A Mutation in the *Bacillus subtilis rsbU* Gene That Limits RNA Synthesis during Sporulation

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ABSTRACT Mutants of *Bacillis subtilis* that are temperature sensitive for RNA synthesis during sporulation were isolated after selection with a ^{32}P suicide agent. Whole-genome sequencing revealed that two of the mutants carried an identical lesion in the *rsbU* gene, which encodes a phosphatase that indirectly activates SigB, the stress-responsive RNA polymerase sigma factor. The mutation appeared to cause RsbU to be hyperactive, because the mutants were more resistant than the parent strain to ethanol stress. In support of this hypothesis, pseudorevertants that regained wild-type levels of sporulation at high temperature had secondary mutations that prevented expression of the mutant *rsbU* gene. The properties of these RsbU mutants support the idea that activation of SigB diminishes the bacterium's ability to sporulate.

IMPORTANCE Most bacterial species encode multiple RNA polymerase promoter recognition subunits (sigma factors). Each sigma factor directs RNA polymerase to different sets of genes; each gene set typically encodes proteins important for responses to specific environmental conditions, such as changes in temperature, salt concentration, and nutrient availability. A selection for mutants of *Bacillus subtilis* that are temperature sensitive for RNA synthesis during sporulation unexpectedly yielded strains with a point mutation in *rsbU*, a gene that encodes a protein that normally activates sigma factor B (SigB) under conditions of salt stress. The mutation appears to cause RsbU, and therefore SigB, to be active inappropriately, thereby inhibiting, directly or indirectly, the ability of the cells to transcribe sporulation genes.

KEYWORDS sigma factor B, RsbU, *Bacillus subtilis*, sporulation

Sporulation is an elaborate developmental process involving the significant change in expression of nearly 600 genes in *Bacillus subtilis*, representing more than 10% of its genome (1). There are at least five RNA polymerase sigma factors (promoter recognition proteins) that mediate this complex change in gene expression. During the formation of the metabolically dormant endospore, two of these sigma factors are active in the forespore (SigF and SigG) and two in the mother cell (SigE and SigK) (2).

The decision to undergo sporulation in response to nutrient deprivation is made in the first 2 h after *B. subtilis* enters the stationary phase. After that time, the cell becomes committed to completing the ordered sporulation program. Spo0A, the master regulator governing the decision to sporulate (3, 4), is activated by phosphorylation carried out by a phosphorelay system involving five kinases and two phosphorelay proteins, Spo0B and Spo0F (5). Only the active phosphorylated Spo0A form (Spo0A~P) directly contacts promoters, resulting in the induction of some genes and the repression of others. Under some conditions, Spo0A~P is subject to inactivation by dephosphorylation by the dedicated phosphatase, Spo0E (4, 5).

In 1973, before any of the sporulation sigma factors had been discovered, we

FIG 1 Proposed model of Spo^ts and ethanol resistance phenotypes of mutants DR33 and DR38. In the absence of environmental stress, RsbW blocks SigB activity and phosphorylates RsbV, thereby inactivating RsbV as an antagonist of RsbW. Under ethanol, salt, or heat stress, RsbU is activated by RsbT and dephosphorylates RsbV, allowing RsbV to bind to and inactivate RsbW. As a result, SigB becomes active. (See the work of Hecker et al. [6] for details.) Hyperactive RsbU phosphatase having a Ser26→Pro alteration dephosphorylates RsbV~P in the absence of strong environmental stress, leading to stationary-phase activation of SigB.

designed a genetic selection aimed at identifying the genes that encode the then-hypothetical sigma factors. We incubated mutagenized cells of *B. subtilis* that were sporulating at 48°C in a medium containing $^{32}PO_4$ of very high specific activity, stored the cells at $-70°C$ for 2 months, and then isolated surviving mutants that were temperature sensitive for RNA synthesis and sporulation. Although at the time, we were able to characterize the mutant phenotypes, which we assumed reflected mutations in either sporulation sigma factors or interacting RNA polymerase core subunits, we were unable to map the mutations. In 2015, however, when whole-genome sequencing had become standard practice, we revisited these mutant strains and discovered that two of them had an identical mutation in *rsbU*, a gene whose product is an indirect activator of the stress-responsive sigma factor, SigB.

