The *mthA* Mutation Conferring Low-Level Resistance to Streptomycin Enhances Antibiotic Production in *Bacillus subtilis* by Increasing the S-Adenosylmethionine Pool Size

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Certain *Str^r* mutations that confer low-level streptomycin resistance result in the overproduction of antibiotics by *Bacillus subtilis*. Using comparative genome-sequencing analysis, we successfully identified this novel mutation in *B. subtilis* as being located in the *mthA* gene, which encodes S-adenosylhomocysteine/methylthioadenosine nucleosidase, an enzyme involved in the S-adenosylmethionine (SAM)-recycling pathways. Transformation experiments showed that this *mthA* mutation was responsible for the acquisition of low-level streptomycin resistance and overproduction of bacilysin. The *mthA* mutant had an elevated level of intracellular SAM, apparently acquired by arresting SAM-recycling pathways. This increase in the SAM level was directly responsible for bacilysin overproduction, as confirmed by forced expression of the *metK* gene encoding SAM synthetase. The *mthA* mutation fully exerted its effect on antibiotic overproduction in the genetic background of *rel⁺* but not the *rel* mutant, as demonstrated using an *mthA relA* double mutant. Strikingly, the *mthA* mutation activated, at the transcription level, even the dormant ability to produce another antibiotic, neotrehalosadiamine, at concentrations of 150 to 200 µg/ml, an antibiotic not produced (<1 µg/ml) by the wild-type strain. These findings establish the significance of SAM in initiating bacterial secondary metabolism. They also suggest a feasible methodology to enhance or activate antibiotic production, by introducing either the *rsmG* mutation to *Streptomyces* or the *mthA* mutation to eubacteria, since many eubacteria have *mthA* homologues.

Streptomycin (Sm) was first shown to be a particularly potent drug against *Mycobacterium tuberculosis* in 1944 (1), and mutants resistant to Sm were reported as early as 1946 (2). Because of its clinical importance, molecular mechanisms of resistance to Sm have been extensively studied, especially in *M. tuberculosis* (3–6). These mutants could be classified into two distinct types, depending on whether they exhibit high- or low-level Sm resistance. Type I mutants carry a mutation within *rpsL*, which encodes the ribosomal protein S12, or within *rrn*, which encodes 16S rRNA, and exhibit high-level Sm resistance (MIC, >100 µg/ml). In contrast, type II mutants possess the wild-type *rpsL* gene and exhibit low-level Sm resistance (MIC, 5 to 10 µg/ml). Most mutations within S12 that confer resistance to, or dependence on, Sm are known to lead to a hyperaccurate phenotype (5), which compensates for the effect of the drug without affecting the interaction between the drug and the ribosome. A number of mutations within 16S rRNA, including those within the 530 loop, also result in both Sm resistance and hyperaccuracy (7–9). However, the mechanisms underlying low-level resistance to Sm (i.e., type II mutations) have remained obscure because they were thought to be less clinically important.

Some mutations causing low-level resistance have been characterized recently. Using the comparative genome-sequencing (CGS) technique, we successfully determined that low-level resistance is caused by mutations in *rsmG* (rRNA small subunit methyltransferase G), which encodes an S-adenosylmethionine (SAM)-dependent 16S rRNA methyltransferase (10). Analysis of the 16S rRNA by high-performance liquid chromatography (HPLC) showed that the *rsmG* mutant lacked a 7-methylguanosine (m7G) modification (11, 12).

Our laboratory has focused on strain improvement for antibiotic overexpression and has developed a new method to activate or enhance antibiotic production in bacteria. Current methods of

antibiotic production, ranging from classical random approaches to metabolic engineering, are either costly or labor-intensive. In contrast, our methods are characterized by simplicity, i.e., introduction of a mutation by isolating spontaneously developed drug-resistant mutants. Thus, the method requires no induced mutagenesis, providing a rational approach to elicit bacterial capabilities within industrial applications (13–16). Importantly, certain *rpsL* or *rsmG* mutants display a markedly increased ability to produce antibiotics, indicating that bacterial gene expression can be altered dramatically by modulating ribosomal proteins and/or rRNA (17–19).

The mechanisms underlying this remarkable activation have been studied. The *rpsL* mutant ribosomes carrying an amino acid substitution in S12, which confers a high level of resistance to Sm, are more stable than those of wild-type controls, indicating that this increase in stability could enhance protein synthesis during the late growth phase (20). We later found that increased expression of the translation factor ribosome-recycling factor also contributes to the enhanced protein synthesis observed during the late growth phase in the *rpsL* K88E mutant (21). This finding sug-

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FIG 1 Structures of bacilysin and neotrehalosadiamine.

gested that both the greater stability of the 70S ribosomes and the elevated levels of ribosome-recycling factor caused by the *rpsL* K88E mutation are responsible for the enhanced protein synthesis seen during the late growth phase and that this underlies antibiotic overproduction by the *rpsL* K88E mutant (21). In contrast, *rsmG* mutants display markedly enhanced expression of SAM synthetase in *Streptomyces* (10). SAM synthetase activity was shown to be important in initiating antibiotic production, as demonstrated by the fact that overexpression of *metK* (encoding SAM synthetase) stimulates antibiotic production in a *Streptomyces coelicolor* *rsmG* mutant and by the finding that exogenous addition of SAM to the culture medium induces antibiotic biosynthesis in wild-type cells (10, 22).

Bacillus subtilis strains are reported to produce three ribosomal antibiotics, TasA, subtilosin, and sublancin; three nonribosomal antibiotics, surfactin, bacilysin, and plipastatin; and the novel phospholipid antibiotic bacilysocin (23, 24). The dipeptide bacilysin is one of simplest peptide antibiotics produced by *B. subtilis*, consisting of an L-alanine at the N terminus and an unusual amino acid, L-anticapsin, at the C terminus (Fig. 1). Although the biosynthesis of bacilysin has been studied extensively (25, 26), little is known about its regulation. *B. subtilis* may also be able to synthesize the antibiotic neotrehalosadiamine (NTD) (Fig. 1), an aminosugar produced by *Bacillus pumilus* and *Bacillus circulans* (27, 28). Although *B. subtilis*, unlike *B. pumilus* and *B. circulans*, normally does not produce this antibiotic, certain rifampin resistance (*rpoB*) mutations activate the dormant ability to produce NTD, possibly by more efficient transcription from σ^A -dependent promoters resulting from alteration of its ternary structure, since the promoter for NTD-biosynthetic genes (*ntdABC*) was recognized by σ^A (29). Among prokaryotes, *B. subtilis* provides a feasible system for the study of various biological functions, as denoted by the presence of a transformation system and the availability of genomic information courtesy of the completed genome project.

We previously reported that certain *B. subtilis* mutants possess low-level Sm resistance and exhibit a marked increase in antibiotic production (18). However, none of these strains had a mutation in the *rsmG* gene, indicating that another mechanism, fundamentally different from that involving *rsmG* mutation and possibly involving activation of secondary metabolism, is responsible for the low-level Sm resistance of *B. subtilis*. Thus, based on the feasibility of the CGS technique (30), we attempted to identify this novel type of Sm resistance mutation, as well as to clarify the mechanism regulating antibiotic production as a model of bacterial secondary metabolism. This paper describes the identification

and analysis of this novel mutation in relation to antibiotic overproduction and activation of dormant antibiotic-biosynthetic genes.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. All strains were derived from *B. subtilis* strain 168. Spontaneous Sm-resistant mutants were obtained as colonies that grew within 3 days after cells were spread on solid L medium (10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter) containing Sm at a concentration of 10 to 15 μ g/ml. We used a *relA1* mutation with a slightly leaky phenotype, accumulating 1/10 of the amount of ppGpp accumulated by the wild type upon nutritional downshift (31), rather than the *relA*-null mutation, because the latter results in a severe growth defect (32).

Strain KJ04 (*mthA1*) was obtained by transformation with conjugation using *B. subtilis* strain YO-005 (*hisC101*) as a recipient (33). The amino acid auxotrophic marker genes, *hisC* and *trpC*, were cotransformed at high frequency (approximately 70%). The histidine auxotrophic recipient strain YO-005 was transformed with the genomic DNA of strain ST20 with selection for histidine prototrophy. Several Sm-resistant transformants were selected from 100 His⁺ Trp[−] transformants, with DNA sequencing analysis confirming that all Sm-resistant transformants contained the expected *mthA1* mutation. One of these transformants was designated KJ04 and used for further study. To obtain KJ05 (*mthA::pMutinT3*), the DNA fragment containing a partial coding region of the *mthA* gene was amplified using the primers *mthAH*-F and *mthAB*-R (see Table S1 posted at <https://drive.google.com/file/d/0Bwj6L7P2wWCwQXZoc2JsX0k0Umc/edit?usp=sharing>), digested with HindIII and BamHI, and cloned into the corresponding sites of pMutinT3 (34). The resulting plasmid, pMutinT3-*mthA*, was used for transformation, with selection for erythromycin resistance (1 μ g/ml).

