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NOTES

Identification of the RsmG Methyltransferase Target as 16S rRNA Nucleotide G527 and Characterization of *Bacillus subtilis rsmG* Mutants▽

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The methyltransferase RsmG methylates the N7 position of nucleotide G535 in 16S rRNA of *Bacillus subtilis* (corresponding to G527 in *Escherichia coli*). Disruption of *rsmG* resulted in low-level resistance to streptomycin. A growth competition assay revealed that there are no differences in fitness between the *rsmG* mutant and parent strains under the various culture conditions examined. *B. subtilis rsmG* mutants emerged spontaneously at a relatively high frequency, 10^{-6}. Importantly, in the *rsmG* mutant background, high-level-streptomycin-resistant *rpsL* (encoding ribosomal protein S12) mutants emerged at a frequency 200 times greater than that seen for the wild-type strain. This elevated frequency in the emergence of high-level streptomycin resistance was facilitated by a mutation pattern in *rpsL* more varied than that obtained by selection of the wild-type strain.

Mutants resistant to streptomycin (Sm) can be classified into two distinct *str* phenotypes depending on whether they exhibit high- or low-level Sm resistance. The *str* mutations conferring high-level Sm resistance have been known for several decades to occur within *rpsL*, which encodes the ribosomal protein S12 (for reviews, see references 9 and 19). The mutations causing low-level resistance have been characterized only recently (22, 25). By use of comparative genome sequencing, we determined that low-level resistance in *Streptomyces coelicolor* is caused by mutations in *rsmG* (*r*RNA *s*mall subunit *m*ethyltransferase *G*), which encodes an *S*-adenosylmethionine (SAM)-dependent 16S rRNA methyltransferase (22). Analysis of the 16S rRNA by high-performance liquid chromatography (HPLC) showed that the Δ*rsmG* mutant lacked a 7-methylguanosine (m^7G) modification. As the only naturally occurring m^7G in 16S rRNA is at position G527 (*Escherichia coli* numbering system), this was assumed to be the site of RsmG methylation. Similar observations were made for *rsmG*-inactivated mutants of *Escherichia coli*, *Staphylococcus aureus*, *Mycobacterium tuberculosis*, and *Mycobacterium smegmatis* and led us to conclude that loss of a conserved m^7G modification in 16S rRNA confers low-level Sm resistance in bacteria (25). In clinical isolates of *M. tuberculosis*, mutations within the *rsmG* gene were indeed an important cause of Sm resistance (25). In addition to conferring low-level Sm resistance, the *rsmG* mutation in *S. coelicolor* also led to the overproduction of antibiotics and enhanced expression of the SAM synthetase gene (22, 24).

Bacterial rRNAs have many methylated nucleotides. In *E. coli*, for example, there are 10 methylations in 16S rRNA and 14 methylations in 23S rRNA (1). Although the collective importance of these rRNA modifications for protein synthesis has been demonstrated (10, 17, 18), the function of individual methylations is still unclear, since inactivation of the genes encoding their cognate methyltransferases does not affect the cell's viability (1, 3, 20). To study the function of RsmG further, we have chosen *Bacillus subtilis* strain 168; genomic information and numerous tools for genetic, biochemical, and physiological analyses are available for this well-characterized system (7, 27). In the present study, we determined the precise location of the methylation target of *B. subtilis* RsmG, and we report here the physiological effects of inactivating *rsmG* in this species.

Strain construction. The coding region of the *rsmG* gene was disrupted by insertion of a neomycin resistance (*neo*) gene. First, a DNA fragment containing *rsmG* (914 bp) was amplified by PCR using primers *rsmG*-F (5′-GTGAAATATGAAGGATATATTG-3′) and *rsmG*-R (5′-GTATCACCATAATATTACGATC-3′) and was cloned into plasmid pCR2.1 (Invitrogen) to form pCR2.1-*rsmG*. A 1.3-kbp SmaI fragment of *neo* derived from pBEST501 (16) was inserted into the HincII site of pCR2.1-*rsmG*. The resulting plasmid, pCR2.1-*rsmG*::*neo*, was linearized with KpnI and was used to transform *B. subtilis* 168. Neomycin-resistant transformants were selected on LB agar plates (with 3 μg/ml neomycin), and one recombinant, KO-756, was used for further study.

