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The effect of novobiocin on solvent production by *Clostridium acetobutylicum*

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Cells of *Clostridium acetobutylicum* treated with novobiocin, a DNA gyrase inhibitor, produced higher butyrate levels and lower solvent levels with acetone being the most affected. Seven enzyme activities involved in acid and solvent production were analyzed. Among them, only CoA transferase, required for acetone formation and acid uptake, experienced a significant decrease in activity. As in *Escherichia coli* and *Bacillus subtilis*, DNA from *C. acetobutylicum* became less negatively supercoiled in the early stationary phase (solventogenic stage), as shown by analysis of linking number of a reporter plasmid by agarose gel electrophoresis in the presence of chloroquine.

Keywords: supercoiling; enzymes; acetone; butanol; *Clostridium acetobutylicum*

Introduction

The switch from acidogenic, exponential growth to solventogenic, stationary growth in *Clostridium acetobutylicum* is triggered by a combination of low pH and high butyrate concentrations. Possible mechanisms of regulation have been discussed [3,9,15,28]. Although many of the genes involved in the acid and solvent production pathways have been cloned and analyzed [3,9], important factors in their regulation remain to be studied.

In many bacterial species, regulation of multigene systems is achieved by activators/repressors (as in nitrogen utilization) and sigma factors (as in sporulation). There has also been increasing evidence that DNA supercoiling and bending may play a role in genetic regulation. Supercoiling is maintained in cells by a group of enzymes called DNA topoisomerases [30]. The two dominant activities are topoisomerase I (topo I) and topoisomerase II (DNA gyrase), which catalyze the removal and introduction of DNA supercoils, respectively. Their activities can change in response to environmental factors: temperature [13], osmolarity [7,14,17], oxygen tension [34], and pH [18]. A number of proteins, such as histone-like proteins [8], are also believed to affect supercoiling. Drugs such as novobiocin and nalidixic acid affect supercoiling by inhibiting DNA gyrase [1,11,22,33,35]. Based on this finding, quinolone antibiotics are used as antibacterial agents [16].

With *Escherichia coli*, there is no correlation between growth rate and linking number [2] during exponential growth. The linking number, the total number of times the two strands of the closed circular plasmid DNA wind around each other, is composed of the twisting number (number of turns of duplex DNA) and the writhe (supercoiled turns) and can be analyzed by gel electrophoresis [19]. Phosphate starvation and heat shock cause no change in linking numbers, but plasmid DNAs isolated from cells in the stationary phase are less negatively supercoiled, and so are those from cells subjected to glucose starvation [2]. In *Bacillus subtilis*, supercoiling changes during different growth phases [26]: (a) in the vegetative stage, the average linking number compared to the relaxed form of a 4.6-kb reporter plasmid is −34; (b) in the stationary phase, the average is −30 (ie less supercoiled than in the vegetative stage); (c) in the endospore stage, the average is −47 in the forespore or the mature spore and −30 in the mother cell. The sharp increase in negative supercoils in the forespore and the mature spore is caused largely by the forespore-specific small acid-soluble proteins (SASP) [25]. The magnitude of the increase in negative supercoils is lower in SASP mutants [26]. Sporulation can be blocked by gyrase inhibitors [29].

This study addresses two questions: (a) is solvent production affected by topoisomerase inhibitors?; and (b) does supercoiling change when *C. acetobutylicum* undergoes the solventogenic switch?

Materials and methods

Strains and growth conditions

Cells of *C. acetobutylicum* ATCC 824 (type strain) were grown in reinforced soluble medium (RSM) [6] or on reinforced clostridial agar (RCA) (Difco, Detroit, MI, USA) plates at 37°C under anaerobic conditions. *E. coli* ER2275 (*mcr*$^-$) (New England BioLabs, Beverly, MA, USA) cells had been transformed with pAN1 [23] and were grown in Luria-Bertani medium (LB) at 37°C under aerobic conditions. Media were supplemented with chloramphenicol (32 μg ml^{-1}) or erythromycin (200 μg ml^{-1} for *E. coli*, 40 μg ml^{-1} for *C. acetobutylicum*) in order to maintain specific plasmids.

