAGCGGAATGCTTTCATCCTAAACCA-IaB	6695–6723
ErmR	TTATCTGGAACATCTGTGGTATGGC	6780–6756

[a] Nucleotide position in 16S rRNA gene of *E. coli*, in pLS1 plasmid (NCBI NC001380), in pT181 (NC001393), in pC194 plasmid (NCBI NC002013), and in pMTL500Eres plasmid.
[b] IaB, Iowa Black quencher.

3. Results and discussion

3.1. Plasmid copy number in B. cereus

The four plasmids used in this study have vast host ranges and were previously tested in a variety of hosts. However, none of them have been tested in *B. cereus*. All these plasmids have a medium or high plasmid copy number in their original host. Nevertheless, the copy number of a specific plasmid varies from host to host.

PCN per chromosome was measured in stationary growth phase culture in the presence of antibiotic to ensure that each cell possesses plasmids. The results indicate that pLS1 and pMTL500Eres have low plasmid content in *B. cereus* (two copies per chromosome) and that pC194 and pT181 have high plasmid copy number per chromosome in this host (100 and 220, respectively, data not shown).

3.2. Plasmid stability in vegetative cells

A poor plasmid stability leading to plasmid loss could have a strong effect on PCN measurement. Therefore, we evaluated the stability of the four plasmids before investigating the PCN in spores and over the bacterial life cycle. We first measured the plasmid stability in *B. cereus* during bacterial growth over 100 generations. Our results show that the pLS1 and pT181 plasmids are rapidly lost in absence of antibiotic as selection pressure (less than 50% remains after 20 generations) (Fig. 1). The plasmid pC194 is stable during more than 20 generations (100% remained after 20 generations, 58% after 100 generations). For the theta replicating plasmid pMTL500Eres, all the cells possessed the plasmid after 100 generations.

It was previously described that some regions of pMTL500Eres enhance the plasmid segregational stability (Swinfield et al., 1991). The great stability of this plasmid in *B. cereus* demonstrates that, even with a lower copy number compared with his original host, this partitioning mechanism is still functioning in *B. cereus*. In contrast, pLS1, pC194, and pT181 plasmids do not possess known active partitioning mechanism and are presumed to be transferred randomly to the daughter cells. The kinetic of plasmid loss for pLS1 in *B. cereus* is consistent with a low plasmid copy number distributed randomly (Summers, 1991). Besides, the great stability of pC194 is compatible with a random distribution of a high copy number plasmid. However, high copy number is not always related with plasmid segregational stability. As example, the plasmid pT181 have a high copy number in *B. cereus* but can be lost very quickly in this bacterial host. Previous authors hypothesized that a poor regulation of the plasmid copy number increase the metabolic charge, leading to growth slowdown of cells carrying plasmid compare to plasmid free cells and thus resulting in plasmid loss (Friehs, 2004). Moreover, it was demonstrated for many randomly-distributed plasmids that a higher copy number and a poor multimer resolution mechanism could lead to high plasmid instability (Friehs, 2004; Herman-Antosiewicz and Wegrzyn, 1999; Summers et al., 1993).

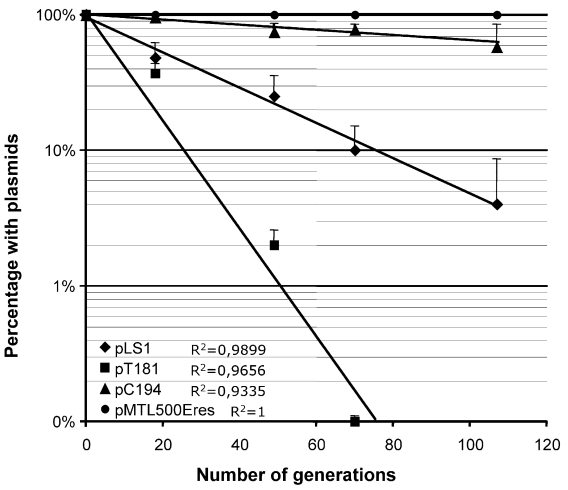

Fig. 1. Plasmid stability over 100 generations in TSB growth medium without antibiotic.

