

Methylation of Spore DNA in *Bacillus coagulans* Strain 26

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The modification status of DNA throughout the life cycle of *Bacillus coagulans* strain 26 was analysed by restriction analysis with methylation-sensitive enzymes. A significant fraction of the GATC sequences (*dam* target) in spore DNA contain *N*⁶-methyladenine, a modification that is lacking during the vegetative phase. From the modulation of the modification pattern of GATC sites, the existence of a *de novo* methylase may be inferred. Spore DNA was more sensitive than vegetative cell DNA to *Bam*HI, *Hpa*I, *Sal*I and *Xho*I, indicating that the sites for these enzymes are modified during the vegetative growth phase.

INTRODUCTION

Prokaryotic DNA contains 5-methylcytosine (5mC), *N*⁶-methyladenine (*N*⁶mA) (Vanyushin *et al.*, 1968) and *N*⁴-methylcytosine (Ehrlich *et al.*, 1985) as modified bases, which are included (at least the former two) in specific sequences of four or more nucleotides which vary from species to species. In different strains of *Bacillus subtilis*, for example, up to six different sequences have been described which include 5mC (Jentsch, 1983). Although a relationship has been demonstrated between the methylation of specific sequences and the restriction–modification (R/M) phenomenon (Günthert & Trautner, 1984) and other functions have also been attributed to methylation (Hattman, 1981; Razin & Friedman, 1981), such as the discrimination of parent and daughter strands for proofreading during DNA synthesis in *Escherichia coli* (Pukkila *et al.*, 1983), the true biological significance of most DNA methylation and the diversity of modified specific sequences remain to be elucidated.

The role played by DNA methylation in *B. subtilis*, in which both 5mC and *N*⁶mA have been reported (Ganesan, 1979), is uncertain. Modulation of the modification pattern seems to be related to the physiological state of the cells (Günthert & Trautner, 1984; Hattman, 1981), and hence populations which are competent for genetic transformation exhibit high levels of methylation activity (Ganesan, 1979). However, neither the genetic determinants, nor the specificity of the sequences affected by such activity, have been determined (Günthert & Trautner, 1984). An increase has also been observed in methylation of cytosine in *B. subtilis*, *Bacillus megaterium* and *Bacillus brevis* during sporulation (Günthert, 1975; Guha, 1985), considered by many researchers as a primitive form of cellular differentiation, though up till now only speculations have been made regarding the true role of methylation in the regulation of sporulation.

In this work, prompted by our interest in sporulation in *B. coagulans* strain 26, we present data concerning the analysis of the methylation of specific sequences during the life cycle of this micro-organism.

METHODS

Micro-organisms and culture conditions. *B. coagulans* strain 26 was isolated in our laboratory (Esteban *et al.*, 1983). *B. subtilis* CECT 39 was from the Colección Española de Cultivos Tipo; *B. subtilis* NRRL B1490 and *Bacillus macerans* NCLB 7588 were gifts from Dr F. G. Priest, Heriot-Watt University, Edinburgh, UK. In each

Abbreviations: *N*⁶mA and mA, *N*⁶-methyladenine; 5mC and mC, 5-methylcytosine; R/M, restriction–modification.

case the growth medium was YED (Esteban *et al.*, 1983). Spores were isolated after 8 h incubation at 37 °C in the resuspension medium described by Sterlini & Mandelstam (1969); DNA purified from older spores was degraded and inadequate for restriction analysis.

Purification of DNA. DNA was purified from cells at different stages of growth following the method of Marmur (1961) with minor modifications which included isopycnic centrifugation in a CsCl gradient. DNA from spores was purified from a homogeneous preparation (100%) according to the technique of Sargent (1980*b*). The quality of each preparation was determined by the ratios A_{260}/A_{280} (1.8–1.9) and A_{260}/A_{230} (2.0–2.3) (Marmur, 1961).