Inhibitor dosage determination

Varying concentrations of nalidixic acid and novobiocin (Sigma, St Louis, MO, USA) were added to *C. acetobutylicum* ATCC 824 10-ml RSM cultures, and growth was monitored by absorbance at 600 nm. A dosage that

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increased doubling time by ~15% was chosen for subsequent studies.

Gas chromatography

Cells were grown to exponential phase, and the culture was then divided into two. Novobiocin was added to one, while the other served as the control. Samples were removed at 2-h intervals. After growth and pH measurements, a sample of the cell culture was centrifuged in a microcentrifuge at 20 000 × *g* for 20 min, and 1 ml of the growth medium was stored at −20°C for later analysis by gas chromatography (GC). The supernatant phase was acidified and analyzed using a Varian gas chromatograph equipped with a Chromosorb 101 column (Alltech, Deerfield, IL, USA). The eluted products were monitored with a flame-ionization detector connected to an integrator (Waters, Milford, MA, USA).

Enzyme assays

Cells were grown anaerobically at 37°C in pH-unregulated RSM cultures to exponential phase. Novobiocin, when added, was at a final concentration of 8 μM. Incubation was continued to 10 h. Aliquots of 200 ml were taken at the time points indicated. Cells were pelletted by centrifugation, resuspended in 9 ml MOPS buffer (pH 7.0) plus 1 mM mercaptoethanol [27], and lysed by two passages through a French pressure cell (SLM Aminco Instruments, Urbana, IL, USA). The cell debris was then removed by centrifugation to produce the crude extract. Protein determination was conducted using the Protein Assay Dye Reagent (Bio-Rad, Hercules, CA, USA) with bovine albumin as standard [4]. Enzyme assays for phosphotransbutyrylase (PTB), butyryl kinase (BK), acetoacetyl-coenzyme A : acetate/butyrate : coenzyme A-transferase (CoA transferase), NADH- and NADPH-dependent butanol dehydrogenase (BDH), and NADH- and NADPH-dependent butyraldehyde dehydrogenase (BAD) were performed according to Clark *et al* [6], and the specific activities for each sample were determined. One unit of activity refers to one nmol of the monitored molecule converted per minute.

Transformation

E. coli cells were transformed according to Chung and Miller [5]. *C. acetobutylicum* ATCC 824 cells were transformed with methylated plasmids from *E. coli* by electroporation according to Mermelstein and Papoutsakis [23].

Plasmid DNA isolation

The reporter plasmid used in this study was the 8.7-kb shuttle vector pSYL2 [21], which has the macrolide, lincosamide, and streptogramin B (MLS) resistance genes and two origins of replication: the ColE1 origin functional in *E. coli* and the origin derived from a *C. butyricum* cryptic plasmid functional in *C. acetobutylicum*. Methylation by the ϕ3T I methyltransferase has been shown to protect plasmid DNA from restriction by the *Cac824*I endonuclease present in *C. acetobutylicum* [23]. The pAN1 plasmid present in *E. coli* ER2275 is a pACYC184 derivative that replicates via the p15A origin of replication and contains the chloramphenicol resistance gene and the gene encoding the methyltransferase, which will methylate co-harboring plasmids.

Plasmids from *E. coli* were isolated by the Qiagen (Studio City, CA, USA) Plasmid DNA Preparation Kit according to the manufacturer's instructions. Plasmids from *C. acetobutylicum* were obtained using a modified alkaline lysis method. At the appropriate growth phase, 200 ml of a culture were centrifuged at 6000 × *g* at 4°C for 15 min. The cell pellet was then washed once in 50 ml of resuspension buffer (6.7% (w/v) sucrose, 50 mM Tris-HCl (pH 8.0), 1 mM EDTA), pelleted, and resuspended again in 19 ml of the buffer. To the cell suspension, 5 ml of lysozyme solution (40 mg ml^{-1} of lysozyme in 25 mM Tris-HCl (pH 8.0)) was added. After a 30-min incubation at 37°C, the cells were pelleted again for 15 min. The DNA was then isolated using the Qiagen Plasmid Kit.

