Arch Microbiol (1990) 153:355–359

Archives of
Microbiology

The *Bacillus subtilis menCD* promoter is responsive to extracellular pH

Kevin F. Hill*, John P. Mueller, and Harry W. Taber**

Department of Microbiology and Immunology, Albany Medical College, Albany, NY 12208, USA

Received June 7, 1989/Accepted November 11, 1989

Abstract. Activation kinetics of a *Bacillus subtilis* menaquinone biosynthetic gene promoter (the *menCD* promoter) were measured during growth and sporulation, with the aid of a *menCD'-lacZ* translational gene fusion. Transient maximal activation was seen shortly after the end of exponential growth in unbuffered complex medium containing a low glucose concentration. These activation kinetics were correlated with transient acidification of the medium under conditions permitting TCA cycle function during the post-exponential period, while mutations that blocked TCA cycle function (*cit* mutants) were associated with sustained acidification and promoter activation during this period. In *cit*$^+$ strains, buffering of the medium to pH 5.7 caused sustained maximal activation, while buffering to pH 7.2 prevented enhancement of activation. The *menCD* promoter appears to be responsive to extracellular acidic pH.

Key words: Menaquinone – *men* genes – *Bacillus subtilis* – Promoter – pH

Menaquinone (MK) is a lipophilic redox quinone which forms part of the electron transport chain of *Bacillus subtilis* (Taber 1980). It is synthesized from an intermediate in the common aromatic amino acid pathway, chorismate, through a series of MK-specific reactions that follows the isomerization of chorismate to isochorismate. Regulation of MK concentration appears to be an important event during the sporulation process in bacilli (Farrand and Taber 1974; Escamilla et al. 1988). Enzymes necessary for MK biosynthesis are encoded by the *men* gene cluster, located at approximately 273 degrees on the *Bacillus subtilis* chromosome (Taber et al. 1981; Meganathan et al. 1981; Miller et al. 1988a). The *men* gene cluster has been isolated by molecular cloning (Miller et al. 1988a), and appears to contain at least two transcriptional units (unpublished data). The *menC* and *menD* genes have been localized provisionally to one unit, under control of a single sigma-A promoter, the *menCD* promoter (K. Hill et al. 1988, Tenth International Spores Conference). In order to understand how changes in *men* gene expression affect cellular MK concentrations, activation of the *menCD* promoter was measured during growth and sporulation (Miller et al. 1988b). Utilization of the *menCD* promoter (as measured with a *menCD'-lacZ* transcriptional fusion and by steady-state *men* transcript accumulation) was maximal at approximately 0.5 h ($T_{0.5}$) after the end of exponential growth, and declined rapidly to $< 10\%$ maximal activity at $T_{4.0}$. Inclusion of 5% glucose in the culture medium virtually abolished the post-exponential decline, while having no effect on the timing or amplitude of maximal activity (Miller et al. 1988b). While pursuing the mechanism by which a high glucose concentration promotes sustained activation of the *menCD* promoter, we noted that the pH of the medium declined to 5.7 at $T_{0.5}$ and remained low during the post-exponential period. By contrast, growth in low (0.1%) glucose containing medium resulted in a transient pH decline to 5.7 at $T_{0.5}$ with a subsequent rise back to neutrality. This pH decrease was presumed to result from the accumulation of glycolytic end products, which in the presence of glucose could not be further metabolized, due to catabolite repression of the tricarboxylic acid (TCA) cycle genes (*cit* genes). The accumulation was temporary during growth in 0.1% glucose, but was sustained in 5% glucose because of incomplete consumption of the carbon source. We hypothesized that the regulation of the *men* genes may be linked to an accumulation and excretion of acidic glycolytic end products or TCA cycle intermediates, and designed experiments to test this hypothesis.