3.3. Plasmid stability during sporulation

We evaluated the plasmid stability during the sporulation of *B. cereus* with and without antibiotic as selection pressure in the YLHG sporulation medium. Our results indicate that *B. cereus* grows 48 h and divides for ~8 generations before the appearance of spores in this medium. The use of selection pressure increases the amount of spores with plasmid only for pT181 (55% instead of less than 1%) (Fig. 2). In both conditions, less than 5% of spores possess the pLS1plasmid in contrast with more than 90% for pC194 and pMTL500Eres (Fig. 2). pT181 and pLS1 plasmids are lost during the growth in YLHG medium as well as during sporulation (Fig. 2). Given that the spores are in dormant state, the antibiotic cannot apply a selection onto the spores that do not inherit the plasmid. So, the selection pressure in the culture media is not enough to ensure stable inheritance of plasmids into the spores.

As shown in Figs 1 and 2, the plasmids pLS1 and pT181 are lost more quickly in the YLHG sporulation medium than in TSB. Indeed, for both plasmids, the loss after eight generations in YLHG without antibiotic corresponds to the loss after 50 generations in TSB. Previous studies have shown that the culture media have a marked influence on plasmid segregational stability (Friehs, 2004). In YLHG, the starvation and chemical agents used to stimulate sporulation could be responsible for plasmid loss.

Our results demonstrate that the plasmid stability instead of the PCN have the strongest influence on the percentage of spore with plasmids. Indeed, pT181 have a high copy number in *B. cereus* and is transferred in only 55% of forespores. Moreover, pMTL500Eres have a low copy number in *B. cereus* and was found in 100% of the spores. This suggests the importance of investigating the plasmid stability prior to the study of plasmid-carried character of spores-forming species. Furthermore, if the use of plasmid without selection pressure is necessary, the plasmid stability must be assessed in all the culture media, taking into account that the kinetic of plasmid loss varies with growth conditions.

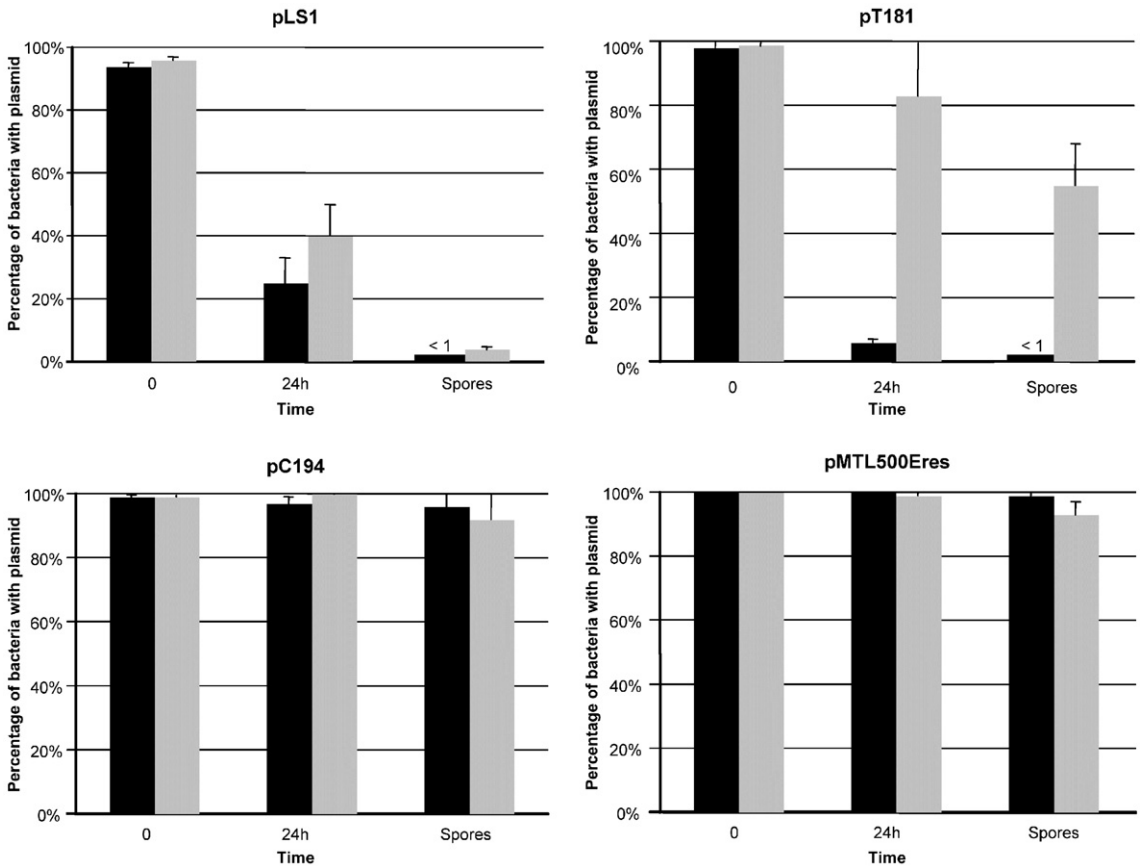

Fig. 2. Stability of all studied plasmids during sporulation in YLHG medium with (gray) and without (black) antibiotic. The cellular state was monitored by microscopy.