SigB is the primary respondent to stressful physiological signals (e.g., phosphate limitation, salt stress, ethanol shock, acid shock, etc.) that may occur during growth or in the stationary phase. SigB can activate the expression of about 150 genes (6). The activity of SigB, however, is affected by a complex multifactor regulatory system (Fig. 1). Posttranslational regulation is effected by the anti-sigma factor, RsbW, which binds to and inactivates SigB, and by RsbV, which binds to and inactivates RsbW. However, RsbW can phosphorylate RsbV, preventing the latter interaction. Phosphorylation of RsbV can be overcome by RsbU, a phosphatase that converts RsbV~P to its active RsbV form, which then binds RsbW, freeing SigB. RsbU is active only under stressful conditions in which a multicomponent macromolecular structure composed of RsbR, RsbS, and RsbT proteins is activated; under such conditions, RsbT binds to and activates the RsbU phosphatase (7–14).

Our finding that mutations in *rsbU* cause sporulation to be temperature sensitive is consistent with interaction between the stress-responsive and sporulation regulatory pathways and supports a model in which activation of SigB interferes with sporulation (15, 16).

(Our initial findings were presented at the 1975 Cold Spring Harbor meeting on RNA polymerase [17].)

RESULTS

Rationale for enrichment of mutants defective in transcription specifically during sporulation with ^{32}P as a suicide agent. When cells of *B. subtilis* are in the growing phase, significant enrichment for mutants defective in transcription would

probably not occur by utilizing $^{32}PO_4$ as the lethal agent, because incorporation of $^{32}PO_4$ into DNA and subsequent DNA decomposition would be the primary killing events. Sporulating cells, however, do not synthesize significant amounts of DNA after the second hour of stationary phase (T_2) (18). It was probable, therefore, that during sporulation the incorporation of $^{32}PO_4$ would be primarily into RNA and that subsequent radioactive decomposition would kill wild-type cells active in transcription. Mutant cells defective in transcription during sporulation might be preferentially spared.

$^{32}PO_4$ suicide protocol. In order to increase incorporation of radioactive phosphate, a culture of *B. subtilis* strain NCTC 3610 mutagenized with 2-aminopurine was synchronized for sporulation in modified medium 121B, without addition of K_2HPO_4 (for descriptions of all media, see Materials and Methods). (Since growth and sporulation of strain 3610 at 48°C under these conditions were normal, we presume that another component of medium 121B, such as technical Casamino Acids, supplied sufficient phosphate.) At T_2 of sporulation (2 h after the exponential growing phase), a sample of the culture was transferred to a flask containing 2 mCi carrier-free $H_3{}^{32}PO_4$ and incubated at 48°C until T_4. The cells were then washed, resuspended in 15% glycerol-containing medium, and stored in aliquots at −70°C.

Aliquots were thawed and plated at 37°C up to 62 days after freezing, at which point 95% of the ^{32}P had decayed. Survivors were screened for temperature sensitivity by replicating onto Difco sporulation medium (DSM) plates incubated at 48°C and 37°C. Survivors exhibiting an unpigmented and/or translucent colonial morphology at 48°C were tested for sporulation at 48°C in liquid 121B medium. The fraction of survivors identified as temperature sensitive for sporulation (Spo^ts) is shown for different time points in Fig. 2. The sporulation frequencies at different temperatures for two Spo^ts mutants, DR33 and DR38, are shown in Table 1.

RNA synthesis during sporulation. The 34 Spo^ts mutants (Fig. 2) were assayed for transcription during sporulation using a whole-cell assay in which cells exposed to toluene become permeable to the precursors and cofactors essential for RNA synthesis. Cells were grown in medium 121B at 37°C, synchronized for sporulation, harvested both during exponential growth and at T_2 of sporulation, and tested for RNA synthesis at both 45°C and 30°C. Although the activities of 31 of the Spo^ts mutants were comparable to that of the wild-type strain, three mutants exhibited relatively diminished RNA synthesis. Figure 3 indicates that when harvested during exponential phase, the mutant strain DR38 showed the same ability as wild-type cells to synthesize RNA at both 30°C and 45°. However, when harvested at T_2 of sporulation, activity was diminished compared to that of the wild type at 30°C, and an even stronger defect was apparent when assayed at 45°C. Transcription assays of strain DR33 showed similar results. A third mutant, DR15, revealed diminished RNA synthesis when assayed at both 45°C and 30°C and was therefore not pursued further.