Offprint requests to: H. W. Taber

Present addresses: * Department of Pediatrics, Bethesda Naval Hospital, Bethesda, MD 20814, USA; ** Department of Microbiology and Molecular Biology, Tufts University Health Sciences Campus, Boston, MA 02111, USA

Table 1. *Bacillus subtilis* strains

Strain	Genotype	Source
RB352	*metC3 glyB133 trpC2*	This laboratory
RB987	*ϕ(men'-lacZ) cat metC3 glyB133 trpC2*	RB352 × pAI200
RB949	*metC3 glyB133 trpC2 citK109*	L. Sonenshein (SF109)
RB989	*ϕ(men'-lacZ) cat metC3 glyB133 trpC2 citK109*	RB949 × pAI200
RB954	*citB75 trpC2*	BGSC[a] (IA120)
RB1008	*ϕ(men'-lacZ) citB75 trpC2*	RB954 × pAI200
RB1	*trpC2*	This laboratory
RB1007	*trpC2*	RB954 × DNA of RB1
RB1009	*ϕ(men'-lacZ) trpC2*	RB1007 × pAI200
RB629	*sdhC12 trpC2*	L. Hederstedt (KA98012)
RB986	*ϕ(men'-lacZ) sdhC12 trpC2*	RB629 × pAI200
RB992	*ϕ(men'-lacZ) trpC2*	RB986 → Sdh^+ by reversion

[a] BGSC, *Bacillus* Genetic Stock Center, Ohio State University, Columbus

Methods

Bacterial strains and plasmids. Bacterial strains used in this work are listed in Table 1. The *Escherichia coli* plasmid vector pJF751 was obtained from J. Hoch. The plasmid pAI200 contains *Bacillus subtilis men* DNA and is described in the text. *B. subtilis* strains containing chromosomally integrated single copies of pAI200 were generated by transformation of appropriate recipients and selection for chloramphenicol resistance.

Culture media LB medium was used for routine culture of *B. subtilis* (LB is 1% [wt/vol] tryptone, 0.5% yeast extract, and 0.5% NaCl). Chloramphenicol, when needed, was added to 5 μg/ml to select for *cat*-containing plasmids. *B. subtilis* growth and sporulation experiments were done in 2× nutrient sporulation (NS) medium (Schaeffer et al. 1965).

DNA manipulations and transformation. DNA manipulations were carried out as described by Maniatis et al. (1982). Chromosomal DNA was isolated from *B. subtilis* strains as described by Saunders et al. (1984), and preparative isolation of plasmid DNA from *B. subtilis* was carried out by the alkaline lysis procedure of Birnboim and Doly (1979). *B. subtilis* strains were transformed by the method of Piggot (1985). Restriction enzymes and DNA modification enzymes were purchased from Boehringer Mannheim and used as recommended by the supplier.

Southern hybridization experiments. DNA to be analyzed was digested with the appropriate enzyme and electrophoresed through a 0.9% agarose gel. The DNA was then denatured and transferred to nitrocellulose by the method of Southern (1975), following the modifications described by Silhavy et al. (1984). Radioactive probes were prepared by the nick translation technique of Rigby et al. (1977) and used in hybridizations at a specific activity of 2×10^6 dpm/ml.

Measurement of β-galactosidase activity. Bacteria were cultured for assay in highly aerated 2× NS broth at 37°C. Cells were grown overnight in 10 ml LB media in 120 ml Erlenmeyer flasks at 28°C and used to inoculate 100 ml of 2× NS broth in 1.0 liter flasks. At the appropriate times during growth and sporulation, 1.0 ml samples were withdrawn, centrifuged, and frozen in liquid nitrogen. The specific activity of β-galactosidase was determined using the substrate *o*-nitrophenol-β-D-galactoside (ONPG) (Miller 1972). One unit of enzyme hydrolyzes 1 μmol of ONPG per min per OD_{600} unit. In determining levels of *menCD* promoter-directed β-galactosidase synthesis, the background level (0–1 U) of ONPG-hydrolyzing activity observed in isogenic parent cells lacking a gene fusion was subtracted from the levels of enzyme activity measured in the fusion-bearing strain. Each experiment was carried out at least three times, and typical data showing the time-dependent kinetics are presented in the *Results* section. These kinetics were reproducible, although absolute activity values varied slightly from experiment to experiment.