Restriction conditions and densitometry. All digestions with restriction enzymes were done under the conditions recommended by suppliers. λ DNA or pBR322 were used as internal controls to check that digestion was complete. Digested DNA was electrophoresed in horizontal agarose gels (0.7–1.25, w/v). The expected average size of fragments produced by digestion with a particular enzyme was calculated assuming that all nucleotides are randomly distributed as a function of the mean mol % G + C of the micro-organism (39.9% for *B. coagulans* strain 26; Esteban *et al.*, 1983), assuming also a random distribution of the sites along the genophore. The observed size, i.e. the mean absorbance value (mode) was determined from the densitograms. All experiments were done at least twice to ensure reproducibility of results. Given the lack of appropriate isoschizomers to test the methylation status of the sites under study, we used enzymes with identical CG content in their recognition sites to compare the average size of the restriction fragments. We assumed that all dinucleotides have the same frequency. In double digestions these additional controls were conducted with two different enzymes. In no case were any significant differences observed between the expected size and the observed size for these controls. Fragment sizes were estimated by plotting the reciprocal of the migrated distance against the M_r of standard markers (λ HindIII or similar).

Average molecular sizes of distributions were estimated from the densitograms (absorbance/size) of photographs of the gels (negatives) obtained by transmission densitometry using a Shimadzu Dual-Wavelength TLC Scanner CS-910.

RESULTS

Distribution of sites for BamHI, HpaI, SalI and XhoI in DNA of B. coagulans strain 26

The predominance of high- M_r restriction fragments that were observed when DNA of *B. coagulans* strain 26 was analysed with *Bam*HI, *Hpa*I, *Sal*I and *Xho*I seemed to indicate that this DNA had few sites for these enzymes (Fig. 1). Spore DNA was more sensitive to digestion with the same enzymes, indicating that these sites were modified during the vegetative growth phase (see the legend of Fig. 1) and were demethylated during the sporulation process. As shown in Table 1, the demethylated *Sal*I and *Xho*I regions have a similar average size (2.32 kbp), comparable to that of the *Bam*HI region (2.1 kbp), and in all three cases it was significantly greater than that of the *Hpa*I region (1.65 kbp).

In all cases, the appearance of a demethylated region in spore DNA was accompanied by a decrease in the region of greatest M_r of the digests (Fig. 1A and lanes S and 12 of Fig. 1B); the sizes of these high- M_r fragments ranged between 15 and 48 kbp. Analysis of the densitograms obtained showed a log-normal distribution, from which it was possible to infer a clustering of the sites in the genome. When the DNAs were co-digested with *Sal*I and *Xho*I (Fig. 1C), the observed size of the demethylated fraction (1.71 kbp) did not vary as much from the predicted value (1.16 kbp) as might have been expected from the results of the single digestions, pointing to a clustering of *Sal*I and *Xho*I sites in the demethylated fraction. Analysis of the size distributions of DNA doubly digested with *Bam*HI + *Hpa*I, *Bam*HI + *Sal*I, *Bam*HI + *Xho*I, *Hpa*I + *Sal*I and *Hpa*I + *Xho*I showed a similar behaviour, pointing to the clustering of these sites in the demethylated fraction of the spore DNA also (data not shown).

Distribution of the GmATC modification in spore DNA

The modification of the GATC sites in the genophore of *B. coagulans* strain 26 was studied by using the isoschizomers *Sau*3A (cleaves GATC, and is insensitive to adenine methylation), *Mbo*I (cleaves at GATC when the sequence is not methylated) and *Dpn*I (cleaves only when GATC is methylated on both strands). DNA purified during different stages of the life cycle of the micro-organism was resistant to *Dpn*I, except the DNA from spores, which showed partial sensitivity (Fig. 2). The observed size of this *Dpn*I-sensitive fraction (methylated on both strands) was 1.3 kbp. Control DNA from vegetative cells, not digested with *Dpn*I, presented the