Strains with disrupted *rsmG* were complemented with an active copy of *rsmG* using plasmid pAPNC213 (21). This vector integrates specifically into the *aprE* locus and allows regulated expression of the target gene from the isopropyl-β-D-thio-

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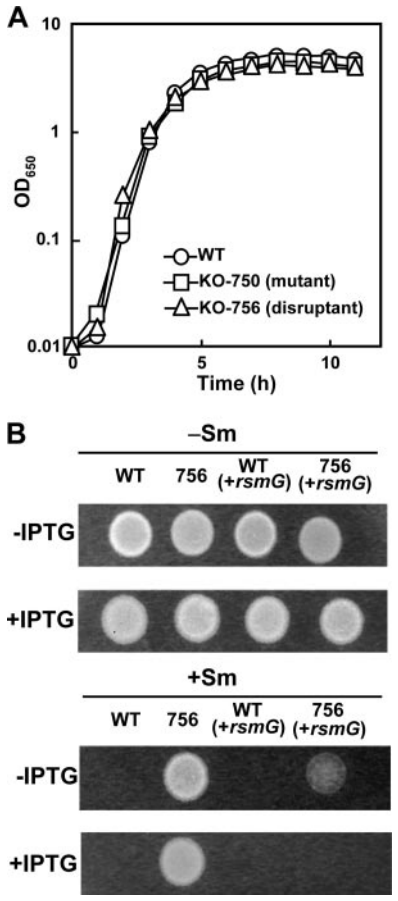

FIG. 1. Growth and susceptibility to Sm of the *B. subtilis* wild-type (WT), *rsmG* frameshift (KO-750), and *rsmG* disrupted (KO-756) strains. (A) Strains were grown in LB medium at 37°C with shaking. (B) Wild-type and KO-756 (756) cells were grown to stationary phase, and 5-μl samples were spotted onto LB medium plates (with or without 30 μg/ml Sm and with or without 2 mM IPTG) and then incubated at 37°C for 12 h. OD_{650}, optical density at 650 nm.

galactopyranoside (IPTG)-inducible *spac* promoter. The full length of the coding region for *rsmG* was amplified by PCR using primers rsmGcom-F (5′-GAGGATCCCCGAGTAGAAAGGATGACGGC-3′; BamHI site underlined) and rsmGcom-R (5′-CATCCCGGGATTTTGATGAAAATATGATG-3′; SmaI site underlined). A BamHI-SmaI fragment containing the *rsmG* gene was inserted into pAPNC213 that had been treated with the same enzymes, generating pAPNCrsmG. The resulting plasmid, pAPNCrsmG, was used to transform the *B. subtilis rsmG* disruptant KO-756. Transformants were selected for plasmid-encoded resistance using 100 μg/ml spectinomycin; one of the spectinomycin-resistant transformants was used for complementation testing of *rsmG*.

Disruption of *rsmG* results in low-level Sm resistance in *B. subtilis*. *rsmG* mutations have previously been shown to cause low-level Sm resistance in *E. coli*, *M. tuberculosis*, and *S. coelicolor* (22, 25). Similarly, disruption of *rsmG* in *B. subtilis* KO-756 (*rsmG*::*neo*) caused increased resistance to Sm (up to 100 μg/ml in LB medium). This resistance phenotype was eliminated by introduction of an active *rsmG* gene into KO-756 followed by induction with IPTG (Fig. 1B). In this complemented strain, the Sm MIC returned to the level for the wild-type strain (10 μg/ml in LB medium), unambiguously demonstrating a causal relationship between loss of *rsmG* activity and acquisition of low-level Sm resistance. We noted that disruption of *rsmG* conferred no resistance to any of the other antibiotics that we tested, including kanamycin, kasugamycin, spectinomycin, gentamicin, thiostrepton, lincomycin, chloramphenicol, erythromycin, and fusidic acid (the MICs in LB medium were 2, 1,500, 30, 0.3, 0.01, 10, 1, 0.05, and 0.02 μg/ml, respectively).