To overexpress the *metK* gene, its complete coding region was amplified using the primers *metK*-F and *metK*-R and cloned into pCR2.1. The

TABLE 1 Bacterial strains and plasmids used in the study

Strain or plasmid	Genotype or description	Source or reference
<i>B. subtilis</i> strains		
168	<i>trpC2</i>	Laboratory stock
YO-005	<i>hisC101</i>	33
ST20	<i>trpC2 mthA1</i>	This study
KJ04	<i>trpC2 mthA1</i> (transformation; ST20→168)	This study
KJ05	<i>trpC2 mthA::pMutinT3</i>	This study
KJ06	<i>trpC2 pUB18-metK</i>	This study
K2-007	<i>trpC2 relA1</i>	31
KO-1234	<i>trpC2 mthA1 relA1</i> (transformation; KJ04→K2-007)	This study
KO-673	<i>trpC2 rsmG</i>	12
<i>E. coli</i> strains		
BW25113	Standard strain of <i>E. coli</i> K-12	Keio Collection
JW0155	<i>mthA</i> disruptant of BW25113	Keio Collection
Plasmids		
pCR2.1	Cloning vector for PCR product	Invitrogen
pCR2.1- <i>mthA</i>	pCR2.1 containing <i>mthA</i> gene	This study
pMutinT3	Integration vector	34
pMutinT3- <i>mthA</i>	pMutinT3 containing the 5' region of the <i>mthA</i> gene	This study
pUB18	Multicopy vector	Laboratory stock
pUB18- <i>metK</i>	pUB18 containing the <i>metK</i> gene	This study

cloned fragment was fully sequenced to confirm its correctness and then digested with EcoRI and subcloned into the corresponding site of pUB18, a derivative of pUB110 with an M13mp18 multiple-cloning site, generating pUB18-metK. The resulting plasmid, pUB18-metK, was used to transform *B. subtilis* 168 with selection for kanamycin resistance (5 µg/ml).

B. subtilis strains were grown at 37°C with vigorous shaking in L medium or NG medium (10 g nutrient broth, 10 g glucose, 2 g NaCl, 5 mg CuSO₄ · 5H₂O, 7.5 mg FeSO₄ · 7H₂O, 3.6 mg MnSO₄ · 5H₂O, 15 mg CaCl₂ · 2H₂O, and 9 mg ZnSO₄ · 7H₂O per liter), a medium developed for antibiotic production by *B. subtilis* (18).

Detection of the *mthA* gene mutation. The *mthA* gene was PCR amplified from *B. subtilis* genomic DNA using the primers *mthA*-F and *mthA*-R (see Table S1 posted at <https://drive.google.com/file/d/0Bwj6L7P2wWCwQXZoc2JsX0k0Umc/edit?usp=sharing>). The PCR products were directly sequenced using an ABI Prism 310 Genetic Analyzer.

Determination of susceptibility to Sm. Cells were grown in L medium for 18 h and diluted 200-fold to approximately 10⁶ cells/ml. Five-microliter aliquots of cell suspension were spotted onto L agar plates containing various concentrations of Sm and incubated at 37°C for 18 h. Susceptibility to Sm or other antibiotics was expressed as the MIC.

Antibiotic production by *B. subtilis* strains. *B. subtilis* strains were grown in NG medium at 37°C with vigorous shaking. Antibiotic production was determined by the paper disk-agar diffusion assay using *Staphylococcus aureus* 209P as a test organism (35). Briefly, 50-µl aliquots of culture supernatant obtained after centrifugation were applied to 8.0-mm paper disks (Advantec), which were placed onto half-strength Mueller-Hinton agar (Difco) plates inoculated with *S. aureus* 209P and incubated for 15 h at 37°C. One unit of bacilysin was defined as the amount of bacilysin that produced a halo 1 mm wide around the disk. To assess the production of neutrehalosadiamine, strains were grown in 57N medium (29) for 24 h and assayed as described above, except for the inclusion of 1 mM glucosamine in the Mueller-Hinton assay plate to negate the effect of any bacilysin that might be produced.

Determination of SAM level. The level of intracellular SAM in *B. subtilis* wild-type (168) and mutant strains was determined by reverse-phase high-pressure liquid chromatography, as described previously (22, 36). Cells grown to various growth phases were harvested, transferred to a petri dish containing 10 ml of 1 M formic acid, and allowed to stand at 4°C for 1 h. The formic acid was collected, filtered through a membrane filter (pore size, 0.45 µm), and lyophilized. The lyophilized samples were dissolved in a small amount of water and analyzed with a Capcell-Pak C₁₈ column (4.6 by 250 mm; Shiseido, Tokyo, Japan). To normalize the number of picomoles of SAM per milliliter of culture to the number of picomoles per number of cells, intracellular SAM levels were expressed as pmol/optical-density (OD) unit, where 1 OD unit was defined as the number of cells that would produce an optical density at 650 nm (OD₆₅₀) of 1 if suspended in 1 ml.

Determination of polyamines. Polyamines were identified as described previously (37, 38). Aliquots of bacterial cultures were pelleted, washed, and extracted with 0.2 M perchloric acid. The polyamines were subsequently danylated and extracted with toluene for analysis by thin-layer chromatography (TLC) on silica gel G plates (Merck) developed in ethyl acetate-cyclohexane (2:3 [vol/vol]). The spots were visualized under UV light.

Identification of neutrehalosadiamine. Neutrehalosadiamine produced by *mthA* mutants was identified by ¹H (500 MHz) and ¹³C (125 MHz) nuclear magnetic resonance (NMR) spectra recorded on a JEOL ECP-500 spectrometer in D₂O.

RT-qPCR. Strains were grown in NG medium at 37°C until the OD₆₅₀ reached 0.5 (exponential growth phase), 1.5 (transition between exponential and stationary phases), or 3.0 (stationary phase). Total RNA was prepared as described previously (39), with contaminating DNA removed by incubation of 2 µg total RNA with 2 U of DNase I (Invitrogen) for 15 min at 25°C. The RNAs were reverse transcribed using a High Capacity RNA-to-cDNA kit (ABI) according to the manufacturer's instructions.

After termination of the reaction by incubation for 5 min at 95°C, samples were analyzed using a 7300 real-time quantitative PCR (RT-qPCR) system (ABI) and Thunder Bird SYBER qPCR Mix (Toyobo, Osaka, Japan). Amplification of the 16S rRNA gene was used as an internal control (see Table S1 posted at <https://drive.google.com/file/d/0Bwj6L7P2wWCwQXZoc2JsX0k0Umc/edit?usp=sharing>).

RESULTS

Development of *B. subtilis* mutants with low-level Sm resistance. Low-level Sm-resistant mutants of *B. subtilis* developed spontaneously at a high frequency of 10⁻⁵ to 10⁻⁶. When selected with an Sm concentration of 10 times the MIC, most of these mutants had mutations in the *rsmG* gene (12). Few *rsmG* mutations, however, were detected when selected with an Sm concentration at 3 times the MIC. Importantly, many of the mutants with slight resistance to Sm, as selected at 3 times the MIC, overproduced bacilysin, suggesting an as yet unidentified type of Sm resistance mutation.

Identification of mutations conferring low-level Sm resistance. As we have successfully identified novel mutations using the CGS technique, a method (30) that uses microarray-based DNA sequencing to identify single-nucleotide polymorphisms (SNPs) and insertion-deletion sites within the genome, we utilized the method to identify the mutation in the *B. subtilis* genome conferring low-level Sm resistance. Genomic DNA was obtained from the mutant strain ST20, with low-level Sm resistance (Table 2) and bacilysin overproduction, and from the parental strain, 168. Using the CGS technique, we identified a putative insertion-deletion site within the gene *mthA*, which encodes the enzyme S-adenosylhomocysteine (SAH)/methylthioadenosine (MTA) nucleosidase. Direct sequencing showed that the mutation consisted of an 11-bp deletion. Strikingly, *mthA* mutations were found in many of the colonies that developed spontaneously on the plates containing an Sm concentration of 3 times the MIC (listed in Table 2 as KO-1225 to KO-1233). The frequency of appearance of *mthA* mutations among these colonies was as high as 2% to 5%. The *mthA* mutations were characterized by the frequent appearance of deletions that resulted in stop codons just downstream of the mutations (Table 2). All of these *mthA* mutants overproduced bacilysin (about 3-fold compared to the wild-type strain) (data not shown).