Plasmid linking number determination

A series of topoisomer standards was generated using pSYL2 isolated from *E. coli* and mixed with topoisomerase I (Promega, Madison, WI, USA) in the presence of different concentrations of ethidium bromide [19]. Reaction conditions were: 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol at 37°C for 1 h (in 40 μl). The reaction products were then purified [19] and analyzed on a 0.7% (w/v) TBE agarose gel at 3–4 V cm^{-1} for 18 h. The linking numbers of the bands were determined following Southern hybridization and phosphoimager analysis [19]. Compressed bands were resolved by adding chloroquine (Sigma) to the gel and to the running buffer. The mobility of pSYL2 isolated from three kinds of *C. acetobutylicum* ATCC 824 cells was determined by comparing it to the topoisomer standards. A 600-ml culture was allowed to grow to exponential phase (culture A); and a 200-ml sample of this culture was allowed to grow for another 10 h (culture B). Therefore, cultures A and B correspond to cells in the acidogenic phase or early solventogenic phase. Plasmids isolated from these cells were analyzed as outlined above and compared to the standards [19].

Southern hybridization

The probe, pSYL2, was radiolabeled as for Northern hybridization. DNA molecules were separated on an agarose gel, nicked on an ultraviolet transilluminator, and blotted onto Hybond N+ membrane by downward alkaline transfer for 1.5 h using 0.4 M NaOH [20]. The blotted membrane was rinsed briefly in 2 × SSC, pre-washed in 5 ml of Rapid Hybridization Buffer (Amersham, Arlington Heights, IL, USA) at 42°C for 15 min, hybridized with the radiolabeled probe in 5 ml of fresh buffer at the same temperature for 1.5 h, and washed three times with 100 ml of wash buffer (40 mM phosphate buffer (pH 7.2), 0.1% SDS) at the same temperature for 30 min. The washed membrane was wrapped in Saran wrap and exposed to an imaging plate. The band with the highest intensity among the population in each sample was used to determine the mean linking number difference [19].

Results

Topoisomerase inhibitor dosage determination

The effects of gyrase inhibitors, which generally decrease negative supercoils, have not been reported for *C. acetobutylicum*, although the effects of various protein and nucleic acid inhibitors have been published [31]. Nalidixic acid and novobiocin in the μM range slowed the growth of *C. acetobutylicum*, suggesting that they are effective against gyrase in this organism. Although treated cells grew more slowly, they reached the same final absorbance as the untreated cells if allowed to continue growing. To quantify the difference in growth by the different concentrations of either inhibitor, the doubling time (t_D) was calculated for each growth curve during exponential growth. The minimal concentration of novobiocin that produced a modest increase in t_D was 8 μM, while addition of 150 μM nalidixic acid was required for a similar effect on growth. It was thought that the high concentration of nalidixic acid required might lead to non-specific effects, so novobiocin was used in subsequent experiments.

Growth and pH

Untreated cells grew faster than novobiocin-treated cells, and their pH profiles differed too. When the growth of the untreated cells slowed, the pH of the medium stopped decreasing and began to rise, while the pH kept decreasing for cells treated with 8 μM novobiocin (Figure 1), suggesting that either the treated cells were having a longer acidogenic stage or they were not taking up the acids as rapidly.

Metabolite analysis

Fermentation products from the same time points as in the studies on pH showed differences between treated and untreated cells (Figure 2). Untreated cells showed a decrease in acetate and butyrate concentration after growth had slowed down, indicating the slowdown of acid production and the beginning of acid uptake. During this time, solvent formation began. Cells treated with 8 μM novobiocin accumulated slightly more butyrate and showed slower removal of both acetate and butyrate. At the same time, less solvents were being produced, especially acetone.

Enzyme activities

Novobiocin exhibited no significant effect on many enzymes in the acid and solvent production pathways: PTB, BK, NADH-dependent BDH, NADH-dependent BAD, and NADPH-dependent BAD (Table 1). On the other hand, a small decrease in NADPH-dependent BDH and a greater decline in CoA transferase activity were observed. When novobiocin was added to the crude extract of the untreated cells, CoA transferase activities were not affected, indicating that novobiocin did not directly inhibit CoA transferase at the protein level *in vitro*. Whether novobiocin could somehow directly affect CoA transferase *in vivo* was unknown. The larger reduction in acetone production correlated with the reduction in CoA transferase activity.