Although mutations that confer drug resistance often have a biological cost causing mutant bacteria to grow more slowly (2), the *rsmG* mutant KO-756 (and KO-750 [see Table 2]) grew as well as parent strain 168, both in LB medium (Fig. 1A) and in other media (not shown), and this result is consistent with earlier studies on *E. coli* and *S. coelicolor* (22, 25). Moreover, growth of the *rsmG* frameshift mutant KO-750 together with wild-type strain 168 in a competition assay (11) revealed no differences in the relative cell numbers (after five cycles of cultivation with reinoculation every 24 h or after 10 cycles of cultivation with reinoculation every 12 h) in various media, including LB medium (data not shown). Nor were there were differences in *rsmG* mutant or wild-type growth when the competition assay was performed using sterilized soil with four cycles of cultivation with reinoculation every 2 weeks. Finally, no differences between the parent and *rsmG* mutant strains were detected with respect to sensitivity of growth to high (55°C) or low (10°C) temperatures. These growth experiments demonstrate that the *rsmG* mutants are as fit as the wild-type strain under the various culture conditions tested. This finding contrasts with previous work on several other 16S rRNA methylases (1, 3, 20), which showed that knockout mutants were less fit than the wild-type strain.

We previously reported that mutation of *rsmG* in *E. coli* did not lead to higher levels of accuracy in translation, and this contrasts with the results for most of the high-level-Sm-resistance mutations, such as those in *rpsL*, that have been characterized in *E. coli*. This was interpreted to indicate that *E. coli* ribosomes lacking RsmG methylation might have a reduced affinity for Sm (25). However, in the case of the *rsmG* mutations of *B. subtilis*, a detectable increase in translational accu-

TABLE 1. Characterization of translation in mutant ribosomes in vivo

Strain	In vivo readthrough induction ratios for Glu-105 codon of LacI[a]	
	GAA (native)	UGA
168 (wild type)	34 ± 9.9	6.0 ± 2.7
WL2 (*rpsL* [K56N])	33 ± 8.9	0.99 ± 0.13
KO-750 (*rsmG*)	33 ± 10	2.2 ± 0.19
KO-756 (*rsmG*::*neo*)	27 ± 4.1	2.2 ± 0.36

[a] The level of translational accuracy was estimated in vivo in a UGA readthrough system that measures the regulation of *lacZ* by LacI (13). Briefly, codon 105 of *lacI* was replaced by an opal codon (UGA); readthrough of the opal codon is required to generate the full-length LacI protein, which would then repress the expression of *lacZ*. For measurement of UGA readthrough, cells were grown to an optical density at 650 nm of 0.5 in Spizizen's salts minimum medium in the presence of IPTG (10 mM) or in the absence of IPTG prior to measurement of β-galactosidase activity (13). The UGA readthrough levels were expressed as the induction ratio (the β-galactosidase activity of the culture supplemented with IPTG divided by the activity of the culture without IPTG).

racy was indeed observed in a readthrough induction assay, although the accuracy was not increased as much as that observed for an *rpsL* (K56N) mutant (Table 1).

RsmG methylates the N7 position of G527 in 16S rRNA. We recently showed that *E. coli* RsmG catalyzes a SAM-dependent m^7G modification in *E. coli* 16S rRNA (25). This observation was confirmed here for *B. subtilis* using reversed-phase HPLC analysis of the rRNA nucleosides, which showed that RsmG also catalyzes an m^7G modification within 16S rRNA (data not shown). Since only one m^7G modification, at G527, is found in the 16S rRNA of *E. coli*, these findings suggest that RsmG modifies the same position in *B. subtilis* (nucleotide G535 in the *B. subtilis* sequence). We tested this possibility here by determining the exact target site of the RsmG methyltransferase.

Total RNA was isolated as previously described (6) from the *B. subtilis* wild-type strain, the *rsmG* frameshift mutant KO-750, and the mutant strain complemented with an active *rsmG* gene. The RNAs were cleaved at N7-methylguanosine positions by reduction with $NaBH_4$, followed by β-elimination with acetic acid-aniline (26, 33); a tRNA carrier, hypermodified at N7 of guanosines by dimethyl sulfate treatment, was added to enhance cleavage at the N7-methylated guanosines in the rRNA (34). The rRNAs were scanned using a series of primers by reverse transcriptase extension (32). The only difference seen was in the termination of reverse transcriptase immediately upstream of nucleotide G527 (G535 in *B. subtilis*) on the 16S rRNA templates (Fig. 2). The band formed in the wild-type sample indicates that there was N7 methylation at this guanosine; there was no such termination after loss of RsmG activity in the *rsmG* mutant KO-750, although the N7-methylation signal returned in the strain complemented with an active *rsmG* gene (Fig. 2). In combination, the HPLC and primer extension data conclusively demonstrate that RsmG is responsible for N7 methylation at position G527 in 16S rRNA.