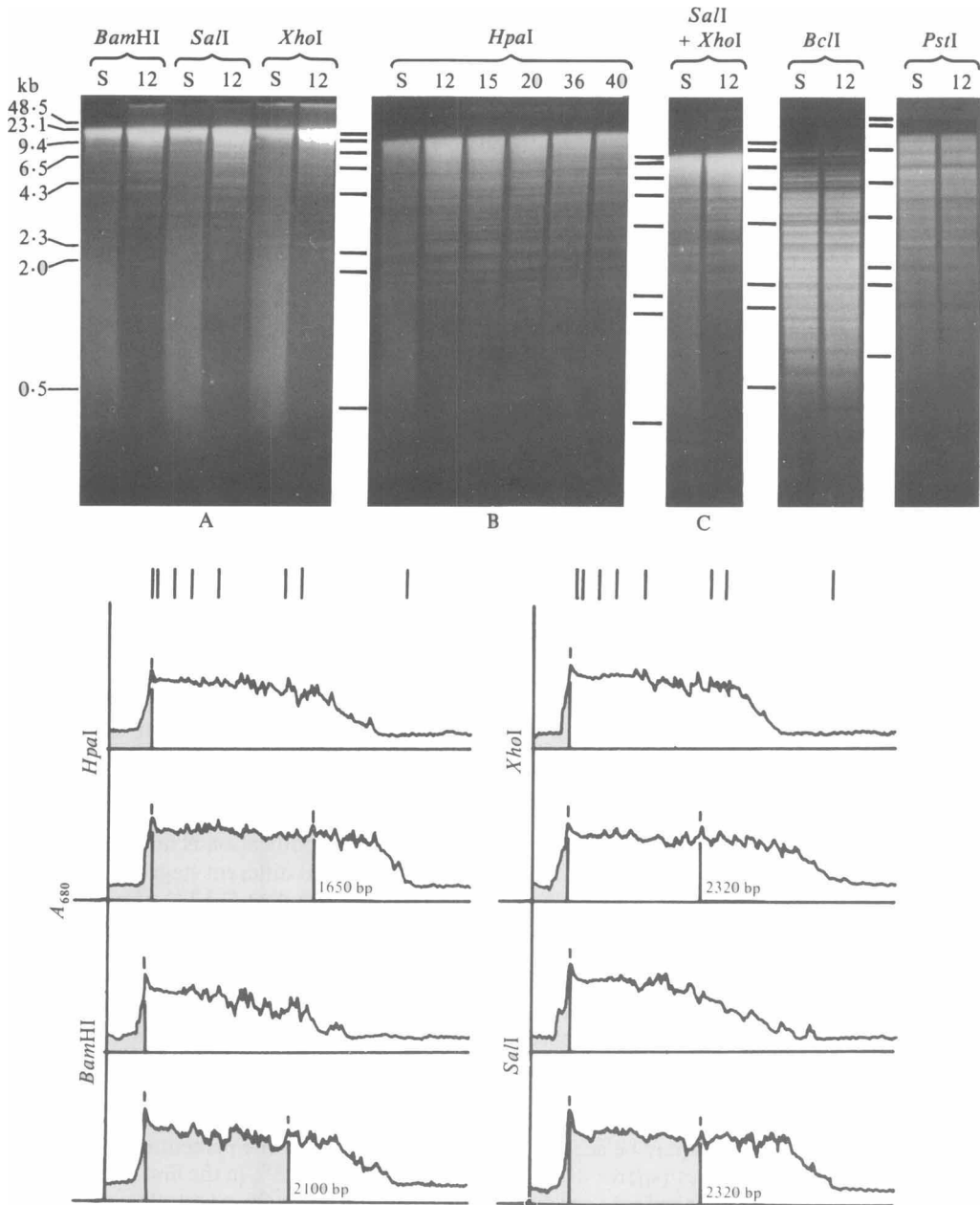


Fig. 1. Reduction in methylation of *Bam*HI, *Hpa*I, *Sal*I and *Xho*I sites (5mC and N^6 mA) in spore DNA of *B. coagulans* strain 26. DNA from spores (S), and from vegetative cells in 12, 15, 20, 36 and 40 h cultures, was digested with *Bam*HI, *Sal*I, *Xho*I (A) and *Hpa*I (B), and co-digested with *Sal*I and *Xho*I (C). *Bcl*I and *Pst*I were used as controls (see Table 1). All the patterns of spore DNA show an increased sensitivity to endonuclease cleavage, indicating demethylation in these sites during spore differentiation. Densitometric tracings are shown below. The semibimodal distributions obtained from spore DNA were interpreted by overlapping them with corresponding distributions from vegetative cell DNA.