Because NADH-dependent and NAD(P)H-dependent BAD activities are unstable in *C. acetobutylicum* ATCC 824 *in vivo* based on rifampicin and chloramphenicol studies [31], and a gene encoding NADH-dependent BAD activity (*adhE*, *aad*) has been found in the same operon as the genes encoding CoA transferase [10,24], it is surprising to find that BAD activities were not affected by novobiocin. However, it is still unclear how many genes may encode BAD activities, so the regulation of BAD activity may be complex [9].

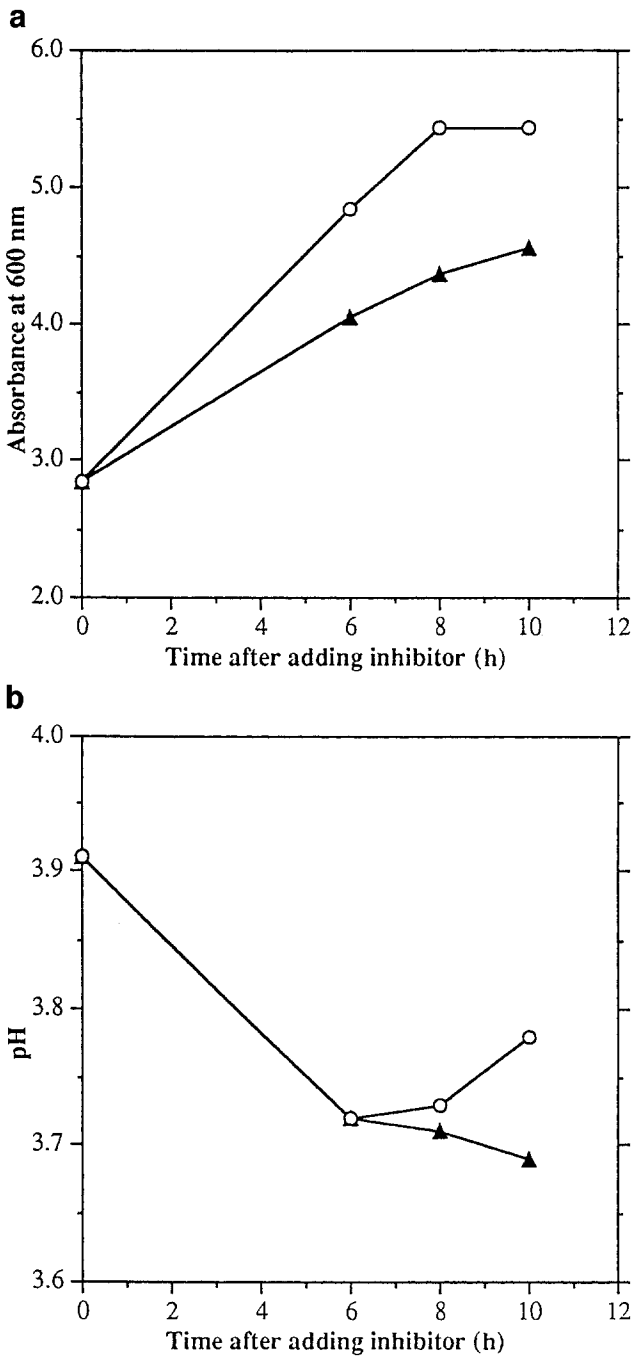

Figure 1 Effects of novobiocin on growth and pH. Cells were grown to exponential phase and then treated with 8 μM novobiocin for 6–10 h. Aliquots were taken every 2 h. (a) Growth was monitored by absorbance at 600 nm. (b) pH was determined at the same time points. (○) control (untreated cultures); (▲) cultures treated with 8 μM novobiocin.