To confirm the causal relationship between the identified mutation and Sm resistance or bacilysin overproduction, we constructed the strain KJ04 (*trpC mthA*) by transformation (Table 1). Similar to the original mutant, ST20, the *mthA* transformant KJ04 showed increased resistance to Sm (Fig. 2) and overproduction of bacilysin (2.5-fold higher than the wild type) (Fig. 3A and C). Bacilysin has been reported to interfere with glucosamine synthesis (40). As expected, antibacterial potency was negated completely by the addition of glucosamine to the assay plate (Fig. 3C), indicating that the observed antibiotic activity was due to bacilysin. Similarly, disruption of *mthA* using pMutinT3, resulting in the disruptant KJ05 (Table 1), increased resistance to Sm (Fig. 2). Disruption of the *mthA* gene in *Escherichia coli* also caused slight resistance to Sm (MIC, 8 µg/ml compared with 4 µg/ml for the wild type BW25113), as determined using L medium and Mueller-Hinton medium (data not shown). These results indicate that the *mthA* mutation was responsible for both increased Sm resistance and bacilysin overproduction.

We previously reported that certain low-level Sm-resistant

TABLE 2 Locations of mutations in the *B. subtilis* *mthA* gene and resulting amino acid changes in the MthA protein

Strain	Sm concn (μg/ml) used for selection	Position of mutation in <i>mthA</i> gene ^a	Amino acid change	Resistance to Sm (μg/ml) ^b
168 (parent)		— ^c		10
ST20	30	577-ATCAGAGCGTT-587→11-bp deletion	Frameshift	50
KO-1225	30	590 C→A	197 Ser→stop codon	50
KO-1226	30	380-GCATCACTGAAGA-392→13-bp deletion	Frameshift	50
KO-1227	30	386 G→T	130 Glu→stop codon	50
KO-1228	30	223 G→A	75 Gly→Ser	50
KO-1229	30	44-624→581-bp deletion	Frameshift	50
KO-1230	30	418 C→T	140 Gln→stop codon	50
KO-1231	30	259-G→1-bp deletion	Frameshift	50
KO-1232	30	548 G→A	183 Cys→Val	50
KO-1233	30	293- ATGATGTAGACG-304→12-bp deletion	4-Amino-acid deletion	50
KO-274	5 ^d	26 T→G	9 Met→Arg	50
KO-275	5 ^d	446 G→A	149 Asp→Gly	50
KO-277	5 ^d	542 A→G	181 Gln→Arg	50
KO-278	5 ^d	Insertion of C after 213 C	Frameshift	50

^a Numbering is from the first nucleotide of the start codon.

^b Determined after 18 h of incubation at 37°C on L agar plates.

^c Wild-type *mthA* gene.

^d Sm resistance was selected using NG medium (18), in which the Sm MIC for the parent strain, 168, was 1 μg/ml.

mutants show an increased ability to produce antibiotic(s) (18). The results described above suggested that those antibiotic-over-producing strains also had mutations in *mthA*. Strikingly, all strains tested (KO-274 to KO-278 in Table 2) had a mutation in *mthA*, confirming the association between *mthA* mutations and antibiotic overproduction.

Characterization of the *mthA* mutant. The *mthA* transformant KJ04 grew more slowly (Fig. 3A) and formed smaller and browner colonies with mountain form on L agar plates (Fig. 4A) than the parental strain, 168. When cultured in liquid L medium, KJ04 cells were filamentous, with cross walls (Fig. 4B). The high-level Sm resistance (*rpsL*) mutations often confer resistance to Sm concentrations greater than 1,000 μg/ml (12, 18). In contrast, the *mthA* mutants were resistant only to Sm concentrations of 50 μg/ml, less than the resistance of *rsmG* mutants (100 μg/ml), and displayed cross-resistance to kanamycin and paromomycin, but not to the antibiotics (chloram-

phenicol, erythromycin, lincomycin, and thiostrepton) that act on the 50S subunit of the ribosome (Table 3). The *mthA* mutants sporulated as well as the wild-type strain, 168 (sporulation frequency, 90% to 95%), as determined microscopically, when cultured in Schaeffer's sporulation medium for 2 days. The MthA protein, encoded by *mthA*, catalyzes two steps (SAH→SRH and MTA→MTR) in the SAM-recycling pathway of *B. subtilis* (Fig. 5). The *mthA* mutation did not cause severe growth defects, even in the chemically defined medium. This growth defect was not repaired by supplementation with methionine, cysteine, or homocysteine (see Fig. S1 in the supplemental material), indicating that the observed growth defect was not due to limitations in these amino acids. Polyamines play an important role in microorganisms, including *B. subtilis*, especially in macromolecular syntheses and in the modulation of translation accuracy (41, 42). Since SAM-recycling pathways are closely involved in polyamine synthesis (Fig. 5), we measured intracellular concentrations of spermidine, spermine, and putrescine and found that none differed significantly in the mutant and parental strains (see Fig. S2 in the supplemental material). Moreover, the addition of spermidine, putrescine, methionine, or cysteine did not affect the resistance of these strains to Sm (data not shown), indicating that polyamines are not relevant to the changes caused by *mthA* mutations.

Unlike antibiotic production, *mthA* mutations did not alter the expression of enzymes, such as α-amylase and proteases, as determined by RT-qPCR analysis of the *amyE* gene, which encodes α-amylase, and the *bpr*, *epr*, *mpr*, *nprB*, *nprE*, and *wprA* genes, which encode proteases (data not shown). Although high-level Sm-resistant (*rpsL*) mutants were found to emerge at 200-fold-higher frequency from *rsmG* mutants than from the wild type (12), similar results were not observed in *mthA* mutants (data not shown).

Transcriptional analysis of the bacilysin-biosynthetic gene.

The biosynthesis of bacilysin is controlled by a polycistronic operon (*ywfBCDEFG*) and a monocistronic operon (*ywfH*) (23), both considered structural genes for bacilysin biosynthesis. We therefore analyzed the transcription of the *ywfB* gene, which may

FIG 2 Sm susceptibility of the *mthA* mutant. The *B. subtilis* strains 168 (wild type [WT]), KJ04 (*mthA1* transformant), and KJ05 (*mthA* disruptant) were grown to stationary phase in L medium at 37°C. Approximately 2×10^8 cells were diluted with distilled water, and 5-μl aliquots of the cell suspension were spotted onto medium containing 0, 10, or 20 μg/ml of Sm, followed by incubation at 37°C for 12 h.

FIG 3 Growth, antibiotic production, and transcriptional analysis of parental (168) and *mthA* mutant (KJ04) strains. (A) Strains were grown in NG medium at 37°C. Antibiotic production was determined by the paper disk-agar diffusion method and expressed as the size of the inhibition zone around the disk (diameter, 8 mm). Growth (closed symbols) and antibiotic production (open symbols) of strain 168 (circles) and strain KJ04 (triangles) are shown. (B) Transcriptional analysis of the *ywfB* gene involved in bacilysin biosynthesis. The strains were grown as for panel A, and the levels of expression of the *ywfB* gene were determined by real-time qPCR. (C) Strains 168 (wild type), KJ04 (*mthA*), K2-007 (*relA*), and KO-1234 (*relA mthA*) were grown for 15 h as for panel A, and bacilysin production was determined by the paper disk-agar diffusion method. (D) Transcriptional analysis of the *relA* gene involved in ppGpp synthesis. Strains were grown as for panel A, and levels of expression of the *relA* gene were determined by real-time qPCR. The error bars indicate the standard deviations of the means of three or more samples.

FIG 4 Morphological appearance of parental (168) and *mthA* mutant (KJ04) strains. (A) Colony morphology observed after 5 days of culture on L agar plates. (B) Microscopic observation (magnification, $\times 1,000$) of cells grown to mid-growth phase (5 h) in L medium.

encode the enzyme that synthesizes the anticapsin moiety (23), by real-time quantitative PCR. Although expression of *ywfB* was detected during the late growth phase (OD₆₅₀ = 5) in wild-type strain 168, high expression was detected during the early growth phase (OD₆₅₀ = 0.5) in the *mthA* mutants, with mutant expression during the late growth phase being much higher than in the wild type (Fig. 3B). Similar results for expression of *ywfB* were obtained when amplification of the *sigA* gene was used, instead of the 16S rRNA gene, as an internal control (data not shown), accounting for the increased production of bacilysin by the *mthA* mutant (Fig. 3A and C). The stringent response, one of the most important adaptation systems in bacteria (43), is closely involved in initiating secondary metabolism (13, 44). This response depends on the transient increase in hyperphosphorylated guanosine nucleotides ([p]ppGpp), which are synthesized from GDP or GTP by the *relA* gene product (ppGpp synthetase) in response to nutrient limitation (43). Transcription of both *ywfBCDEFG* and *ywfH* is enhanced directly or indirectly under conditions that elicit the stringent response (23). Introduction of a *relA* mutation into the wild-type 168 or *mthA* mutant strain severely inhibited bacilysin production (Fig. 3C), indicating that the *mthA* mutation can fully exert its effect on antibiotic overproduction in the genetic background of *relA*⁺. Therefore, it was possible that the *mthA* mutation, like a certain thiostrepton resistance (*tsp*) mutation in *Streptomyces* (19), increased *relA* expression, leading to enhanced ppGpp synthesis and eventual bacilysin overproduction. We