Nucleotide position 527 is invariably a guanosine in bacteria, and database searches of the available genome sequences further revealed that all bacteria have a homologue of *rsmG*. The identity of this nucleotide and its methylation are thus highly conserved and can be inferred to be functionally important. Nucleotide G527 is situated within a hairpin loop (the so-called 530 loop) that is one of the most highly conserved features of 16S rRNA, and mutations in this loop have been associated with resistance to Sm (29). This region of 16S rRNA is situated close to the ribosomal protein S12, and both of these ribosomal components play a major role in translational fidelity (4, 5, 23, 28, 29, 31). The previous studies coincide with the findings obtained here showing that loss of G527 methylation confers Sm resistance.

Emergence of high-level Sm resistance in *B. subtilis rsmG* mutants. Spontaneous mutations that lead to high-level Sm resistance (an approximately 100-fold increase in the MIC) generally emerge at a low frequency in bacteria (10^{-11} to 10^{-9}), with the majority of these mutations occurring within *rpsL* (8). Consistently, in *B. subtilis* wild-type strain 168 (MIC in LB medium, 10 μg/ml), spontaneous mutants conferring high-level Sm resistance (MIC, at least 1,000 μg/ml) arose at a low frequency, between 2×10^{-11} and 8×10^{-10}. In contrast, mutants with low-level Sm resistance (MIC, 100 μg/ml) emerged at a much higher frequency, in the range from 3×10^{-7} to 4×10^{-6}. Most of these mutants (19 out of 21) contained changes in *rsmG*, and these changes were in many cases frameshift mutations that resulted in a stop codon immediately downstream of the mutation site (Table 2). Strikingly, but consistent with previous observations for *E. coli* and *S. coelicolor* (22, 25), the *B. subtilis rsmG* mutants (and the *rsmG* disruption mutant KO-756) produced spontaneous mutants showing resistance to a high level of Sm (5,000 μg/ml) at a frequency on the order of 10^{-6} or 10^{-7}. The data for *rsmG* mutant KO-750 are shown in Table 3 and show that there was a 500- to 2,000-fold-greater frequency of mutation to high-level Sm resistance than that observed for the wild-type strain. About one-third (68 out of 190) of the high-level-Sm-resistant *rsmG* mutants were found to have a mutation in *rpsL* (Table 3), but a majority (122 out of 190) had no mutation in either the *rpsL*, *rpsD* (encoding ribosomal protein S4), or *rpsE* (encoding