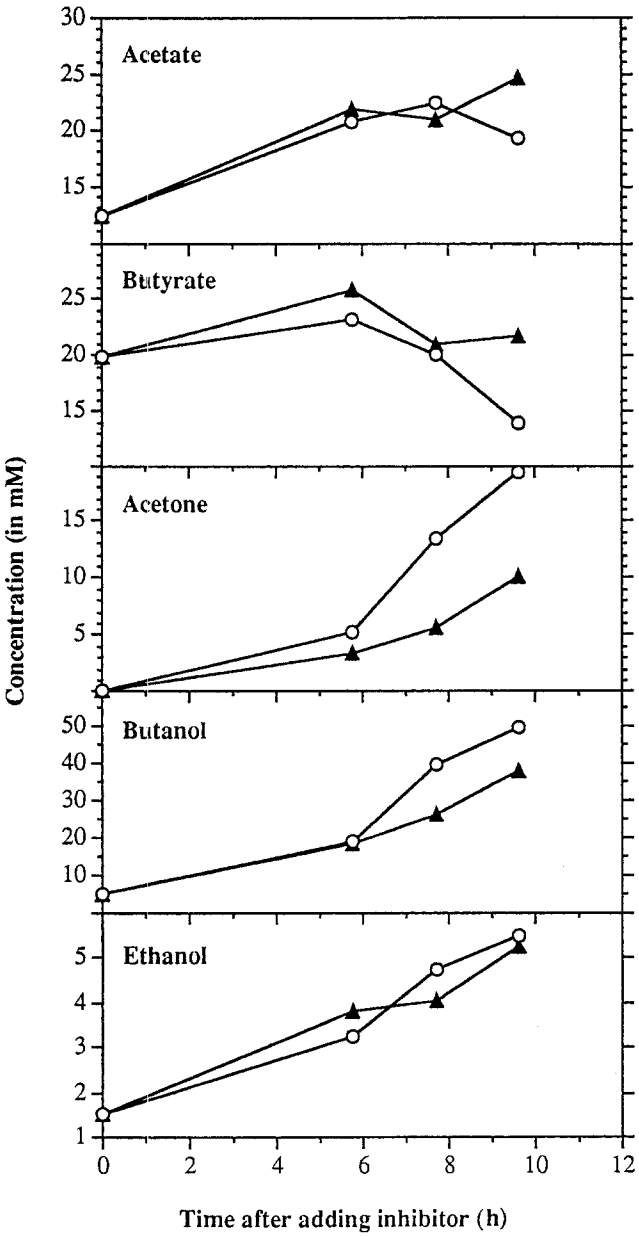

Figure 2 Effects of novobiocin on acid and solvent production. The fermentation products of the same cells as in Figure 1 were analyzed by gas chromatography. (○) control; (▲) cultures treated with 8 μM novobiocin. Samples were analyzed from at least three cultures as described in Materials and Methods, and a representative plot is shown. The reproducibility (standard deviation) of the measurements was generally ±7% of the value shown on the graph.

Plasmid linking number determination

Topoisomers of pSYL2 were generated by treatment with topo I in the presence of different concentrations of ethidium bromide and resolved on agarose gels. Each topoisomer standard or each sample isolated *in vivo* showed multiple bands, and this heterogeneity has been attributed to thermodynamic fluctuations of the DNA during rejoining by topoisomerases [19]. Bands corresponding to topoisomers with low and high linking numbers were not resolved. To resolve compressed bands, chloroquine was added to the gel and the running buffer, giving higher resolution than ethidium bromide. When chloroquine was present at 0.4–2.0 μM, the compressed bands were resolved. Adjacent bands differed by one supercoil, corresponding to a linking number difference of 1 [19]. The band showing the highest intensity according to phosphoimaging analysis was used to determine the mean linking number.

Methylated pSYL2 was electroporated into *C. acetobutylicum* and isolated during different growth phases. The isolated plasmids were all highly negatively supercoiled and showed the same mobility in gels containing no chloroquine. When chloroquine was present, the bands were resolved. When these plasmid samples were linearized by *Eco*RI, they showed the same mobility in chloroquine-containing gels. When intact, the plasmid from the acidogenic phase (corresponding to the same growth phase as 0 h in Figures 1 and 2) showed a higher mobility than from the early solventogenic phase (corresponding to the same growth phase as 10 h in Figures 1 and 2), indicating that it was more negatively supercoiled. Compared to the standards (Figure 3), pSYL2 DNA isolated from the acidogenic phase showed a linking number difference of approximately −63, while the same plasmid from the early solventogenic phase showed a value of −47.

Discussion

The effect of novobiocin on solvent production suggests that supercoiling changes may contribute to the regulation of genes in the acid and solvent pathways. The reduction, upon entering stationary phase, in (a) the rise of pH, (b) the removal of acids, and (c) the production of solvents (especially acetone) suggests a possible influence on the solvent switch. This observation can be explained by a reduction of activity of CoA transferase, which is an essential enzyme in both acid uptake and acetone production. This effect of novobiocin is similar to the effects of nutritional factors which can affect the butanol/acetone ratio [12].