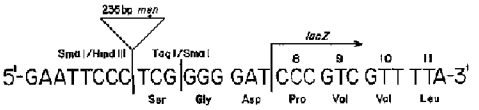

Fig. 1. Structure of the *menCD'-lacZ* fusion joint in plasmid pAI200. The 235 bp insert contains the *menCD* promoter, transcriptional start site, ribosomal binding site, translational start site, and the first 32 codons of the most proximal open reading frame. This is fused in-frame with the eigth codon of *lacZ*

Results

Construction of a menCD'-lacZ translational gene fusion

In order to avoid any complicating effects of *Escherichia coli* translational signals contained within the *menCD'-lacZ* transcriptional fusion used previously (Miller et al. 1988b), a translational gene fusion was constructed. A 235 bp fragment including the *menCD* promoter and the first 32 codons of the most proximal open reading frame was isolated by treatment of plasmid pAI46 (Miller et al. 1988a) with *Hin*dIII and *Taq*I, and filled-in with the Klenow fragment of DNA polymerase I to yield blunt ends. This fragment was inserted into the *Sma*I site of the integration vector pJF751 (Ferrari et al. 1985) to create pAI200, containing an in-frame *menCD'-lacZ* protein fusion. The structure of the fusion joint was confirmed by plasmid DNA sequencing, and it is shown in Fig. 1. Plasmid pAI200 was then integrated by transformation into several *Bacillus subtilis* strains (Table 1), and the integrants confirmed as single-copy constructs by Southern hybridization. Expression of *menCD'-lacZ* was measured during growth and sporulation in 2× nutrient sporulation (NS) medium containing 0.1% glucose, as previously described (Miller et al. 1988b). In wild type strains, the translational fusion showed an activity profile identical with the transcriptional fusion (data not shown), consistent with the hypothesis that *menCD* expression is controlled at the transcriptional level (Miller et al. 1988b).

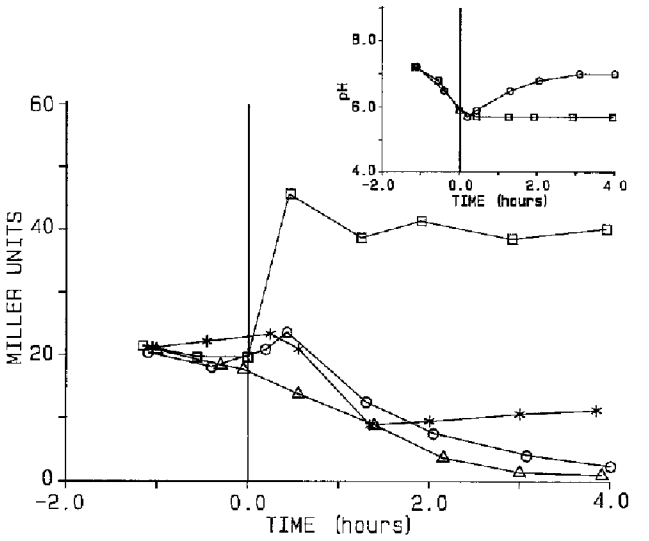

Fig. 2. Activity profiles of β-galactosidase in *Bacillus subtilis citK* and *cit*$^+$ integrants of pAI200, containing the *menCD'-lacZ* fusion during growth in unbuffered and buffered sporulation media. The inset shows pH values during the same time periods. Symbols: □, strain RB989 (*citK*), unbuffered medium; ○, strain RB987 (*cit*$^+$), unbuffered medium; *, strain RB989, buffered medium (pH 7.2); △, strain RB987, buffered medium (pH 7.2). T_0 = measured end of exponential growth phase