3.4. Variations of the PCN over B. cereus life cycle

We observed that germination and sporulation of *B. cereus* ATCC14579 are affected by the presence of antibiotics, even for antibiotic resistant strains (data not shown). Indeed, as observed by former authors, the passage from spore to vegetative cells is totally inhibited in presence of chloramphenicol (Hu et al., 2007; Steinberg et al., 1965). Moreover, the use of all tested antibiotics delayed the spore-forming process of several days. Consequently, the PCN measurement over *B. cereus* life cycle must be performed without antibiotics in the culture media. This experiment was carried out with strains containing pC194 and pMTL500Eres, which are well conserved for the duration of this experiment (~7 generations in TSB and ~8 in YLHG).

Our results show that the PCN per chromosome varies widely during bacterial life cycle. The PCN ranges from 10 to 150 copies for pC194 (Fig. 3), and from 1 to 4 copies per chromosome for pMTL500Eres (Fig. 4). For both plasmids, the PCN per chromosome is at its maximum during stationary growth phase and at its minimum during the germination. For both plasmids, the highest PCN reached into YLHG sporulation medium was lower than in TSB.

The PCN is usually compared with the number of genomes. Therefore, changes in the number of genomes influence the measured PCN. In fast growing bacteria, such as *E. coli*, each cell contains several bacterial chromosome during the active replication phase (Cooper and Helmstetter, 1968). Assuming that the genetic material in spores is minimal, the germination process could include extensive chromosome replication before the beginning of the active growth phase. That could explain, at least in part, the very low PCN per chromosome measured for pC194 and pMTL500Eres during germination (Figs. 3 and 4). It could also explain the drop of the PCN when cells in stationary growth phase are inoculated in fresh YLHG medium and enter in active growth phase (Figs. 3 and 4).

It is interesting to note that the PCN of pC194 reaches its maximum in the early stationary growth phase and pMTL500Eres, during the late stationary growth phase. This difference could reside in the copy number regulation mechanism of both plasmids. Indeed, the plasmids of the pC194 family regulate their copy number by the production of an anti-sense RNA that inhibits the translation of the plasmid replication initiator (del Solar et al., 1998). For their part, the plasmids of the pAMβ1 family, in which belong pMTL500Eres, used a combination of an anti-sense RNA and of a repressor protein (del Solar and Espinosa, 2000; del Solar et al., 1998).

High copy number plasmids can rely on random distribution to ensure that each daughter cell inherits of at least

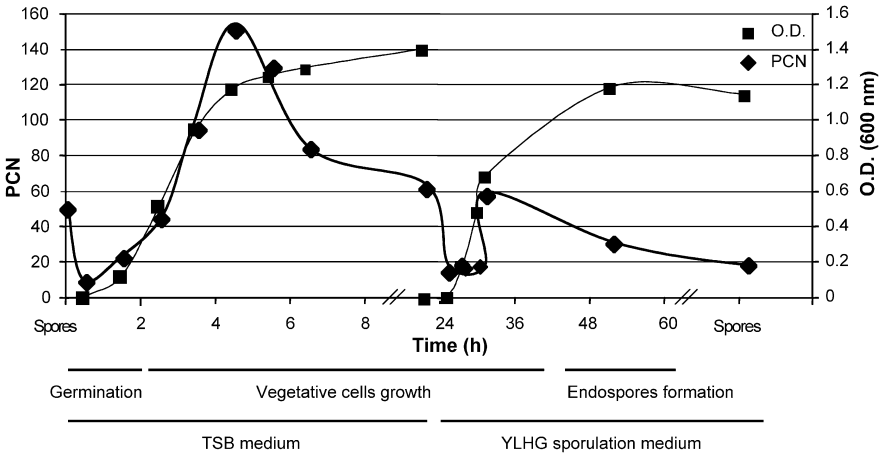

Fig. 3. pC194 PCN variations over the life cycle of *B. cereus*. Spores were incubated in TSB to induce germination and growth and further inoculated in YLHG medium to induce sporulation. The cellular state was monitored by microscopy.