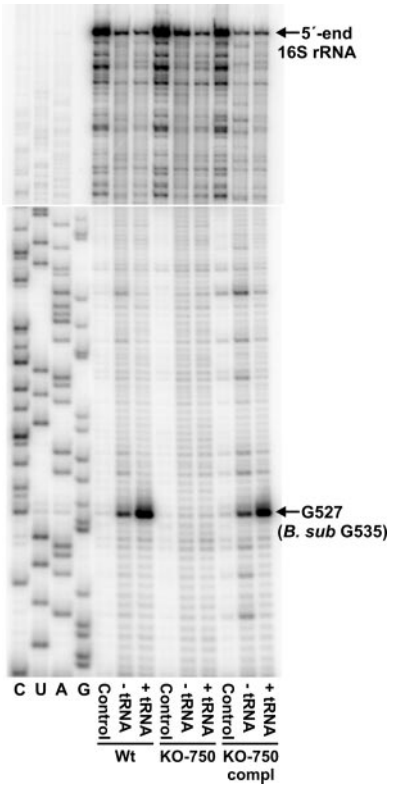

FIG. 2. Identification of the site of RsmG methylation: gel autoradiogram of primer extension of the rRNAs from *B. subtilis* wild-type strain 168 (Wt), *rsmG* frameshift mutant KO-750, and the mutant strain complemented with an active copy of *rsmG* (KO-750 compl). The oligodeoxynucleotide primer 5′-CCTGCGAGCCCTTTACGCC-3′ was hybridized to 16S rRNA nucleotides 567 to 585 (*E. coli* numbering) and extended with reverse transcriptase. Control, untreated samples; − tRNA, the rRNAs were treated with $NaBH_4$ and aniline to break the phosphodiester backbone at m^7G methylation sites (26, 33); + tRNA, rRNAs were treated with $NaBH_4$ and aniline after addition of dimethyl sulfate-modified tRNA to enhance cleavage at m^7G methylation sites (34). The position of the RsmG methylation site at nucleotide G527 (corresponding to G535 in the *B. subtilis* 16S rRNA sequence) is indicated. Wild-type rRNA was used as the template for the dideoxy sequencing reactions (lanes C, U, A, and G). The top panel shows the upper part of the gel autoradiogram extending to the 5′ end of the rRNA.

TABLE 2. Locations and identities of the mutations in the *rpsL* and *rsmG* genes

Strain	Sm concn (μg/ml) used for selection	Position of mutation in *rpsL* or *rsmG*[a]	Amino acid substitution	Resistance to Sm (μg/ml)[b]
Wild-type 168		—[c]		10
		rpsL		
KO-670	1,000	167A→G	Lys56→Arg	3,000
KO-671	1,000	167A→C	Lys56→Thr	5,000
KO-672	1,000	166A→C	Lys56→Gln	5,000
		rsmG		
KO-673	50	186,187TT→A	Frameshift (Val70→stop codon)	100
KO-674	50	499G-541C→Δ	Frameshift (Gly167→stop codon)	100
KO-675	50	326T→A	Frameshift (Leu109→stop codon)	100
KO-676	50	242G→T	Gly81→Val	100
KO-677	50	574G→Δ	Frameshift (Val214→stop codon)	100
KO-678	50	231 (AGCGGG) insertion	Gly-Ala insertion at position 77	100
KO-679	50	313C→T	Arg105→Trp	100
KO-680	50	125C→A	Thr42→Asn	100
KO-681	50	360T→TT	Frameshift (Asp126→stop codon)	100
KO-682	50	231 (AGCGGG) insertion	Gly-Ala insertion at position 77	100
KO-683	50	558A→Δ	Frameshift (Val214→stop codon)	100
KO-684	50	659A→Δ	Frameshift	100
KO-685	50	231A-236G→Δ	Ala78, Gly79→Δ	100
KO-707	50	558A→Δ	Frameshift (Val214→stop codon)	100
KO-708	50	310A→AAA	Frameshift (Glu110→stop codon)	100
KO-709	50	231A-236G→Δ	Ala78, Gly79→Δ	100
KO-710	50	606G-644T→Δ	Leu203-Ile215→Δ	100
KO-711	50	233C→A	Ala78→Glu	100
KO-712	50	528G-708A→Δ	Ala177-Pro236→Δ	100
KO-713	50	ND[d]		100
KO-714	50	ND		100
KO-750[e]	50			100
KO-756[f]				100

[a] Numbering from the start codon (ATG) of the open reading frame.
[b] Determined 16 h after incubation on LB agar at 37°C.
[c] Wild-type *rpsL* and *rsmG* genes.
[d] ND, mutations were not detected within the *rsmG* gene.
[e] KO-750 is the result of replacing the *rsmG* gene in strain 168 with the mutant copy from KO-673 by transformation.
[f] Strain KO-756 is an *rsmG* disruptant (*rsmG*::*neo*) of strain 168. Selection was carried out with 100 μg/ml spectinomycin.

ribosomal protein S5) gene. Importantly, the *rsmG rpsL* double mutants displayed a pattern of *rpsL* mutations more varied than the patterns derived directly by selection of the wild-type strain. For example, the mutations Lys101→Glu, Pro104→Thr, and Pro104→Arg are mutations that are novel or are found only rarely in high-level-Sm-resistant isolates. From a clinical microbiology viewpoint, the increased frequency and variety of these high-level-Sm-resistance mutations in the *rsmG* strains are significant; however, the underlying physiological mechanism by which they occur remains to be clarified. We can rule out the possibility that RsmG functions as an antimutator-like protein, since the *rsmG* mutation in *E. coli* does not affect the frequency at which mutants resistant to antibiotics other than Sm emerge (25).