When cells were grown at 48°C, the restrictive temperature for sporulation in the mutants, RNA synthesis activity of strain DR38 harvested during exponential phase was again similar to that of wild-type cells but was diminished compared to that of wild-type cells when harvested at T_2 and assayed at either 30°C or 45°C (data not shown). Together, these results were consistent with the hypothesis that strains DR33 and DR38 have a stationary-phase defect in RNA synthesis that was responsible for the Spo^ts phenotype.

Classical genetic studies of strain DR38. Spontaneous Spo^+ derivatives of strain DR38 were isolated by heating at 80 to 85°C samples derived from cultures at $T_{8.5}$ and plating for survivors. The frequency of appearance of Spo^+ derivatives was 10^{-6}, consistent with a single mutation being responsible for the DR38 phenotype. A culture of DR92, a Spo^+ derivative of strain DR38, harvested at T_2 of sporulation, was indistinguishable from the wild type when assayed in the permeable cell system at both 45°C and 30°C (data not shown). This result suggests that a single mutation in strain DR38
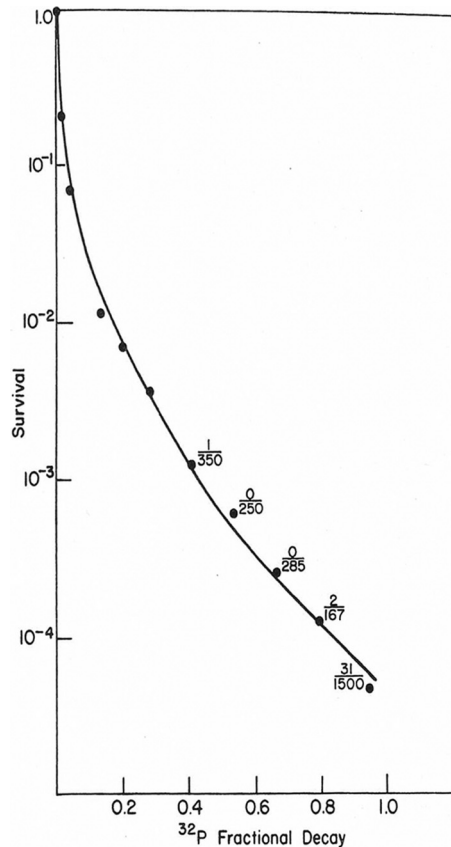
FIG 2 Survival curve of a ^{32}P-labeled sporulating culture. Aliquots of a culture that had incorporated $^{32}PO_4$ during sporulation were thawed at various times after freezing and plated for colony formation at 37°C. Time of storage at −70°C is expressed as the fractional decay of ^{32}P (0.5 decay = 14.31 days). Control experiments with unlabeled cultures showed that loss of viability due to freezing, storage, and thawing was not significant (>75% survival for a time equivalent to 95% ^{32}P decay). Random survivors were tested for the Spo[ts] phenotype by replica plating and heat tests for sporulation at 48°C. The fractions in the figure indicate the proportion of Spo[ts] mutants detected among the survivors for given time points. Republished from reference 17 with permission.

was responsible for both the Spo[ts] phenotype and the transcriptional defect during sporulation.

B. subtilis strain 3610 was chosen as the parent strain for this study because of its very efficient sporulation at 48°C and because it was, at the time, the principal strain in which the sporulation-related modification of RNA polymerase activity had been detected (19, 20). Strain 3610 is not competent for transformation, however, and mapping our Spo[ts] mutations proved to be impossible at that time using classical approaches (data not shown). As an alternative approach, we assessed the impact of the *spo*(Ts) mutation on the transcription apparatus by testing for potential compen-

TABLE 1 Spore formation by asynchronous cultures of 3610 and Spo[ts] mutants DR33 and DR38[a]