Activity of the menCD promoter in TCA cycle mutants

Since mutational blocks in *cit* genes should mimic the repressive effect of growth in 5% glucose, we measured *menCD* promoter activity in *cit* mutant strains. Three TCA cycle mutants were used and compared with isogenic *cit*$^+$ derivatives. Strains RB986, RB989 and RB1008 were deficient in succinate dehydrogenase (*sdhC*), 2-ketoglutarate dehydrogenase (*citK*) and aconitase (*citB*), respectively (Table 1). Kinetics of β-galactosidase activity were measured as a function of growth and sporulation for each of these strains, and are reported in Miller units. Cells were cultured in unbuffered 2× NS medium containing 0.1% glucose (initial pH 7.2). Throughout the exponential and post-exponential phase, the pH of the culture supernatant was measured. In separate experiments, cells were cultured in the same sporulation medium buffered at pH 7.2 with 0.1 M MOPS. The results for the *citK*/*cit*$^+$ isogenic pair are shown in Fig. 2. In unbuffered medium, the *cit*$^+$ strain showed maximal β-galactosidase activity at $T_{0.5}$ and declined thereafter. These kinetics correspond to those previously noted with a transcriptional *menCD'-lacZ* fusion (Miller et al. 1988b). The *citK* strain also showed maximal activity at $T_{0.5}$, but the amplitude was much greater, and there was very little decline from this maximum to $T_{4.0}$. Changes in the pH of the medium were different for the two strains as well; growth of the *cit*$^+$ strain caused acidification of the medium to pH 5.7 by $T_{0.25}$, after which the pH immediately began to increase, reaching 7.0 by $T_{3.0}$; however, continued incubation of the *citK* strain after $T_{0.25}$ did not result in a return of medium pH to neutrality; instead, it remained at 5.7. The effect on *menCD* promoter activation of buffering the growth media at pH 7.2

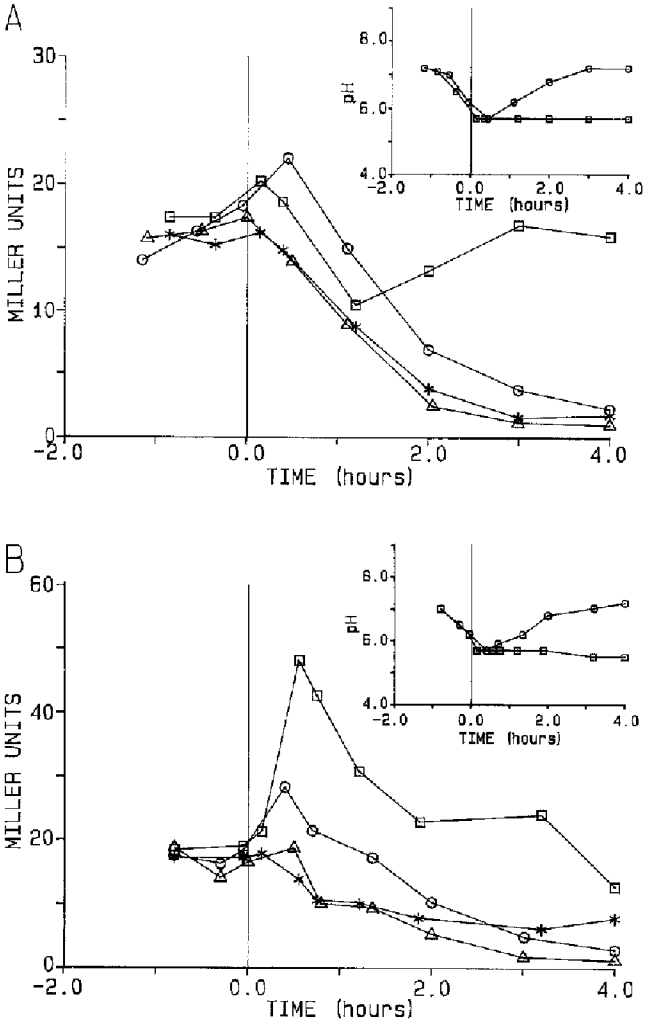

Fig. 3A, B. Activity profiles of β-galactosidase in *B. subtilis citB*, *cit*$^+$, *sdhC*, and *sdh*$^+$ integrants of pAI200, containing the *menCD'-lacZ* fusion, during growth in unbuffered and buffered sporulation media. As in Fig. 2, the insets show pH values. Symbols, **A** □, strain RB1008 (*citB*), unbuffered medium; ○, strain RB1009 (*cit*$^+$), unbuffered medium; *, strain RB1008, buffered medium; △, strain RB1009, buffered medium. Symbols, **B** □, strain RB986 (*sdhC*), unbuffered medium; ○, strain RB992 (*sdh*$^+$), unbuffered medium; *, strain RB986, buffered medium; △, strain RB992, buffered medium. T_0 as in Fig. 2

also is shown in Fig. 2. In the *cit*$^+$ strain, the rise in β-galactosidase activity at $T_{0.5}$ was abolished, while in the *citK* strain, the high sustained activation was considerably reduced (pH measurements, not depicted in Fig. 2, showed that buffering with 0.1 M MOPS was adequate).