Table 1. Overrepresentation of the demethylated sites on spore DNA in *B. coagulans* strain 26

Restriction enzyme*	Assayed DNA	Expected size (bp) (A)	Observed size (bp) (B)	Percentage over-representation (C)†
<i>Bam</i> HI GGATmCC	Spore	6991	$\left\{ \begin{array}{l} 23130 \\ 2100 \\ 23130 \end{array} \right.$	232
	12 h			
<i>Hpa</i> I GTTmAAC	Spore	3081	$\left\{ \begin{array}{l} 23130 \\ 1650 \\ 23130 \end{array} \right.$	86
	12 h			
<i>Sal</i> I GTmCGAC	Spore	6991	$\left\{ \begin{array}{l} 23130 \\ 2320 \\ 23130 \end{array} \right.$	201
	12 h			
<i>Xho</i> I CTmCGAG	Spore	6991	$\left\{ \begin{array}{l} 23130 \\ 2320 \\ 23130 \end{array} \right.$	201
	12 h			
<i>Bcl</i> I TGmATCA	Spore	3081	3200	0
	12 h			
<i>Pst</i> I mCTGCAG	Spore	6991	7300	0
	12 h			
<i>Sal</i> I + <i>Xho</i> I GTmCGAC CTmCGAG	Spore	3495	$\left\{ \begin{array}{l} 23130 \\ 1706 \\ 23130 \end{array} \right.$	104
	12 h			

* Methylation of the recognition sequence as shown renders DNA insensitive to the enzyme. *Bcl*I and *Pst*I were used as contrastive controls of unmodified sites for 2 G or C and 4 A or T (*Bcl*I) and 4 G or C and 2 T or A (*Pst*I).

† Calculated using the following equation: $C = [(A - B)/B] \times 100$.

same appearance as the DNA shown in Fig. 2, lanes 3–7, indicating that there is no DNA methylated in both strands at the GATC sites during the vegetative growth phase.

The absence of modification of *Bcl*I (TGmATCA) sites, as determined from the distribution of the restriction fragments (Table 1), and the identity of the restriction fragments between spores and vegetative cells (Fig. 1) indicate that the GmATC modification is not flanked, at 5' and 3', respectively, by T and A. From analysis of DNA purified at different stages of vegetative growth using *Sau*3A and *Mbo*I it is possible to infer a lack of *N*⁶mA at GATC sites during the exponential growth phase and the beginning of the stationary phase. However, DNA purified during the later stationary phase exhibited a partial resistance to cleavage by *Mbo*I; the DNA from 36 h cultures was more refractory to digestion than that from 40 h cultures (data not shown). From the joint interpretation of the analysis of these DNA samples with *Dpn*I, *Sau*3A and *Mbo*I, it may be seen that as the sporulation process begins, *de novo* methylase activity is induced with GmATC specificity which first modifies one strand, with the appearance of hemimethylated DNA (36 and 40 h), then the antiparallel strand, either before or at the same time as DNA is packaged in the spore. The greater modification observed in the 36 h DNA compared with that of 40 h may be accounted for in this context by the percentage of spores at these times; the value [spores/(spores + vegetative cells)] \times 100 is 28% in the first case and 41% in the second (it should be taken into account that *B. coagulans* strain 26 is very efficient in spore formation).

Electrophoresis of the *Mbo*I restriction fragments of spore DNA revealed two fractions in distributions of GmATC sites (Fig. 3). One fraction has all its GATC sites modified; this region shows slightly greater mobility than undigested DNA with an approximate size of 40 kbp. Furthermore, analysis with *Dpn*I shows that it is a region statistically poor in GATC sites. The second fraction is partially resistant to *Mbo*I and exhibits modified sites intercalated between unmodified ones. The densitograms obtained from the restriction analysis (Fig. 3) show that about 75% of the GATC sites in the second fraction are modified, since the observed average sizes of *Mbo*I and *Sau*3A fragments were 0.52 kbp and 0.13 kbp, respectively.