TABLE 3 Antibiotic susceptibilities of *B. subtilis* wild-type and mutant strains

Antibiotic tested	Major target	MIC (μ g/ml) for strain ^a :		
		168	KJ04 (<i>mthA</i>)	KO-673 (<i>rsmG</i>)
Streptomycin	Ribosome	10	50	100
Chloramphenicol	Ribosome	2	2	2
Erythromycin	Ribosome	0.1	0.1	0.1
Fusidic acid	Ribosome	0.3	0.3	0.3
Kanamycin	Ribosome	0.5	2	0.5
Kasugamycin	Ribosome	1,500	1,500	1,500
Lincomycin	Ribosome	30	30	30
Neomycin	Ribosome	0.2	0.3	0.2
Paromomycin	Ribosome	0.3	1	0.3
Tetracycline	Ribosome	8	8	8
Thiostrepton	Ribosome	0.03	0.03	0.03
Puromycin	Protein synthesis	20	20	20
Rifampin	RNA polymerase	0.3	0.3	0.3
Cefalexin	Cell wall	0.2	0.2	0.2

^a Determined after a 20-h incubation at 37°C on L agar plates.

FIG 5 Outline of SAM-recycling pathways in *B. subtilis*. The genes and enzymes involved in SAM recycling are as follows: *metI*, cystathionine γ -synthase; *metC* and *patB*, cystathionine β -lyase; *metE*, methionine synthase; *metK*, SAM synthetase; *speD*, SAM decarboxylase; *speE*, spermidine synthase; *mthA*, SAH/MTA nucleosidase (*mthA* is synonymous with *mtnA*); *mtnK*, methylthioribose kinase; the *mtnA* and *mtnWXBD* gene products, involved in the MTR-to-KMBA recycling pathway; *mtnE*, aminotransferase; *luxS*, S-ribosylhomocysteine hydrolase; *yrhA* (*mccA*), cystathionine β -synthase; *yrhB* (*mccB*), cystathionine γ -lyase and homocysteine γ -lyase; SAM (S-adenosylmethionine); SAH (S-adenosylhomocysteine); KMBA (α -keto- γ -methyl-thiobutyric acid); MTA (methylthioadenosine); MTR (methylthioribose); and SRH (S-ribosylhomocysteine). AI-2 indicates autoinducer-2. The figure was drawn on the basis of work by Hullo et al. (53).

found, however, that the transcription of *relA* was somewhat decreased in the *mthA* mutant (Fig. 3D).

Increase in SAM pool size is responsible for bacilysin overproduction. In *Streptomyces* spp., SAM is involved in triggering the onset of secondary metabolism; overexpression of *metK* which encodes SAM synthetase, or addition of SAM into the medium causes antibiotic overproduction (22, 45). SAM metabolism was partially blocked by the *mtaA*-null mutation in *B. subtilis*, because MthA catalyzes two steps (SAH→SRH and MTA→MTR) in the SAM-recycling pathway (Fig. 5). Consequently, the *mtaA* mutation may increase the SAM pool size, eventually activating secondary metabolism. To address this hypothesis, we measured SAM pool size in wild-type and mutant cells grown to various growth phases. As expected, SAM pool sizes were 2-fold larger in the *mtaA* mutant than in the wild type (Fig. 6A). The expression of *metK* was not increased in the *mtaA* mutant but rather somewhat decreased (Fig. 6B), suggesting that the mutation increases SAM pool size by blocking SAM recycling.

To confirm that SAM initiates antibiotic production in *B. subtilis*, we attempted the forced expression of the *metK* gene in wild-type cells by utilizing the multicopy vector pUB18, in which the *metK* gene was integrated. As expected, the SAM pool size was 1.5 to 1.8 times greater in the resulting transformant, KJ06, harboring pUB18-*metK*, than in the parental strain throughout all growth phases (Fig. 7). KJ06 also showed enhanced production of bacitracin (10 units), similar to the *mtaA* mutant KJ04, but did not show elevated resistance to Sm (data not shown). These results indicate

FIG 6 Intracellular SAM level and transcriptional analysis of the *metK* gene in wild-type (168) and *mtbA* mutant (KJ04) strains. (A) The strains were grown in NG medium at 37°C to an OD₆₅₀ of 0.5, 1, or 1.5. Intracellular SAM levels were determined by reverse-phase high-performance liquid chromatography. (B) Transcriptional analysis of the *metK* gene. Strains were grown in NG medium as for panel A. Total RNAs were extracted from the cells and used for real-time qPCR analysis. The transcription level of *metK* was normalized relative to the amount of 16S rRNA in each RNA sample. The error bars indicate the standard deviations of the means of three or more samples.

that the increased SAM pool size is responsible for the increased production of bacilysin, but not for low-level resistance to Sm. Since the *rsmG*-null mutation confers low-level resistance to Sm (12), we hypothesized that the *mthA* mutation may severely decrease the level of expression of *rsmG*, making the *mthA* mutant slightly resistant to Sm. This possibility was ruled out, however, as

FIG 7 Effect of *metK* overexpression on the intracellular SAM level. Strains were grown in NG medium at 37°C to an OD₆₅₀ of 0.5, 1, or 2. Intracellular SAM levels were determined by reverse-phase high-performance liquid chromatography. The error bars indicate the standard deviations of the means of three or more samples.

FIG 8 NTD production and transcriptional analysis in parental (168) and *mthA* mutant (KJ04) strains. (A) Strains were grown in S7N medium at 37°C for 24 h, and NTD production was determined by the paper disk-agar diffusion method. (B) Transcriptional analysis of the *ntdA* gene. The strains were grown as for panel A for 12, 16, or 20 h, and the level of expression of the *ntdA* gene was determined by real-time qPCR. The error bars indicate the standard deviations of the means of three or more samples.

the level of expression of *rsmG* was as high in KJ04 as in the parental strain (see Fig. S3 in the supplemental material), although these results do not completely exclude the possibility that *mthA* mutation inhibited RsmG activity at the posttranslational level.

***mthA* mutation activates even dormant genes.** *B. subtilis* 168 can produce the antibiotic NTD, although the genes responsible for its biosynthesis are dormant (i.e., silent) under ordinary culture conditions (29). Although the physiological condition(s) that permits the expression of these genes has not been determined, the S487→L mutation in the *rpoB* gene, which encodes the β -subunit of RNA polymerase, can activate the NTD-biosynthetic genes, resulting in the production of 500 to 1,000 $\mu\text{g/ml}$ NTD (29). Wild-type *B. subtilis* produces less than detectable levels ($<1 \mu\text{g/ml}$). We therefore assessed whether the *mthA* mutation accompanied by an increase in the SAM level can activate these dormant genes. The wild-type (168) and *mthA* mutant (KJ04) strains were grown for 24 h in S7N medium, developed for NTD production (29), and NTD production was assayed by the disk-agar diffusion method using Mueller-Hinton assay plates containing 1 mM glucosamine to negate the effects of bacitracin. Strikingly, the *mthA* mutant produced 150 to 200 $\mu\text{g/ml}$ NTD, whereas none was produced by the wild type (Fig. 8A). Isolation of the antibacterial compound, followed by ^1H NMR and ^{13}C NMR analyses, confirmed that it was NTD (see Fig. S4A and B in the supplemental material) (46).

NTD biosynthesis is controlled by the polycistronic gene *ntdABC* for NTD biosynthesis and a monocistronic gene, a positive regulator of *ntdABC* (29). Transcriptional analysis of the *ntdA* gene showed that the *mthA* mutation resulted in the expression of *ntdA* mRNA during the late growth phase (16 to 20 h) (Fig. 8B), accounting for the burst of NTD production. Thus, modulation of the SAM pool size not only enhanced the production of antibiotics, but activated the expression of dormant genes.

DISCUSSION

We previously reported that antibiotic production by *B. subtilis* is markedly activated by introducing certain low-level Sm resistance mutations, which differ from *rsmG* and *rpsL* mutations (12, 18). In the present study, we used the CGS technique to successfully identify this third mutation, which confers low-level Sm resistance, at least in *B. subtilis* and *E. coli*. Our principal findings were as follows: (i) the third mutation conferring low-level Sm resistance was located in the *mthA* gene, which encodes SAH/MTA nucleosidase; (ii) the *mthA* mutation increased intracellular SAM by arresting the SAM-recycling pathways; (iii) the increased SAM level directly or indirectly enhanced antibiotic production and activated even dormant genes involved in secondary-metabolite biosynthesis; and (iv) the activation of antibiotic production was apparently achieved at the transcriptional level, not as a methyl donor for antibiotic biosynthesis, by markedly enhancing the expression of biosynthetic genes.