Temp (°C)	No. of spores (10^8/ml) for strain:		
	3610	**DR33**	**DR38**
48	1.8	0.014	0.015
45	5.7	0.40	0.50
42	6.3	0.80	0.90
37	6.0	2.3	3.3
30	2.9	2.4	3.0

[a]For cultures at 37 to 48°C, heat resistance was determined at T_{17} to T_{24}. For cultures at 30°C, heat resistance was determined at T_{36}. Data are from reference 17.
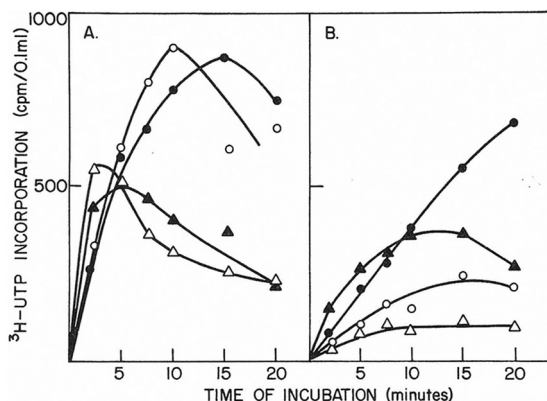
FIG 3 RNA synthesis in toluene-treated cells of strains 3610 and DR38. Cells of 3610 and DR38 grown in medium 121B at 37°C were harvested at the indicated times, made permeable to the substrates of RNA polymerase, and assayed for RNA synthesis as described in Materials and Methods. (A) Vegetative cells; (B) sporulating cells ($T_{1.7}$). Symbols: white circles, DR38 assayed at 30°C; white triangles, DR38 assayed at 45°C; black circles, 3610 assayed at 30°C; black triangles, 3610 assayed at 45°C. Republished from reference 17 with permission.

satory effects of mutations in genes encoding known components of RNA polymerase. Lipiarmycin is a direct inhibitor of RNA polymerase (21, 22); resistance mutations lie in the *rpoC* gene, which encodes the β' subunit (23). Of 10 spontaneous, lipiarmycin-resistant derivatives of strain DR38, 3 sporulated at 48°C at >20% of the wild-type frequency, measured at T_{17} of sporulation, a more than 20-fold increase in sporulation efficiency compared to that of strain DR38. The partial suppression of the Spo[ts] defect by mutations in *rpoC* suggested that the *spo*(Ts) mutation in DR38 might reside in a gene encoding a subunit of RNA polymerase or a closely associated protein. We also isolated spontaneous rifampin-resistant derivatives of strains DR33 and DR38 that presumably have mutations in *rpoB*, the gene encoding the β subunit of RNA polymerase (24). These rifampin-resistant derivatives sporulated at lower frequencies at both 30°C and 48°C, a result that would be consistent with interaction of the *rsbU* and *rpoB* mutations, but the *rpoB* mutations may cause a decrease in sporulation independently of *rsbU*.

Whole-genome sequencing of 3610, DR33, and DR38. Forty years after the isolation of these Spo[ts] mutants and their antibiotic-resistant derivatives, the exact nucleotide changes in strains DR33 and DR38 were discovered by whole-genome sequencing (Table 2). No alteration was found in any gene coding for an RNA polymerase core subunit or sigma factor in either strain. However, both strains contained an identical mutation in the *rsbU* gene, which encodes a phosphatase that is known to be a regulator of SigB, the sigma subunit of the stress response regulon. Codon 26 of *rsbU* was changed to encode proline instead of serine. There were also four additional nucleotide differences between 3610 (wild type) and DR33, but only one of the four

TABLE 2 Sequence changes in wild-type, mutant, and revertant strains

| Strain (type) | Change(s) in gene or regulatory region | | | | |
	rsbU	*rsbT*	*rsbT-rsbU* promoter region	*ywiC*	*ythQ*
3610 (wild type)					
DR33 (mutant)	Ser26→Pro			Ile237→Val	
DR38 (mutant)	Ser26→Pro			Ile237→Val	Lys336→Glu
DR38R1 (pseudorevertant)[a]	Ser26→Pro	Frameshift creates nonsense codon		Ile237→Val	Lys336→Glu
DR38R2 (pseudorevertant)	Ser26→Pro		TATAAT −10 region→TGTAAT	Ile237→Val	Lys336→Glu
DR38R3 (pseudorevertant)	Ser26→Pro, Gln94→nonsense codon			Ile237→Val	Lys336→Glu

[a]For DR38R1, the deletion of an A residue within codon 68 of *rsbT* creates a nonsense mutation in codon 72. The resulting block in translation may be polar on *rsbU* downstream.