The effects of novobiocin differ from those of rifampicin [31], which decreases NADH-dependent and NADPH-dependent BAD activities but not CoA transferase activity. This suggests that the effects of novobiocin were not due to a general blockage of transcription. In addition, the slightly different effects of novobiocin on acetone production and butanol production suggest that these two processes may be regulated by different mechanisms. It has been proposed that the alcohol production pathways are induced by accumulated NAD(P)H [15]. NAD(P)H would then be removed during alcohol production. The acetone production pathway may additionally respond to the presence of accumulated acids, so that the acids can be converted to their CoA derivatives during acetone production, thereby reducing acid stress [32].

The linking number studies showed that, as in *E. coli* and *B. subtilis*, DNA from *C. acetobutylicum* became less negatively supercoiled in the early stationary stage. DNA isolated from this phase did not show the dramatic increase in supercoils seen in *B. subtilis* forespores [25,26]. Plasmid DNA isolated from *C. acetobutylicum* grown to the late solventogenic/endospore stage did become highly supercoiled and was more supercoiled than plasmid DNA isolated from the acidogenic stage. Thus, changes in super-

Table 1 Effect of novobiocin on enzyme activities

Enzyme assay[a]	Activities (units mg^{-1})	
	Control culture	Culture with 8 μM novobiocin
Enzymes involved in butyrate production		
Phosphotransbutyrylase	16000 ± 1000	17000 ± 1100
Butyrate kinase	3090 ± 131	3300 ± 160
Enzymes involved in butanol production		
NADH-dep. butanol dehydrogenase	11 ± 2.6	16 ± 3.2
NADPH-dep. butanol dehydrogenase	31 ± 3.7	20 ± 4.1
NADH-dep. butyraldehyde dehydrogenase	54 ± 1.6	61 ± 2.4
NADPH-dep. butyraldehyde dehydrogenase	43 ± 1.6	42 ± 2.4
Enzyme involved in acetone production and acid uptake		
CoA transferase	860 ± 140 890 ± 65[b]	400 ± 22

[a]Crude extracts from the same cell culture at the 10th hour as in Figure 2 were analyzed for the enzyme activities shown. The assays were performed three times as described in Materials and Methods. The standard deviation for each value is indicated.
[b]Control extract + 8 μM novobiocin added during the enzyme assay.

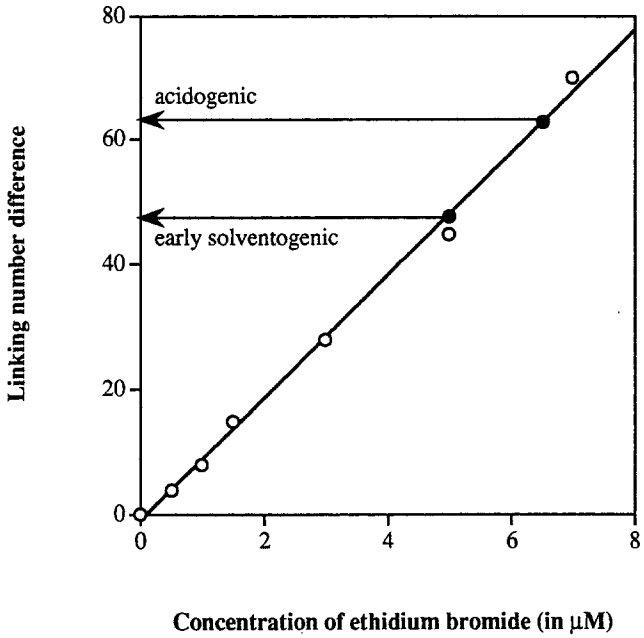

Figure 3 Linking number differences of pSYL2. Topoisomer standards were generated by treating pSYL2 with topo I in the presence of different concentrations of ethidium bromide. The linking number differences from relaxed DNA (pSYL2 treated with topo I in the absence of ethidium bromide) (○) were plotted against ethidium bromide concentration to obtain a standard curve. Plasmids isolated from cells in the acidogenic phase, and early solventogenic phase were separated by electrophoresis on the same gel and compared to the topoisomer standards; their linking number differences (●) are also shown.

coiling do accompany the transition to the solventogenic phase, and these changes should be considered in developing more detailed models of regulation.

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