The high frequency of emergence of spontaneous *mthA* mutants is likely due to the dispensability of the gene, allowing cells to remain viable. Our finding that SAM plays an essential role in triggering the onset of secondary metabolism is not unprecedented. For example, *rsmG* mutants in *S. coelicolor* exhibit enhanced expression of the *metK* gene encoding SAM synthetase, accompanied by increased protein synthesis during the late growth phase, eventually leading to the overproduction of antibiotics (10). Thus, increases in SAM synthetase and protein synthesis activity resulting from the *rsmG* mutation likely activated secondary metabolism in *S. coelicolor* (10). In *B. subtilis*, *rsmG* mutations did not result in increased antibiotic production, consistent with findings that these mutants did not show increased protein synthesis during the late growth phase or increased SAM synthetase activity (12). Thus, in contrast to *Streptomyces*, secondary metabolism in *B. subtilis* is not activated by *rsmG* mutations. Rather, mutations in *mthA*, not *rsmG*, increase intracellular SAM, but by a completely different mechanism. In *S. coelicolor*, *rsmG* mutations enhance *metK* expression, whereas in *B. subtilis*, *mthA* mutations block SAM-recycling pathways, with both leading to increased SAM levels (Fig. 9). The causal relationship between antibiotic production and the SAM level in *B. subtilis* was confirmed by forced expression of *metK* in the wild-type strain. As reviewed by Parveen and Cornell (42), the importance of SAH/MTA nucleosidase encoded by the *mthA* gene is currently increasing because a comprehensive analysis of its various roles demonstrates that it is an integral component of the activated methyl cycle, which recycles adenine and methionine through SAM-mediated methylation reactions. Inhibition of this enzyme by certain substrate analogues limits synthesis of autoinducers and hence causes reduction in biofilm formation and may attenuate virulence in certain bacteria.

Mutations in *rsmG* have been shown to cause low-level Sm resistance in *E. coli*, *M. tuberculosis*, *S. coelicolor*, and *B. subtilis* (10–12, 47). Loss of the m7G modification in 16S rRNA results in resistance to Sm, providing a molecular basis for *rsmG* mutation-induced Sm resistance. In contrast, the mechanism by which *mthA* mutations cause low-level Sm resistance is presently unclear, especially since an increased SAM level did not cause Sm resistance. As the *mthA* mutation-mediated Sm resistance in *B. subtilis* (and in *E. coli*) was slight, the resistance was likely due to a secondary effect of the mutation. Figure 9 shows a comparison of

FIG 9 Schematic showing the signal transduction pathways in *B. subtilis* and *Streptomyces* spp., from the *mthA* or *rsmG* mutation to the enhancement of antibiotic production or the activation of silent genes. The scheme was based on the work presented here and previous studies in *B. subtilis* (12, 18, 23, 29, 31) and *Streptomyces* spp. (13, 44, 48).

B. subtilis and *Streptomyces* signal transduction systems, starting from the *mthA* and *rsmG* mutations, respectively, to the ability to enhance antibiotic production or activate silent genes (10, 12, 13, 23, 29, 44, 48). Interestingly, *Streptomyces* spp. and *Zymomonas* spp. have no *mthA* homologue, whereas many bacteria (e.g., *E. coli*, *S. aureus*, *Salmonella enterica*, *Thermus thermophilus*, *Helicobacter pylori*, and *Listeria monocytogenes*) do. Initiation of secondary metabolism in *Streptomyces* is characterized by involvement of autoinducers, such as A factor (48).

SAM is synthesized from methionine and ATP by an SAM synthetase encoded by the *metK* gene. SAM is highly reactive and plays a central role in many cellular functions (42, 49, 50). In contrast to *Streptomyces* cells (22), extracellular SAM cannot be incorporated by *E. coli* or *B. subtilis* cells (51, 52). The major steps for SAM recycling in *B. subtilis* have been recently characterized by Hullo et al. (53). The intracellular SAM level should be tightly regulated in native *B. subtilis* cells, since a 2-fold increase markedly altered cellular physiology and morphology, as shown in the present study. The filamentous form of the *mthA* mutant accompanied by elevated SAM (Fig. 4) was striking, due to recent findings that *E. coli* cells starved for SAM are very long, suggesting that SAM and methylation are important for cross wall formation (54). The results described here, together with previous findings in *rsmG* mutants, establish the significance of SAM in initiating antibiotic production in bacteria, indicating an intrinsic role for SAM in microbial secondary metabolism (55). In addition, intro-

duction of a multicopy plasmid containing the *Streptomyces spectabilis metK* gene into *Streptomyces lividans* was found to induce antibiotic production (45), and the addition of SAM to the culture medium increased antibiotic production by *S. coelicolor*, *Streptomyces griseus*, and *Streptomyces griseoflavus* (22, 56). SAM is the methyl donor for the methylation of cytosine and adenosine bases in DNA, rRNA, and tRNA; of various proteins; and of small molecules important for both lower and higher organisms (42, 57, 58). Thus, SAM synthetase is likely essential for the viability of *E. coli* (59) and *B. subtilis* (60), with overexpression in these bacteria causing methionine auxotrophy, perhaps by depleting the intracellular methionine pool, either by consuming it to synthesize SAM or by repressing the methionine-biosynthetic genes. In *E. coli*, SAM was shown to be a corepressor of the methionine regulation system (49). Although there is no evidence to date for the existence of a methyltransferase involved in the regulation of antibiotic production, SAM-dependent protein methylation may play a role in controlling the activity of the regulatory proteins encoded by these developmental genes. It may be possible that the increased SAM pool size stimulated SAM-dependent methylation of RelA (or YjbM/YwaC), resulting in enhancement of ppGpp synthesis, followed by acceleration of antibiotic production. Alternatively, DNA or RNA methylation may be involved in the expression of these regulatory genes. For example, the extent of methylation of a particular macromolecule (e.g., a particular DNA region) could determine the probability that a second macromol-

ecule (e.g., a repressor or activator) can bind to the first, significantly altering gene expression, similar to findings in *E. coli* (61). In addition to being a methyl donor, SAM may be directly involved in regulating antibiotic synthesis as a corepressor or inducer, as shown for methionine regulon expression in *E. coli* (49). It is also noteworthy that SAM is a precursor for certain autoinducers, which control many different processes, including antibiotic production, biofilm formation, and virulence (62–64). Autoinducer 2 (AI-2), a furanosyl borate diester, is produced from SAM in *B. subtilis* (Fig. 5) (53). Although *mthA* mutation likely did not enhance AI-2 production in this case, it is possible that the elevated level of SAM caused enhanced production of another, yet unknown autoinducer (if any), eventually accelerating the secondary metabolism.

ppGpp is crucial in triggering the onset of antibiotic production in *Streptomyces* spp., whereas morphological differentiation is triggered by a reduction in GTP (13, 44, 65). In *B. subtilis*, both GTP and ppGpp are key factors initiating antibiotic production (23). Bacilysin production in *B. subtilis* is apparently controlled by CodY, a GTP-binding protein, because *codY* disruption increased the level of expression of genes involved in bacilysin biosynthesis (23). However, the effect of GTP can be elicited only in *rel*⁺ wild-type cells, not in *relA* mutant-type cells. Neither decoyinine treatment nor *codY* disruption activated transcription from the *ywfBCDEFG* operon responsible for the bacilysin biosynthesis in *relA* mutant cells (23). Thus, ppGpp plays a pivotal role as a positive regulator in antibiotic production by *B. subtilis*, in accordance with the results from the present work showing that the *mthA* mutation fully exerted its effect on antibiotic overproduction in *rel*⁺ but not *relA* mutant cells (Fig. 3C). Since ppGpp is essential for transcription of the *ywfBCDEFG* and *ywfH* genes via the CodY-mediated regulation system, bacilysin production in *B. subtilis* is controlled by a dual regulation system composed of ppGpp and GTP (23), unlike antibiotic production in *Streptomyces* spp. (Fig. 9). Recent advances with respect to ppGpp-RNA polymerase interrelationship have clarified that, unlike *E. coli*, where ppGpp decreases rRNA promoter activity by directly inhibiting RNA polymerase, in *B. subtilis* and *T. thermophilus* (species distantly related to *E. coli*), ppGpp reduces the available GTP pools, thereby modulating rRNA promoter activity indirectly (66–69). In addition, direct regulation of GTP homeostasis by ppGpp was analyzed in detail in *B. subtilis*, demonstrating that two GTP biosynthesis enzymes (Gmk and HprT) are major posttranscriptional targets of ppGpp whose activities are strongly inhibited by ppGpp *in vitro*, while inhibition of IMP dehydrogenase (GuaB) activity by ppGpp is likely at least a minor contributor in *B. subtilis* (70, 71) and perhaps in *T. thermophilus* (67). This situation could be prominent, especially in the absence of starvation (with lower ppGpp levels) rather than under starvation conditions (with higher ppGpp levels). Recent work by Belitsky and Sonenshein (72) clarified the CodY-binding motifs using a motif-searching algorithm. CodY regulates transcription in several ways, including (i) negative or positive regulation by binding within or near a promoter site, (ii) negative regulation by interfering with the binding of a positive regulator, and (iii) negative regulation by acting as a roadblock to RNA polymerase. It is unclear at present what the direct critical target(s) of ppGpp is in initiating bacilysin biosynthesis in *B. subtilis*. The current achievements described above may be helpful in clarifying the mechanisms underlying

regulation of the secondary metabolism in *Bacillus* and related bacteria at the molecular level.