In *S. coelicolor*, *rsmG* mutations conferring low-level Sm resistance result in overproduction of the antibiotic actinorhodin (22, 24, 30). The *S. coelicolor rsmG* mutants exhibit enhanced expression of SAM synthetase, accompanied by increased protein synthesis activity at late growth phase, which eventually leads to overproduction of antibiotics (actinorhodin, undecylprodigiosin, and calcium-dependent antibiotics) (22). It is believed that the increases in SAM synthetase activity and protein synthesis activity caused by the *rsmG* mutation are both linked to the activation of secondary metabolism. In the *B. subtilis rsmG* frameshift mutant KO-750, however, there was no increase in production of either bacilysin or neotrehalosadiamine (data not shown), which are antibiotics that this organism produces at late growth phase (14, 15). Consistent with these results, KO-750 showed neither an increase in protein synthesis at late growth phase nor an increase in SAM synthetase activity (data not shown). Thus, in contrast to the situation in *Streptomyces*, secondary metabolism in *B. subtilis* is not activated by mutation of *rsmG*. Furthermore, the *rsmG* mutation did not affect sporulation, competence, or protease production, at least under usual culture conditions (data not shown), which again contrasts in part with the reduced ability to sporulate that was exhibited by the *S. coelicolor rsmG* mutants (22).

Concluding remarks. In the present study we determined the exact location of the rRNA methylation target for RsmG and thereby further clarified one molecular mechanism underlying low-level Sm resistance. Sm is still an important drug for the treatment of tuberculosis, and our findings provide new insight into the role of rRNA modification in the acquisition of antibiotic resistance. The phylogenetic conservation of RsmG and of the 16S rRNA sequence in the 530 loop suggests that methylation at this rRNA site should confer some selective advantage. Nevertheless, the apparent lack of a disadvantage

TABLE 3. Effect of *rsmG* mutation on the emergence of high-level-Sm-resistant mutants[a]

Strain	Frequency of high-level-Sm-resistant mutants[b]	Position of mutation detected in *rpsL* gene	Amino acid exchange	No. of mutants
Wild-type 168	3×10^{-10}–4×10^{-9}	166A→C	Lys56→Gln	13
		167A→C	Lys56→Thr	10
		167A→G	Lys56→Arg	15
		167A→T	Lys56→Ile	9
		168A→C	Lys56→Asn	14
		311C→T	Pro104→Leu	15
Total				76
KO-750 (*rsmG*)	5×10^{-7}–2×10^{-6}	166A→C	Lys56→Gln	10
		167A→C	Lys56→Thr	5
		167A→G	Lys56→Arg	9
		167A→T	Lys56→Ile	2
		168A→C	Lys56→Asn	5
		168A→T	Lys56→Asn	2
		301A→G	Lys101→Glu	2
		310C→A	Pro104→Thr	1
		311C→T	Pro104→Leu	12
		311C→A	Pro104→Gln	6
		311C→G	Pro104→Arg[c]	1
		313G→A	Gly105→Arg	12
		313G→C	Gly105→Arg	1
Total				68

[a] Selection for high-level resistance was performed with Sm at 5,000 μg/ml (for the *rsmG* mutant) or at 3,000 μg/ml (for the wild type).

[b] To measure the frequency of resistant mutants, single colonies were isolated, and cells originating from each of about 10 clones were examined separately.

[c] The mutant with this mutation required 1,000 μg/ml of Sm for growth.

in cells that no longer can methylate the G527 position clearly prompts a question concerning the biological importance of this modification.

Concerning secondary metabolite production, we previously reported that certain *B. subtilis* mutants possess low-level Sm resistance and exhibit a 10- to 50-fold increase in antibiotic production (12). These mutants have been reevaluated, and consistent with the findings presented here, none of these strains had a mutation in the *rsmG* gene. This indicates that another type of mechanism, fundamentally different from that involving *rsmG* mutation, can be acquired by *B. subtilis* to confer low-level Sm resistance. In contrast to what happens in the *rsmG* mutants, acquisition of low-level resistance to Sm by this unidentified mechanism may be linked with activation of secondary metabolism.

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