To test whether the above results were unique to *citK* strains, similar measurements were carried out on *citB* (Fig. 3A) and *sdhC* (Fig. 3B) strains. Qualitatively similar *menCD* promoter activation kinetics were obtained, although with significant quantitative differences. For example, the *citB* strain showed kinetics similar to the *cit*$^+$ strain until $T_{1.0}$, when the decline was reversed, and promoter activation was sustained (Fig. 3A). The *sdhC* strain, by contrast, was similar to *citK* in having a much higher $T_{0.5}$ maximal activity, but this was then followed

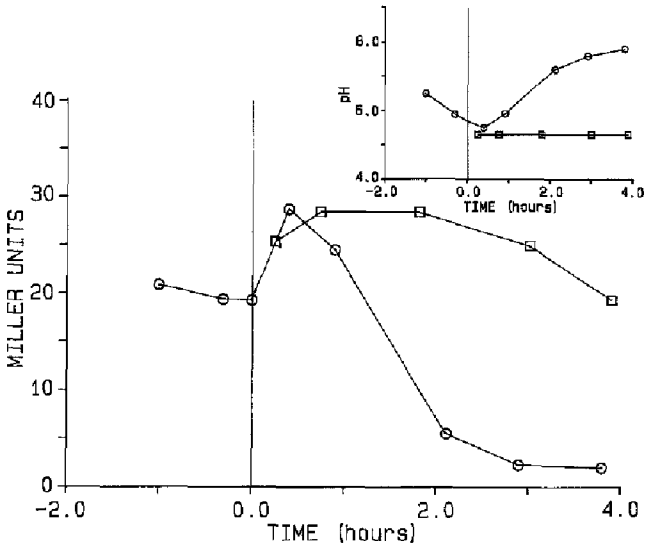

Fig. 4. Effect of growth medium acidification on activity profiles of β-galactosidose in the *cit*$^+$ φ(*menCD'-lacZ*) strain RB987. Symbols: ○, growth in unbuffered sporulation media; □, MES buffer (pH 5.5) added at $T_{0.25}$ to a final concentration of 0.1 M. T_0 as in Fig. 2

by a decline (Fig. 3B). With both *citB* and *sdhC*, growth in unbuffered 2× NS medium resulted in a sustained pH of 5.7 during the post-exponential period (insets, Fig. 3A, B). When comparable experiments were carried out in buffered media (Fig. 3A, B), all strains showed steady declines in β-galatosidase activity during the post-exponential period; that is, there was no maximum observed at $T_{0.5}$.

Effect of acidic buffering on menCD promoter activity

The continued activation of the *menCD* promoter in *cit* strains during the post-exponential period could have been due to a failure to synthesize necessary TCA cycle intermediates. To test this, we examined whether artificial lowering of medium pH by addition of an acidic buffer would cause activity of the *menCD* promoter in a *cit*$^+$ strain to remain at the maximal level. As shown in Fig. 4, strain RB987 was grown in 2× NS medium containing 0.1% glucose to $T_{0.25}$ and divided into two aliquots. One culture was buffered to pH 5.5 with 0.1 M MES, while the other was left unbuffered. The latter showed a normal decline in β-galactosidase activity from $T_{0.5}$ to $T_{4.0}$, while the acidic buffering caused *menCD* promoter activation to remain at a sustained high level.

The changes in β-galactosidase activity observed in these experiments could have resulted from changes in *menCD* promoter activation, or from alterations in either chimeric enzyme or mRNA stability. Enzyme stability was assessed by measuring rates of β-galactosidase decay after treating cell samples with chloramphenicol. An example of the data obtained is shown in Table 2. Rates of decay slowed during the post-exponential period; but differences between cultures with sustained or transiently low pH values were not sufficient to account for the large

Table 2. Rate of decay of β-galactosidase activity in unbuffered and acidically buffered media