Sequencing of the genomes of *Streptomyces*, fungi, and myxobacteria has shown that, although each strain contains genes encoding the enzymes necessary to synthesize a plethora of potential secondary metabolites, only a fraction are expressed during fermentation (73, 74). Methods of activating dormant antibiotic-biosynthetic gene clusters are therefore of interest in both basic and industrial microbiology (16, 75–77). Since the *mthA* mutation activated the expression of dormant genes involved in NTD production, alterations in the intracellular SAM level, by introducing an *rsmG* (in *Streptomyces*) or an *mthA* (in eubacteria) mutation, could be a feasible way to activate the dormant genes, as they require neither induced mutagenesis nor gene-engineering technique. The forced expression of the *metK* gene should also be effective, though labor-intensive. Thus, our method provides a powerful tool for screening novel compounds and for strain improvement to overproduce useful compounds. Clinically, *mthA* mutations appear to be microbiologically insignificant because, unlike *rsmG* mutations, these mutations, despite their high frequency, did not trigger the emergence of mutants with high-level Sm resistance.

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S.T. and J.-Y.K. mainly worked with transcriptional analysis and physiological analysis, respectively. Y.H. conducted structural analysis of NTD, and K.O. designed the research work and wrote the article.

REFERENCES

1. Schatz A, Waksman SA. 1944. Effect of streptomycin and other antibiotic substances upon *Mycobacterium tuberculosis* and related organisms. *Proc. Soc. Exp. Bio. Med.* 57:244–248. <http://dx.doi.org/10.3181/00379727-57-14769>.
2. Klein M, Kimmelman LJ. 1946. The role of spontaneous variants in the acquisition of streptomycin resistance by the *Shigellae*. *J. Bacteriol.* 52:471–479.
3. Honore N, Cole ST. 1994. Streptomycin resistance in mycobacteria. *Antimicrob. Agents Chemother.* 38:238–242. <http://dx.doi.org/10.1128/AAC.38.2.238>.
4. Honore N, Marchal G, Cole ST. 1995. Novel mutation in 16S rRNA associated with streptomycin dependence in *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* 39:769–770. <http://dx.doi.org/10.1128/AAC.39.3.769>.
5. Carter AP, Clemons WM, Brodersen DE, Morgan-Warren RJ, Wimberly BT, Ramakrishnan V. 2000. Functional insights from the structure of the 30S ribosomal subunit and its interactions with antibiotics. *Nature* 407:340–348. <http://dx.doi.org/10.1038/35030019>.
6. Ogle JM, Ramakrishnan V. 2005. Structural insights into translational fidelity. *Annu. Rev. Biochem.* 74:129–177. <http://dx.doi.org/10.1146/annurev.biochem.74.061903.155440>.
7. Montandon PE, Wagner R, Stutz E. 1986. *E. coli* ribosomes with a C912 to U base change in the 16S rRNA are streptomycin resistant. *EMBO J.* 5:3705–3708.
8. Powers T, Noller HF. 1991. A functional pseudoknot in 16S ribosomal RNA. *EMBO J.* 10:2203–2214.
9. Pinard R, Payant C, Melancon P, Brakier-Gingras L. 1993. The 5'