TABLE 3 Survival of strain 3610, Spo^ts mutant DR38, and pseudorevertants of DR38 (DR38R2 and DR38R3) following 2.5-h ethanol challenge

Temp (°C)	% Ethanol	Percentage of survival by CFU			
		3610 (wild type)	DR38 (Spo^ts)	DR38R2 (Spo⁺)	DR38R3 (Spo⁺)
48	6.0	2	67	ND[a]	1
48	6.5	0.07	2.1	0.06	ND
37	9	0.02	3.0	0.1	0.06

[a]ND, not done.

resulted in a codon change. That is, the third codon from the end of the *ywiC* gene, encoding a valine, was replaced by an isoleucine codon.

Strain DR38 contained *rsbU* and *ywiC* mutations identical to those in DR33 and an additional point mutation leading to the replacement of lysine-336 by glutamate in the *ythQ* gene, which encodes an adenosine glycosidase repair enzyme (Table 2). The additional mutation in DR38, which is likely a descendant of DR33, may have arisen as a result of further rounds of DNA replication in the presence of 2-aminopurine. Since strains DR33 and DR38 showed identical behaviors in the transcription assays outlined above, identical frequencies of sporulation, and identical colonial morphologies, it is unlikely that the *ythQ* mutation contributed to the DR38 phenotype.

Response of the Spo^ts mutants to heat and ethanol stresses. RsbU-dependent activation of SigB normally results in a strong, transient response to ethanol that permits enhanced survival. Thus, if the *rsbU* mutation in question impaired RsbU activity, the mutant cells would be defective in eliciting the stress response, resulting in cells more sensitive to ethanol than the wild type. We observed, however, that strain DR38 exhibited enhanced survival compared to the wild type, whether the cultures were exposed to ethanol at 37°C or 48°C (Table 3). The straightforward interpretation of these results is that the mutated form of RsbU has not lost its function as a modulator of the stress response but instead elicits a hyperactive SigB-mediated stress response.

To clarify the relationship between the *rsbU* mutation and the phenotype of strain DR38, we isolated spontaneous Spo⁺ revertants by streaking DSM plates with a heavy inoculum of DR38 cells and incubating the plates at 48°C. Rare opaque colonies were picked as likely Spo⁺ isolates and purified. Three spontaneous, independently isolated derivatives of DR38 sporulated at the wild-type frequency at 48°C: wild-type strain 3610 showed 6.0×10^8 spores per ml, while mutant strain DR38 had 0.003×10^8 spores per ml, but revertant strains DR38R1, DR38R2, and DR38R3 had 6.3×10^8, 6.1×10^8, and 5.8×10^8 spores per ml, respectively. Whole-genome sequencing of these Spo⁺ phenotypic revertants (Table 2) revealed that all three Spo⁺ derivatives were pseudorevertants; each contained the original *rsbU*, *ywiC*, and *ythQ* mutations as well as an additional mutation that resulted in interference with RsbU function. DR38R3 acquired a nonsense mutation in *rsbU* at a site downstream of codon 26, leading to the encoding of a severely truncated RsbU fragment. DR38R1 contained a frameshift mutation early in the *rsbT* gene, which encodes the protein that binds to and activates RsbU as a phosphatase. DR38R2 contained a mutation in the −10 region of the promoter for the operon that includes *rsbT* and *rsbU*, presumably leading to reduced expression of the two proteins needed for activation of SigB. Thus, the Spo⁺ phenotype of the revertants can be ascribed in all three cases to genetic modifications that reduce or eliminate RsbU activity.