Time of addition of chloramphenicol	Decay rate, Miller units per min	
	Unbuffered	Buffered (pH 5.5)
T_0	0.55(5.8)	–
T_1	0.56(6.0)	0.38(5.5)
T_2	0.25(7.0)	0.24(5.5)

As in Fig. 4, strain RB987 was grown in unbuffered sporulation medium with 0.1% glucose, and at T_0 divided into two aliquots. One culture was buffered with 0.1 M MES (pH 5.5), and the other remained unbuffered. At the times indicated (T_0, T_1, T_2), 100 μg/ml chloramphenicol was added, and β-galactosidase activity measured at 3-min intervals for 12 min. Decay rates were estimated graphically. Extracellular pH values are given in parentheses

activity differences observed (Fig. 4). Although *men*-specific mRNA measurements have not been made in this study, it was shown previously that *menCD* promoter activity as measured by β-galactosidase levels reflects mRNA accumulation in unbuffered sporulation medium (Miller et al. 1988b).

Discussion

Our results indicate that decreased extracellular pH is associated with increased activation of the *menCD* promoter; this decreased pH can arise by accumulation of glycolytic end products or other carboxylic acids, or can be induced by buffering the medium to an acidic pH. Furthermore, enhancement of promoter activation can be reversed by extracellular buffering to a neutral pH. Cellular requirements for increased *men* gene transcription and increased MK synthesis at decreased external pH may be related to the bioenergetic role of MK as a component of the electron transport chain, the primary function of which is the extrusion of protons. The intracellular proton concentration is a function both of metabolism and of external pH. When the external pH decreases to values below the intracellular pH, a net diffusion of protons into the cell can occur, and a larger MK concentration in the cytoplasmic membrane may be necessary to maintain proton extrusion rates consistent with intracellular pH homeostasis (Booth 1985). Alternatively, activation of *men* gene expression could be enhanced by the presence of elevated concentrations of carboxylic acids, which might be taken up by cells more readily at acidic pH. That is, pH may be a correlate of promoter activation rather than involved directly in the mechanism of activation. Fisher and Magasanik (1984) showed that aconitase and histidase formation are responsive to cellular levels of 2-ketoglutarate. Similarly, operons in the *men* gene cluster may be regulated positively by glycolytic end products or TCA cycle intermediates. This is presently under study.

Genetic regulatory mechanisms related to environmental pH have not, to our knowledge, previously been described in *Bacillus subtilis*, although *citC'-lacZ* gene fusions show enhanced expression in *cit* mutant strains

(Igo and Losick 1986), suggesting that *ctc* also may be regulated by pH. However, regulation of several *Escherichia coli* envelope protein genes by extracellular pH has been reported recently (Heyde and Portalier 1987; Heyde et al. 1988); transcription only of *ompC* was stimulated at low pH, and this enhanced expression was dependent on the *ompC* promoter. In addition, several genes of unknown function that are responsive to low pH have been identified in *Escherichia coli* (Slonczewski et al. 1987); some of these (*exa* genes) are activated by external acidification; others (*ina* genes) only by acidification of the cytoplasm with weak acids. As we have not yet measured intracellular pH changes in response to extracellular acidification, it is unclear what the proximal signal for activation of the *B. subtilis menCD* promoter may be. Whatever the nature of this signal, these findings taken together with the previously demonstrated interrelationship of pH-mediated gene expression and anaerobiosis in *Salmonella typhimurium* (Aliabadi et al. 1988) and the induction of both heat shock (Taglicht et al. 1987) and SOS (Schuldiner et al. 1986) responses in *E. coli* by alkalinization, suggest that transmembrane pH gradients play significant roles in bacterial gene regulation (Padan and Schuldiner 1987).

Acknowledgements. We would like to thank Linc Sonenshein for helpful suggestions and for strain SF109, Lars Hederstedt for strain KA98012, Jim Hoch for plasmid pJF751 and Jeff Driscoll for valuable discussions. Kevin Hill received support from Public Health Service training grant HL07506 to Albany Medical College. This work was also supported in part by Public Health Service grant GM34931 from the National Institute of General Medical Sciences.