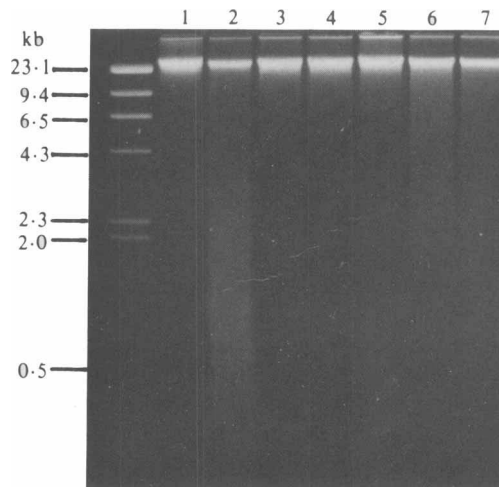


Fig. 2. Extent of methylation of GATC (N^6 mA) sites in spore DNA of *B. coagulans* strain 26. DNA from spores, and from vegetative cells in 12, 15, 20, 36 and 40 h cultures, was digested with *DpnI* (cleaves only when GATC is methylated in both strands). (1) Undigested spore DNA. (2-7) *DpnI*-treated DNA from: spores (2); mid-exponential phase cells, 12 h (3); late exponential phase cells, 15 h (4); early stationary phase cells, 20 h (5); late stationary phase cells, 36 h (6); late stationary phase cells, 40 h (7).

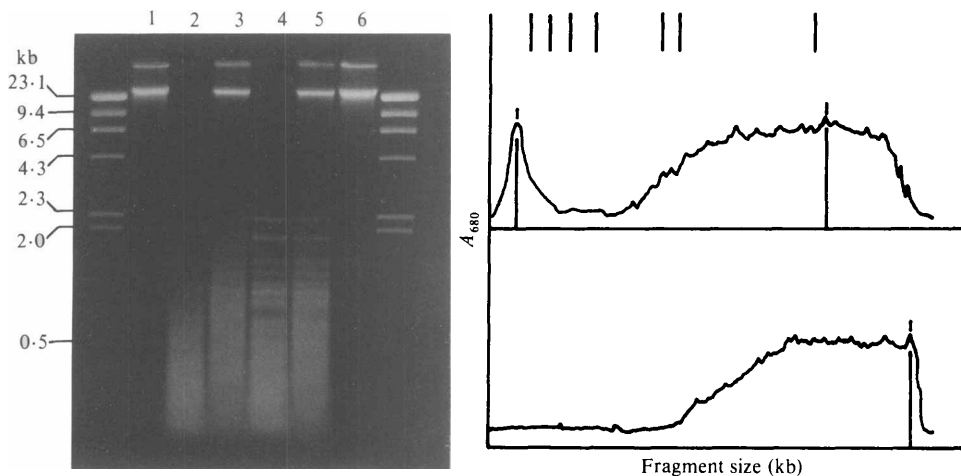


Fig. 3. Distribution of the GmATC sequence in spore DNA of *B. coagulans* strain 26. Spore DNA (1 μ g) was digested with *Sau3A* and *MboI* (2 units per μ g DNA); the digested DNAs were electrophoresed on 1% agarose gels, stained with ethidium bromide and photographed, and the negatives were scanned as described in Methods. (1) and (6) undigested spore DNA; (2) and (4) *Sau3A*-treated spore DNA; (3) and (5) *MboI*-treated spore DNA. λ DNA was added to the DNA samples in lanes, (4) and (5) to test for complete digestion. Densitometric tracings of the negatives obtained of *Sau3A* and *MboI* digests are also shown.

DISCUSSION

Following the idea that the biological significance of DNA methylation must be reflected in the distribution pattern of the modified bases in DNA (Szyf *et al.*, 1982), we studied the modulation of the modification pattern of the genophore of *B. coagulans* strain 26 for the sequences GGATCC, GTTAAC, GTCGAC, CTCGAG and GATC. In all cases a non-random distribution of sites was observed and in the last one there was a non-random

distribution of the modified sites. These non-random distributions suggest that base modification may play a specific role related to the sporulation process. The role of these modifications in sporulation itself, i.e. whether they are the cause or a consequence of sporulation processes, remains to be determined.