DISCUSSION

The N-terminal domain of RsbU protein contains alpha helices located in the region that interacts with helices of the RsbT protein, forming the RsbU/RsbT stress response activator complex (25). This interaction activates the RsbU phosphatase, which converts RsbV~P to the active form of the RsbV anti-anti-sigma factor. RsbV binds to RsbW, the anti-sigma factor, displacing it from the SigB-RsbW inhibitory complex, resulting in activation of SigB (Fig. 1). The RsbU protein in strains DR33 and DR38 contains proline

in place of serine at residue 26, in the region crucial for RsbU-RsbT interaction. Given that proline residues are generally highly disruptive to helical structures, we guessed initially that the *rsbU* mutation in strains DR33 and DR38 reduced the function of RsbU, thereby interfering with the SigB-dependent stress response. In fact, a knockout of the *ctc* gene, which is expressed from a SigB-dependent promoter, was shown to confer a Spo^{ts} phenotype (26), suggesting that a mutation resulting in the loss of function of RsbU could also cause the Spo^{ts} phenotype.

However, our results have led us to conclude that this simple model is incorrect; the Spo^{ts} phenotype of strains DR33 and DR38 appears to be caused by aberrantly hyperactive RsbU. That is, the hyperactive phosphatase encoded by the mutant *rsbU* gene provides a selective advantage in the presence of ethanol and also interferes with the ability of the cells to sporulate, perhaps by causing SigB to compete with sporulation sigma factors for binding to the RNA polymerase core enzyme. Reducing the production or activity of the mutant RbsU restored sporulation. There may be more than one contributor to the observed phenotypes, however. The fact that SigB is normally more active during growth at high temperature than at 37°C may help to explain why the impact of the *rsbU* Ser26Pro mutation is strongest in stationary phase at high temperature.

Previous discoveries have revealed a potential mechanism connecting hyperactivation of SigB and interference with sporulation. It has long been known that addition of ethanol within a 45-min time period around T_1 of sporulation inhibits spore formation (27), presumably through stimulation of the SigB-dependent stress response. More recently, Reder et al. (15, 16) reported that the *spo0E* coding sequence is preceded by a perfect SigB promoter sequence and that ectopic expression of SigB interferes with the onset of sporulation. In a construct in which SigB is expressed from a xylose-inducible promoter, addition of xylose leads to overexpression of *spo0E*. Since the *spo0E* gene encodes a phosphatase that converts phosphorylated Spo0A to its inactive, dephosphorylated form, activation of SigB should be expected to interfere with sporulation gene expression. Thus, the SigB-dependent promoter for *spo0E* appears to be a key meeting point of the stress response and sporulation regulons. It is likely, however, that more than one SigB target contributes to the phenotypes observed here.

MATERIALS AND METHODS

Growth conditions and strains. *B. subtilis* strain NCTC 3610 and mutant derivatives were used. Cells were grown and synchronized for sporulation in medium 121B, containing basal medium 121 (0.08 M NaCl, 0.02 M KCl, 0.02 M NH$_4$Cl, 0.12 M Tris, 1 mM MgCl$_2$, 0.2 mM CaCl$_2$, 0.002 mM FeCl$_3$, 2.5 mM Na$_2$SO$_4$, 0.002 mM ZnCl$_2$; pH adjusted to 7.5 with HCl) supplemented to final concentrations of 0.2% glucose, 0.2% Casamino Acids (Difco technical), 2.5 mM K$_2$HPO$_4$, 0.1 mM MnCl$_2$, 3.4 mM sodium citrate, and 0.15 mM FeCl$_3$ (28). For some experiments, medium 121A (containing basal medium 121 supplemented to final concentrations of 0.8% glucose, 0.02% Casamino Acids [Difco technical], 1 mM K$_2$HPO$_4$, and 0.15 mM ferric citrate) was used. Cells were also grown in nutrient sporulation medium (DSM), consisting of 8 g nutrient broth (Difco), 0.25 g MgSO$_4$·7H$_2$O, and 1 g KCl per liter, pH 7.0 to 7.2, supplemented with 1 μM FeSO$_4$, 1 mM Ca(NO$_3$)$_2$, and 10 μM MnCl$_2$ (29) to test for sporulation in liquid culture. Cells were grown in L broth, containing 10 g tryptone (Difco), 5 g yeast extract (Difco), and 5 g NaCl per liter at pH 7.0, where indicated. CFU were determined using DSM plates containing 1.7% agar.