- proximal helix of 16S rRNA is involved in the binding of streptomycin to the ribosome. *FASEB J.* 7:173–176.
10. Nishimura K, Hosaka T, Tokuyama S, Okamoto S, Ochi K. 2007. Mutations in *rsmG*, encoding a 16S rRNA methyltransferase, result in low-level streptomycin resistance and antibiotic overproduction in *Streptomyces coelicolor* A3(2). *J. Bacteriol.* 189:3876–3883. <http://dx.doi.org/10.1128/JB.01776-06>.
 11. Okamoto S, Tamaru A, Nakajima C, Nishimura K, Tanaka Y, Tokuyama S, Suzuki Y, Ochi K. 2007. Loss of a conserved 7-methylguanosine modification in 16S rRNA confers low-level streptomycin resistance in bacteria. *Mol. Microbiol.* 63:1096–1106. <http://dx.doi.org/10.1111/j.1365-2958.2006.05585.x>.
 12. Nishimura K, Johansen SK, Inaoka T, Hosaka T, Tokuyama S, Tahara Y, Okamoto S, Kawamura F, Douthwaite S, Ochi K. 2007. Identification of the RsmG methyltransferase target as 16S rRNA nucleotide G527 and characterization of *Bacillus subtilis* *rsmG* mutants. *J. Bacteriol.* 189:6068–6073. <http://dx.doi.org/10.1128/JB.00558-07>.
 13. Ochi K. 2007. From microbial differentiation to ribosome engineering. *Biosci. Biotechnol. Biochem.* 71:1373–1386. <http://dx.doi.org/10.1271/bbb.70007>.
 14. Hosaka T, Ohnishi-Kameyama M, Muramatsu H, Murakami K, Tsurumi Y, Kodani S, Yoshida M, Fujie A, Ochi K. 2009. Antibacterial discovery in actinomycetes strains with mutations in RNA polymerase or ribosomal protein S12. *Nat. Biotechnol.* 27:462–464. <http://dx.doi.org/10.1038/nbt.1538>.
 15. Ochi K, Hosaka T. 2013. New strategies for drug discovery: activation of silent or weakly expressed microbial gene clusters. *Appl. Microbiol. Biotechnol.* 97:87–98. <http://dx.doi.org/10.1007/s00253-012-4551-9>.
 16. Ochi K, Tanaka Y, Tojo S. 2014. Activating the expression of bacterial cryptic genes by *rpoB* mutations in RNA polymerase or by rare earth elements. *J. Ind. Microbiol. Biotechnol.* 41:403–414. <http://dx.doi.org/10.1007/s10295-013-1349-4>.
 17. Shima J, Hesketh A, Okamoto S, Kawamoto S, Ochi K. 1996. Induction of actinorhodin production by *rpsL* (encoding ribosomal protein S12) mutations that confer streptomycin resistance in *Streptomyces lividans* and *Streptomyces coelicolor* A3(2). *J. Bacteriol.* 178:7276–7284.
 18. Hosoya Y, Okamoto S, Muramatsu H, Ochi K. 1998. Acquisition of certain streptomycin-resistant (*str*) mutations enhances antibiotic production in bacteria. *Antimicrob. Agents Chemother.* 42:2041–2047.
 19. Wang G, Hosaka T, Ochi K. 2008. Dramatic activation of antibiotic production in *Streptomyces coelicolor* by cumulative drug resistance mutations. *Appl. Environ. Microbiol.* 74:2834–2840. <http://dx.doi.org/10.1128/AEM.02800-07>.
 20. Okamoto-Hosoya Y, Hosaka T, Ochi K. 2003. An aberrant protein synthesis activity is linked with antibiotic overproduction in *rpsL* mutants of *Streptomyces coelicolor* A3(2). *Microbiology* 149:3299–3309. <http://dx.doi.org/10.1099/mic.0.26490-0>.
 21. Hosaka T, Xu J, Ochi K. 2006. Increased expression of ribosome recycling factor is responsible for the enhanced protein synthesis during the late growth phase in an antibiotic-overproducing *Streptomyces coelicolor* ribosomal *rpsL* mutant. *Mol. Microbiol.* 61:883–897. <http://dx.doi.org/10.1111/j.1365-2958.2006.05285.x>.
 22. Okamoto S, Lezhava A, Hosaka T, Okamoto-Hosoya Y, Ochi K. 2003. Enhanced expression of S-adenosylmethionine synthetase causes overproduction of actinorhodin in *Streptomyces coelicolor* A3(2). *J. Bacteriol.* 185:601–609. <http://dx.doi.org/10.1128/JB.185.2.601-609.2003>.
 23. Inaoka T, Takahashi K, Ohnishi-Kameyama M, Yoshida M, Ochi K. 2003. Guanine nucleotides guanosine 5'-diphosphate 3'-diphosphate and GTP co-operatively regulate the production of an antibiotic bacilysin in *Bacillus subtilis*. *J. Biol. Chem.* 278:2169–2176. <http://dx.doi.org/10.1074/jbc.M208722000>.
 24. Tamehiro N, Okamoto-Hosoya Y, Okamoto S, Ubukata M, Hamada M, Naganawa H, Ochi K. 2002. Bacilysocin, a novel phospholipid antibiotic produced by *Bacillus subtilis* 168. *Antimicrob. Agents Chemother.* 46:315–320. <http://dx.doi.org/10.1128/AAC.46.2.315-320.2002>.
 25. Hilton MD, Alaeddinoglu NG, Demain AL. 1988. *Bacillus subtilis* mutant deficient in the ability to produce the dipeptide antibiotic bacilysin: isolation and mapping of the mutation. *J. Bacteriol.* 170:1018–1020.
 26. Koroglu TE, Ogulur I, Mutlu S, Yazgan-Karatas A, Ozcengiz G. 2011. Global regulatory systems operating in bacilysin biosynthesis in *Bacillus subtilis*. *J. Mol. Microbiol. Biotechnol.* 20:144–155. <http://dx.doi.org/10.1159/000328639>.
 27. Tsuno T, Ikeda C, Numata K, Tomita K, Konishi M, Kawaguchi H. 1986. 3,3'-Neotrehalosadiamine (BMV-28251), a new aminosugar antibiotic. *J. Antibiot. (Tokyo)* 39:1001–1003. <http://dx.doi.org/10.7164/antibiotics.39.1001>.
 28. Numata K, Satoh F, Hatori M, Miyaki T, Kawaguchi H. 1986. Isolation of 3,3'-neotrehalosadiamine (BMV-28251) from a butirosin-producing organism. *J. Antibiot. (Tokyo)* 39:1346–1348. <http://dx.doi.org/10.7164/antibiotics.39.1346>.
 29. Inaoka T, Takahashi K, Yada H, Yoshida M, Ochi K. 2004. RNA polymerase mutation activates the production of a dormant antibiotic 3,3'-neotrehalosadiamine via an autoinduction mechanism in *Bacillus subtilis*. *J. Biol. Chem.* 279:3885–3892. <http://dx.doi.org/10.1074/jbc.M309925200>.
 30. Albert TJ, Dailidene D, Dailide G, Norton JE, Kalia A, Richmond TA, Molla M, Singh J, Green RD, Berg DE. 2005. Mutation discovery in bacterial genomes: metronidazole resistance in *Helicobacter pylori*. *Nat. Methods* 2:951–953. <http://dx.doi.org/10.1038/nmeth805>.
 31. Inaoka T, Ochi K. 2002. RelA protein is involved in induction of genetic competence in certain *Bacillus subtilis* strains by moderating the level of intracellular GTP. *J. Bacteriol.* 184:3923–3930. <http://dx.doi.org/10.1128/JB.184.14.3923-3930.2002>.
 32. Wendrich TM, Marahiel MA. 1997. Cloning and characterization of a *relA/spoT* homologue from *Bacillus subtilis*. *Mol. Microbiol.* 26:65–79. <http://dx.doi.org/10.1046/j.1365-2958.1997.5511919.x>.
 33. Inaoka T, Kasai K, Ochi K. 2001. Construction of an *in vivo* nonsense readthrough assay system and functional analysis of ribosomal proteins S12, S4, and S5 in *Bacillus subtilis*. *J. Bacteriol.* 183:4958–4963. <http://dx.doi.org/10.1128/JB.183.17.4958-4963.2001>.
 34. Moriya S, Tsujikawa E, Hassan AK, Asai K, Kodama T, Ogasawara N. 1998. A *Bacillus subtilis* gene-encoding protein homologous to eukaryotic SMC motor protein is necessary for chromosome partition. *Mol. Microbiol.* 29:179–187. <http://dx.doi.org/10.1046/j.1365-2958.1998.00919.x>.
 35. Ochi K, Ohsawa S. 1984. Initiation of antibiotic production by the stringent response of *Bacillus subtilis* Marburg. *J. Gen. Microbiol.* 130:2473–2482.
 36. Payne SH, Ames BN. 1982. A procedure for rapid extraction and high-pressure liquid chromatographic separation of the nucleotides and other small molecules from bacterial cells. *Anal. Biochem.* 123:151–161. [http://dx.doi.org/10.1016/0003-2697\(82\)90636-4](http://dx.doi.org/10.1016/0003-2697(82)90636-4).
 37. Flores HE, Galston AW. 1982. Analysis of polyamines in higher plants by high performance liquid chromatography. *Plant Physiol.* 69:701–706. <http://dx.doi.org/10.1104/pp.69.3.701>.
 38. Sekowska A, Bertin P, Danchin A. 1998. Characterization of polyamine synthesis pathway in *Bacillus subtilis* 168. *Mol. Microbiol.* 29:851–858. <http://dx.doi.org/10.1046/j.1365-2958.1998.00979.x>.
 39. Inaoka T, Satomura T, Fujita Y, Ochi K. 2009. Novel gene regulation mediated by overproduction of secondary metabolite neotrehalosadiamine in *Bacillus subtilis*. *FEMS Microbiol. Lett.* 291:151–156. <http://dx.doi.org/10.1111/j.1574-6968.2008.01450.x>.
 40. Kenig M, Abraham EP. 1976. Antimicrobial activities and antagonists of bacilysin and anticapsin. *J. Gen. Microbiol.* 94:37–45. <http://dx.doi.org/10.1099/00221287-94-1-37>.
 41. Igarashi K, Kashiwagi K. 2010. Modulation of cellular function by polyamines. *Int. J. Biochem. Cell Biol.* 42:39–51. <http://dx.doi.org/10.1016/j.biocel.2009.07.009>.
 42. Parveen N, Cornell KA. 2011. Methylthioadenosine/S-adenosylhomocysteine nucleosidase, a critical enzyme for bacterial metabolism. *Mol. Microbiol.* 79:7–20. <http://dx.doi.org/10.1111/j.1365-2958.2010.07455.x>.
 43. Cashel M, Gentry DR, Hernandez VJ, Vinella D. 1996. The stringent response, p 1458–1496. In Neidhardt FC, Curtiss R, III, Ingraham JL, Lin ECC, Low KB, Magasanik B, Reznikoff WS, Riley M, Schaechter M, Umberger HE (ed), *Escherichia coli and Salmonella: cellular and molecular biology*, 2nd ed. ASM Press, Washington, DC.
 44. Bibb MJ. 2005. Regulation of secondary metabolism in streptomycetes. *Curr. Opin. Microbiol.* 8:208–215. <http://dx.doi.org/10.1016/j.mib.2005.02.016>.
 45. Kim DJ, Huh JH, Yang YY, Kang CM, Lee IH, Hyun CG, Hong SK, Suh JW. 2003. Accumulation of S-adenosyl-L-methionine enhances production of actinorhodin but inhibits sporulation in *Streptomyces lividans* TK23. *J. Bacteriol.* 185:592–600. <http://dx.doi.org/10.1128/JB.185.2.592-600.2003>.
 46. Anjum S, Vetter ND, Rubin JE, Palmer DRJ. 2013. Synthesis of 3,3'-neotrehalosadiamine and related 1,1'-aminodisaccharides using dis-