The partial methylation observed at *Bam*HI, *Hpa*I, *Sal*I and *Xho*I sites may be accounted for in terms of partial methylation *in vivo*; there is growing evidence to suggest that not all potentially methylatable sites are modified as reported by Razin & Friedman (1981). Guha (1985) reported that only some TTmCGAA sites are methylated *in vivo* in *B. subtilis* Marburg 168. Similar observations have been described by Vanyushin & Dobrista (1975) for *B. brevis*. The possible alternative, i.e. that one might be dealing with populations with a significant proportion of mutants deficient in R/M systems, similar to the HsdM⁻ and HsdR⁻ mutants described in *Haemophilus influenzae* (Glover & Piekarowicz, 1972), is rejected by us since DNA analysis of homogeneous spore populations shows similar patterns, pointing to a non-random distribution of these four sites in the genophore of the bacillus; this distribution phenomenon in prokaryotic DNA such as that of *E. coli* is not unfamiliar (Razin & Friedman, 1981).

Both the partial modification of these sites and their asymmetric distribution in the genophore indicate that they are probably not involved in the classic function described for R/M systems. In this sense, it should be noted that for cellular defence, R/M systems with four-base-pair recognition sequences are much more useful than those with six. In this context it is highly significant that the methylases coded by *B. subtilis* phages are able to recognize and modify sequences such as GGCC, CCGG and GCNGC (Günthert & Trautner, 1984). Such ideas seem to be unrelated to results which point to some role other than that assigned to R/M systems for DNA methylation, though it is very interesting to note that the only R/M system whose regulation has been studied in depth shows that there is a close connection between sporulation efficiency and the expression of BsuM restrictase (Fucik *et al.*, 1982) whose specificity, 5'PyTmCGAR3', has recently been determined (Guha, 1985).

There is contradiction between the methylation observed by us (*Bam*HI, *Hpa*I, *Sal*I and *Xho*I) and the results of Günthert (1975) during spore formation in *B. subtilis*, *B. megaterium* and *B. brevis*, in which a marked increase in 5mC was reported. Guha (1985) has stated that during sporulation of *B. subtilis* Marburg 168 a small increase may be observed in amounts of 5mC. Although we still do not know whether the total 5mC and N⁶mA content decreases in the genophore packaged in the spores of *B. coagulans* strain 26, the demethylation observed at these four sites favours the hypothesis that DNA methylation might play a role in sporulation (Freese *et al.*, 1984).

The most surprising aspect of all this is the GmATC modification observed in the DNA of spores of this micro-organism. Although Dreiseikelmann & Wackernagel (1981) noted an absence of *dam* modification in *B. subtilis* Marburg 168, our results might not be in disagreement with theirs, since they analysed modification during the competent state and not during sporulation. However, analysis of spore DNA from *B. subtilis* strains NRRL B1490 and CECT 39 shows the absence of *dam* modification in them. This modification does exist in spores of *B. macerans* NCLB 7588, but it is also observed during the vegetative phase (unpublished observations).

From our point of view there is no relationship between increased activity of prophages (in particular their methylases) during development of competence (Yasbin, 1977; Ganesan, 1979; Trautner *et al.*, 1980) and sporulation. There are some significant differences between the methylase system induced in competent cells, which is perhaps correlated with the activation or derepression of the SOS system (Yasbin, 1977), and that which is associated with the sporulation process, as described here. Even though nutrient starvation induces competence, sporulation and the SOS response, sporulation is regarded as a differentiation process (Sonenshein, 1984), not a physiological state. It involves cell differentiation, rather than a cessation of growth.

An important deduction arising from the modulation of the modification pattern of the GATC sequence during sporulation is the existence of a *de novo* methylase. Since the need has been demonstrated for a complete replication of the genophore during spore formation (Sargent, 1980a; Binnie & Coote, 1983), DNA methylation probably accompanies such replication during

the process which eventually leads to its packaging. The fact that DNA methylated in both strands is not found in vegetative cells, even when analysed in a population with a high proportion of sporulating cells, suggests that methylation at GATC might allow discrimination between the genophore to be packaged and the DNA to be degraded between the daughter cells; this idea, however, is highly speculative.

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