References

Aliabadi Z, Park YK, Slonczewski JL, Foster JW (1988) Novel regulatory loci controlling oxygen and pH-regulated gene expression in *Salmonella typhimurium*. J Bacteriol 170:842–851

Birnboim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl Acid Res 7:1513–1523

Booth IR (1985) Regulation of cytoplasmic pH in bacteria. Microbiol Rev 49:359–378

Escamilla JE, Baguera B, Ramirez R, Garcia-Horsman A, del Arenal P (1988) Role of menaquinone in inactivation and activation of the *Bacillus cereus* forespore respiratory system. J Bacteriol 170:5908–5912

Farrand SK, Taber HW (1974) Changes in menaquinone concentration during growth and early sporulation in *Bacillus subtilis*. J Bacteriol 117:324–326

Ferrari FA, Trach K, Hoch JA (1985) Sequence analysis of the *spo0B* locus reveals a polycistronic transcription unit. J Bacteriol 161:556–562

Fisher SH, Magasanik B (1984) 2-Ketoglutarate and the regulation of aconitase and histidase formation in *Bacillus subtilis*. J Bacteriol 158:379–382

Heyde M, Portalier RC (1987) Regulation of major outer membrane porin proteins of *Escherichia coli* K-12 by pH. Mol Gen Genet 208:511–517

Heyde M, Lazzaroni JC, Magnouloux-Blanc B, Portalier R (1988) Regulation of porin gene expression over a wide range of extracellular pH in *Escherichia coli* K-12: influence of a *tolA* mutation. FEMS Microbiol Lett 52:59–66

Igo MM, Losick R (1986) Regulation of a promoter that is utilized by minor forms of RNA polymerase holoenzyme in *Bacillus subtilis*. J Mol Biol 191:615–624

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Meganathan R, Bentley R, Taber H (1981) Identification of *B. subtilis men* mutants that lack *o*-succinylbenzoyl-coenzyme A synthetase and dihydroxynaphthoate synthase. J Bacteriol 145:328–332

Miller JH (1972) Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Miller P, Rabinowitz A, Taber H (1988a) Molecular cloning and preliminary genetic analysis of the *men* gene cluster of *Bacillus subtilis*. J Bacteriol 170:2735–2741

Miller P, Mueller J, Hill K, Taber H (1988b) Transcriptional regulation of a promoter in the *men* gene cluster of *Bacillus subtilis*. J Bacteriol 170:2742–2748

Padan E, Schuldiner S (1987) Intracellular pH and membrane potential as regulators in the prokaryotic cell. J Membrane Biol 95:189–198

Piggot PJ (1985) Transformation of competent *Bacillus subtilis*. Spores Newsl 8:246–250

Rigby PWJ, Dieckman M, Rhodes C, Berg P (1977) Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J Mol Biol 113:237–251

Saunders CW, Schmidt BJ, Mirot MS, Thompson LD, Guyer MS (1984) Use of chromosomal integration in the establishment and expression of *blaZ*, a *Staphylococcus aureus* β-lactamase gene, in *Bacillus subtilis*. J Bacteriol 157:718–726

Schaeffer P, Millet J, Aubert JP (1965) Catabolic repression of bacterial sporulation. Proc Natl Acad Sci USA 54:704–711

Schuldiner S, Agmon V, Brandsma J, Cohen A, Friedman E, Padan E (1986) Induction of SOS functions by alkaline intracellular pH in *Escherichia coli*. J Bacteriol 168:936–939

Silhavy TJ, Berman ML, Enquist LW (1984) Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Slonczewski JL, Gonzalez TN, Bartholomew FM, Holt NJ (1987) Mud-directed *lacZ* fusions regulated by low pH in *Escherichia coli*. J Bacteriol 169:3001–3006

Southern E (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–517

Taber H (1980) Functions of vitamin K_2 in microorganisms. In: Suttie JW (ed) Vitamin K metabolism and vitamin K-dependent proteins. University Park Press, Baltimore, pp 177–187

Taber HW, Dellers EA, Lombardo LR (1981) Menaquinone biosynthesis in *Bacillus subtilis*: isolation of *men* mutants and evidence for clustering of *men* genes. J Bacteriol 145:321–327

Taglicht D, Padan E, Oppenheim AB, Schuldiner S (1987) An alkaline shift induces the heat shock response in *Escherichia coli*. J Bacteriol 169:885–887