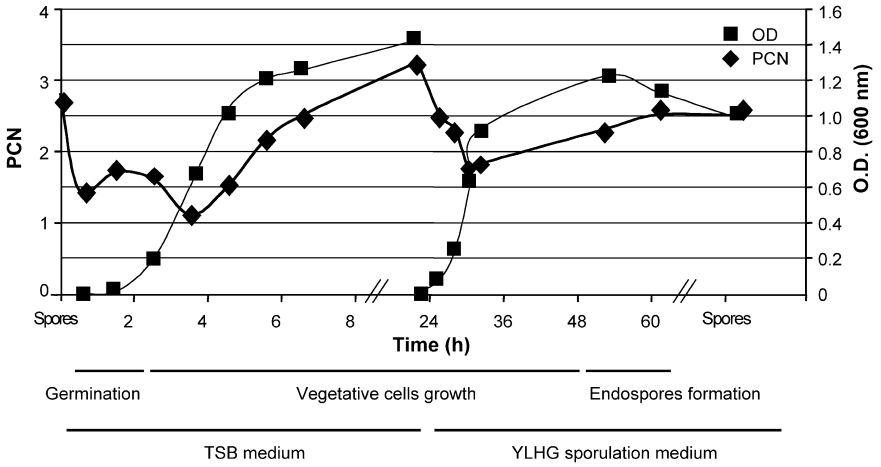

Fig. 4. pMTL500Eres PCN variations over the life cycle of *B. cereus*. Spores were incubated in TSB to induce germination and growth and further inoculated in YLHG medium to induce sporulation. The cellular state was monitored by microscopy.

one copy of the plasmid. However, during sporulation, the bacterial cell divides into two unequal-sized compartments, the mother cell (big) and the forespores (small). Therefore, it is likely that a random distribution of the plasmid leads to less plasmid content into the smaller part.

Previous studies have shown that the PCN per chromosome of pUB110 in spores of *B. subtilis* is 10% of the PCN for vegetative cells (Mason et al., 1988; Mason and Setlow, 1987; Nicholson and Setlow, 1990). The plasmids pUB110 and pC194 use a rolling circle replication mechanism of the same replicon family (del Solar et al., 1998; Osborn, 1999). Therefore, it is possible that the PCN per chromosome of pUB110 in *B. subtilis* varies during bacterial life cycle as we observed for pC194 in *B. cereus*. In that case, the comparison between the PCN per chromosome in spores and vegetative cells would be influenced by the culture media and growth phase. The PCN in vegetative cells is usually measured with stationary growth phase cultures. If we compare the PCN per chromosome of pC194 in stationary growth phase in rich culture media (150) with the PCN per chromosome in spore (20–50), we also obtain a lower plasmid content in spores as observed by former authors (Mason et al., 1988; Mason and Setlow, 1987; Nicholson and Setlow, 1990). Nevertheless, the number of plasmids into vegetative cells in the sporulation medium during the formation of endospores (∼30) is in the same order of magnitude as the plasmid content of spores (∼20). Consequently, the division into unequal-sized compartments during sporulation has no effect on partitioning of the theta replicating plasmid pMTL500Eres as well as the randomly-distributed rolling circle plasmid pC194.

In recent years, improvements in hybridization techniques (Smolina et al., 2007; Zwirglmaier, 2005), image analysis and microscope equipment (Martin et al., 2008) make it possible to detect plasmidic genes with *in situ* methods. However, the sensitivity of these techniques for plasmidic genes is still influenced by the PCN. According to the huge variations of PCN over bacterial life cycle that we observed, spores state and stationary growth phase are the most appropriate stages to measure plasmid-carried

characters using techniques such as fluorescent *in situ* hybridization. Indeed, in these stages, the PCN is relatively high and in a more steady state compared with growth phase cells.

4. Conclusion

In this work, we demonstrated that the use of antibiotic in the culture media is not an important feature to ensure stable inheritance in spores of plasmids carrying antibiotic resistance genes. Characteristics of the plasmid are a more critical factor for stable inheritance, especially in spores. We also established that the PCN of both low and high copy number plasmids is modulated by growth phase as well as culture media over *B. cereus* life cycle. The PCN of the studied low and high copy number plasmids were at their minimum during the germination and at their maximum during the stationary growth phase. Moreover, we revealed that the PCN in spores is related to the PCN during endospores formation. Therefore, the plasmid partitioning during sporulation is not influenced by the unequal-size of forespores and mother cells, even for a plasmid distributed randomly.

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