- armed, armed, and superarmed building blocks. *Tetrahedron* 69:816–825. <http://dx.doi.org/10.1016/j.tet.2012.10.058>.
47. Tanaka Y, Komatsu M, Okamoto S, Tokuyama S, Kaji A, Ikeda H, Ochi K. 2009. Antibiotic overproduction by *rpsL* and *rsmG* mutants of various actinomycetes. *Appl. Environ. Microbiol.* 75:4919–4922. <http://dx.doi.org/10.1128/AEM.00681-09>.
 48. Ohnishi Y, Yamazaki H, Kato JY, Tomono A, Horinouchi S. 2005. AdpA, a central transcriptional regulator in the A-factor regulatory cascade that leads to morphological development and secondary metabolism in *Streptomyces griseus*. *Biosci. Biotechnol. Biochem.* 69:431–439. <http://dx.doi.org/10.1271/bbb.69.431>.
 49. Greene RC. 1996. Biosynthesis of methionine, p 542–560. In Neidhardt FC, Curtiss R, III, Ingraham JL, Lin ECC, Low KB, Magasanik B, Reznikoff WS, Riley M, Schaechter M, Umberger HE (ed), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, DC.
 50. Iwig DF, Booker SJ. 2004. Insight into the polar reactivity of the onium chalcogen analogues of S-adenosyl-L-methionine. *Biochemistry* 43: 13496–13509. <http://dx.doi.org/10.1021/bi048693+>.
 51. Wang S, Arends SJ, Weiss DS, Newman EB. 2005. A deficiency in S-adenosylmethionine synthetase interrupts assembly of the septal ring in *Escherichia coli* K-12. *Mol. Microbiol.* 58:791–799. <http://dx.doi.org/10.1111/j.1365-2958.2005.04864.x>.
 52. Grove TL, Benner JS, Radle MI, Ahlum JH, Landgraf BJ, Krebs C, Booker SJ. 2011. A radically different mechanism for S-adenosyl-methionine-dependent methyltransferases. *Science* 332:604–607. <http://dx.doi.org/10.1126/science.1200877>.
 53. Hulio MF, Auger S, Soutourina O, Barzu O, Yvon M, Danchin A, Martin-Verstraete I. 2007. Conversion of methionine to cysteine in *Bacillus subtilis* and its regulation. *J. Bacteriol.* 189:187–197. <http://dx.doi.org/10.1128/JB.01273-06>.
 54. El-Hajj ZW, Reyes-Lamothe R, Newman EB. 2013. Cell division, one-carbon metabolism and methionine synthesis in a *metK*-deficient *Escherichia coli* mutant, and a role for MmuM. *Microbiology* 159:2036–2048. <http://dx.doi.org/10.1099/mic.0.069682-0>.
 55. Nodwell JR. 2007. Novel links between antibiotic resistance and antibiotic production. *J. Bacteriol.* 189:3683–3685. <http://dx.doi.org/10.1128/JB.00356-07>.
 56. Saito N, Kurosawa K, Xu J, Okamoto S, Ochi K. 2003. Effect of S-adenosylmethionine on antibiotic production in *Streptomyces griseus* and *Streptomyces griseoflavus*. *Actinomycetologica* 17:47–49. http://dx.doi.org/10.3209/saj.17_47.
 57. Chiang PK, Gordon RK, Tal J, Zeng GC, Doctor BP, Pardhasaradhi K, McCann PP. 1996. S-Adenosylmethionine and methylation. *FASEB J.* 10:471–480.
 58. Reisenauer A, Kahng LS, McCollum S, Shapiro L. 1999. Bacterial DNA methylation: a cell cycle regulator? *J. Bacteriol.* 181:5135–5139.
 59. Wei Y, Newman EB. 2002. Studies on the role of the *metK* gene product of *Escherichia coli* K-12. *Mol. Microbiol.* 43:1651–1656. <http://dx.doi.org/10.1046/j.1365-2958.2002.02856.x>.
 60. Yocum RR, Perkins JB, Howitt CL, Pero J. 1996. Cloning and characterization of the *metE* gene encoding S-adenosylmethionine synthetase from *Bacillus subtilis*. *J. Bacteriol.* 178:4604–4610.
 61. van der Woude M, Braaten B, Low D. 1996. Epigenetic phase variation of the *pap* operon in *Escherichia coli*. *Trends Microbiol.* 4:5–9. [http://dx.doi.org/10.1016/0966-842X\(96\)81498-3](http://dx.doi.org/10.1016/0966-842X(96)81498-3).
 62. Miller MB, Bassler BL. 2001. Quorum sensing in bacteria. *Annu. Rev. Microbiol.* 55:165–199. <http://dx.doi.org/10.1146/annurev.micro.55.1.165>.
 63. Miller ST, Xavier KB, Campagna SR, Taga ME, Semmelhack MF, Bassler BL, Hughson FM. 2004. *Salmonella typhimurium* recognizes a chemically distinct form of the bacterial quorum-sensing signal AI-2. *Mol. Cell* 15:677–687. <http://dx.doi.org/10.1016/j.molcel.2004.07.020>.
 64. Gonzalez Barrios AF, Zuo R, Hashimoto Y, Yang L, Bentley WE, Wood TK. 2006. Autoinducer 2 controls biofilm formation in *Escherichia coli* through a novel motility quorum-sensing regulator (MqsR, B3022). *J. Bacteriol.* 188:305–316. <http://dx.doi.org/10.1128/JB.188.1.305-316.2006>.
 65. Braeken K, Moris M, Daniels R, Vanderleyden J, Michiels J. 2006. New horizons for (p)ppGpp in bacterial and plant physiology. *Trends Microbiol.* 14:45–54. <http://dx.doi.org/10.1016/j.tim.2005.11.006>.
 66. Krasny L, Gourse RL. 2004. An alternative strategy for bacterial ribosome synthesis: *Bacillus subtilis* rRNA transcription regulation. *EMBO J.* 23: 4473–4483. <http://dx.doi.org/10.1038/sj.emboj.7600423>.
 67. Kasai K, Nishizawa T, Takahashi K, Hosaka T, Aoki H, Ochi K. 2006. Physiological analysis of the stringent response elicited in an extreme thermophilic bacterium, *Thermus thermophilus*. *J. Bacteriol.* 188:7111–7122. <http://dx.doi.org/10.1128/JB.00574-06>.
 68. Ross W, Vrentas CE, Sanchez-Vazquez P, Gaal T, Gourse RL. 2013. The magic spot: a ppGpp binding site on *E. coli* RNA polymerase responsible for regulation of transcription initiation. *Mol. Cell* 50:420–429. <http://dx.doi.org/10.1016/j.molcel.2013.03.021>.
 69. Zuo Y, Wang Y, Steitz TA. 2013. The mechanism of *E. coli* RNA polymerase regulation by ppGpp is suggested by the structure of their complex. *Mol. Cell* 50:430–436. <http://dx.doi.org/10.1016/j.molcel.2013.03.020>.
 70. Kriel A, Bittner AN, Kim SH, Liu K, Tehranchi AK, Zou WY, Rendon S, Chen R, Tu BP, Wang JD. 2012. Direct regulation of GTP homeostasis by (p)ppGpp: a critical component of viability and stress resistance. *Mol. Cell* 48:231–241. <http://dx.doi.org/10.1016/j.molcel.2012.08.009>.
 71. Kriel A, Brinsmade SR, Tse JL, Tehranchi AK, Bittner AN, Sonenshein AL, Wang JD. 2014. GTP dysregulation in *Bacillus subtilis* cell lacking (p)ppGpp results in phenotypic amino acid auxotrophy and failure to adapt to nutrient downshift and regulate biosynthesis genes. *J. Bacteriol.* 196:189–201. <http://dx.doi.org/10.1128/JB.00918-13>.
 72. Belitsky BR, Sonenshein AL. 2013. Genome-wide identification of *Bacillus subtilis* CodY-binding sites at single-nucleotide resolution. *Proc. Natl. Acad. Sci. U. S. A.* 110:7026–7031. <http://dx.doi.org/10.1073/pnas.1300428110>.
 73. Bentley SD, Chater KF, Cerdeno-Tarraga AM, Challis GL, Thomson NR, James KD, Harris DE, Quail MA, Kieser H, Harper D, Bateman A, Brown S, Chandra G, Chen CW, Collins M, Cronin A, Fraser A, Goble A, Hidalgo J, Hornsby T, Howarth S, Huang CH, Kieser T, Larke L, Murphy L, Oliver K, O'Neil S, Rabinowitsch E, Rajandream MA, Rutherford K, Rutter S, Seeger K, Saunders D, Sharp S, Squares R, Squares S, Taylor K, Warren T, Wietzorrek A, Woodward J, Barrell BG, Parkhill J, Hopwood DA. 2002. Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). *Nature* 417:141–147. <http://dx.doi.org/10.1038/417141a>.
 74. Goldman BS, Nierman WC, Kaiser D, Slater SC, Durkin AS, Eisen JA, Ronning CM, Barbazuk WB, Blanchard M, Field C, Halling C, Hinkle G, Iartchuk O, Kim HS, Mackenzie C, Madupu R, Miller N, Shvartsbeyn A, Sullivan SA, Vaudin M, Wiegand R, Kaplan HB. 2006. Evolution of sensory complexity recorded in a myxobacterial genome. *Proc. Natl. Acad. Sci. U. S. A.* 103:15200–15205. <http://dx.doi.org/10.1073/pnas.0607335103>.
 75. Hopwood DA. 9 June 2008. The tip of the iceberg. American Society for Microbiology. <http://schaechter.asmblog.org/schaechter/2008/06/the-tip-of-the.html>.
 76. Zhu H, Sandiford SK, van Wezel GP. 2014. Triggers and cues that activate antibiotic production by actinomycetes. *J. Ind. Microbiol. Biotechnol.* 41:371–386. <http://dx.doi.org/10.1007/s10295-013-1309-z>.
 77. Tanaka Y, Kasahara K, Hirose Y, Murakami K, Kugimiya R, Ochi K. 2013. Activation and products of the cryptic secondary metabolite biosynthetic gene clusters by rifampin resistance (*rpoB*) mutations in actinomycetes. *J. Bacteriol.* 195:2959–2970. <http://dx.doi.org/10.1128/JB.00147-13>.