Measurement of sporulation. Spore formation was quantitated by incubating a culture sample at 80°C to 85°C for 10 min and determining the titers of survivors on DSM plates at 37°C (29).

Mutagenesis and mutant selection. Using the approach of Georgopoulos (30), a culture of 10^4 cells/ml in medium 121A was exposed to 0.05% 2-aminopurine overnight, washed, and stored frozen in basal medium 121 containing 15% glycerol. A sample of the mutagenized population was used to inoculate medium 121B without added phosphate. When the culture, shaken at 48°C, reached the second hour of stationary phase (T_2), 0.9 ml of culture was transferred to a flask containing 0.1 ml (2 mCi) carrier-free H$_3$^{32}PO$_4$ (the stock solution in 0.02 M HCl had been neutralized with 0.2 M NaOH). The cells were incubated at 48°C until T_4. By that time, 66% of the added ^{32}PO$_4$ had been incorporated into trichloroacetic acid (TCA)-insoluble material. At T_4, the cells were washed with basal medium 121, resuspended in the same medium containing 15% (vol/vol) glycerol, divided into aliquots, and stored at −70°C.

Assay of RNA polymerase activity in cells exposed to cold shock and toluene. To make cells permeable to the substrates of RNA polymerase, we used the following method, described by Fisher et al. (31), that removes any uptake barrier for RNA precursors. A 30-ml culture of vegetative cells growing in medium 121B (90 Klett units) or an equivalent mass of sporulating cells was quick-cooled by adding to 12 ml crushed ice containing 0.05 M Tris-Cl (pH 7.9), centrifuged, washed with ice-cold 0.05 M Tris-Cl

(pH 7.9), and resuspended in 1 ml of a 1% suspension of toluene in ice-cold 0.05 M Tris-Cl (pH 7.9). The cells were vortexed for 20 s and incubated at 0°C for 20 min before assaying. The toluene-treated cells were diluted 5-fold with a reaction mixture (see below). At the indicated time points, 100-μl samples were removed and added to 2 ml 5% TCA containing 0.01 M Na pyrophosphate. Precipitates were collected on Whatman GF/A filters, which were assessed for radioactivity in a Nuclear-Chicago liquid scintillation counter. The reaction mixture contained 0.04 M Tris-Cl (pH 7.9), 0.3 mM GTP, UTP, and CTP, 1.5 mM ATP, 0.2 mM dithiothreitol (DTT), 0.1 M KCl, 0.01 M MgCl$_2$, 0.1 mM disodium EDTA, 0.4 mM phosphate buffer (an equimolar mixture of KH$_2$PO$_4$ and K$_2$HPO$_4$), and 5-[^{3}H]UTP (2 μCi/ml).

Assay to determine ethanol resistance. Ethanol resistance was tested by exposing cells grown in L broth to 6.0 or 6.5% ethanol at 48°C or to 9% ethanol at 37°C for 2.5 h and then measuring survival on DSM agar.

Whole-genome sequencing. Chromosomal DNA was extracted from 1-ml cultures of *B. subtilis* strains grown overnight at 37°C in L broth using a DNeasy kit (Qiagen), according to the manufacturer's protocol for Gram-positive bacteria. From these DNA preparations, Illumina sequencing libraries were constructed using the oligo(C)-tailing HTML (homopolymer tail-mediated ligation) method as previously described (32). Each library was indexed with a unique 8-nucleotide (nt) truseq barcode. The libraries were then pooled and run on a single lane of an Illumina HiSeq 2500 sequencing apparatus. The resulting reads were demultiplexed using a Q30 quality threshold, and all subsequent data analysis was performed using CLC Genomics Workbench v4.7.2. The sequencing reads were first filtered for quality using a limit of 0.004. The filtered reads were then assembled to two reference genomes: CP003329 (*Bacillus subtilis* strain 6051-HGW; equivalent to NCTC 3610) and KF365913 (plasmid pBS32). The resulting reference assemblies were then used as input for single nucleotide polymorphism (SNP) and deletion/insertion polymorphism (DIP) detection.

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