JOURNAL OF BACTERIOLOGY, Nov. 1996, p. 6361–6365 Vol. 178, No. 21
0021-9193/96/$04.00+0

Identification and Characterization of Transcripts from the Biotin Biosynthetic Operon of *Bacillus subtilis*

JOHN B. PERKINS,* STANLEY BOWER, C. LINDA HOWITT,
R. ROGERS YOCUM, AND JANICE PERO

OmniGene Bioproducts, Inc., Cambridge, Massachusetts 02138

Received 31 May 1996/Accepted 14 August 1996

Northern (RNA) blot analysis of the *Bacillus subtilis* biotin operon, *bioWAFDBIorf2*, detected at least two steady-state polycistronic transcripts initiated from a putative vegetative (P_{bio}) promoter that precedes the operon, i.e., a full-length 7.2-kb transcript covering the entire operon and a more abundant 5.1-kb transcript covering just the first five genes of the operon. Biotin and the *B. subtilis birA* gene product regulated synthesis of the transcripts. Moreover, replacing the putative P_{bio} promoter and regulatory sequence with a constitutive SP01 phage promoter resulted in higher-level constitutive synthesis. Removal of a *rho*-independent terminator-like sequence located between the fifth (*bioB*) and sixth (*bioI*) genes prevented accumulation of the 5.1-kb transcript, suggesting that the putative terminator functions to limit expression of *bioI*, which is thought to be involved in an early step in biotin synthesis.

Biotin biosynthesis in *Escherichia coli* is regulated at the level of transcription by a classical repressor-operator mechanism (5, 10, 12). The repressor is encoded by the *birA* gene, and when complexed with its corepressor, biotinoyl-5′-AMP, BirA binds to an operator that overlaps the promoters for divergent *bioA* and *bioBCDF* operons and represses transcription from both promoters (reviewed in reference 10). Binding is cooperative and involves two holorepressor monomers binding to the two palindromic half sites of the operator (1). Transcription of the *bio* genes is controlled from this bidirectional promoter-operator sequence, and the detection of additional, internal promoters has not been reported. This regulatory model has been confirmed by several methods, including isolation of mutations in the operator region or the *birA* gene that deregulated biotin production (4, 5, 9, 15, 17, 21), DNA binding studies of purified BirA to the *E. coli bio* operon, and DNA protection (5, 11, 23).

In gram-positive bacteria, biotin synthesis has been studied extensively in two species, *Bacillus sphaericus* (14, 16, 22) and, more recently, *Bacillus subtilis* (8, 6, 7). In *B. sphaericus*, the genes are located in two separate operons, *bioXWF* and *bioDAYB*. Characterization of the regulatory apparatus controlling expression of these genes included the isolation of constitutive biotin-producing mutations that map either to a conserved 15-bp inverted repeat preceding the two operons (i.e., possible operator mutants) or to another site(s) on the chromosome unlinked to either operon (25). Recently, our research group has cloned and sequenced the biotin biosynthetic genes of *B. subtilis* (7, 8). *B. subtilis* contains at least six *bio* genes that are organized in a single operon, *bioWAFDB Iorf2*; a seventh open reading frame (*orf2*) of unknown function is located at the end of the *bio* operon. Four of the genes, *bioA*, *-B*, *-D*, and *-F*, show strong similarity to genes of the same name from *B. sphaericus* and *E. coli*, and *bioW* shows strong similarity to *bioW* of *B. sphaericus*. The *bioI* gene encodes a cytochrome P-450-like enzyme that appears to be involved in pimelic acid synthesis, an early intermediate in biotin synthesis, and *orf2* is not essential for biotin biosynthesis (7). On the basis of sequencing data, transcription of these genes appeared to be controlled by a putative vegetative (σ^A) promoter sequence (P_{bio}) at the beginning of the operon and a regulatory sequence adjacent to the promoter which shows similarity to the regulatory regions of the *B. sphaericus* and *E. coli bio* operons. *B. subtilis* also has a *birA* homolog that is not linked to the *bio* operon. Point mutations in *birA* deregulated biotin production (6).

Here we report on the identification of *B. subtilis bio*-specific transcripts by Northern (RNA) blot hybridization and the identification of sites in the *bio* operon that regulate these transcripts.

Identification of *bio*-specific RNA transcripts. On the basis of the DNA nucleotide sequence of the cloned biotin operon of *B. subtilis*, *bioWAFDBIorf2*, transcription of the seven genes is believed to be initiated from a single putative vegetative (σ^A) promoter sequence, P_{bio} (TTGACA-N_{17}-TATATT), about 84 bp upstream from the first structural gene of the operon, *bioW* (Fig. 1) (7). A potential transcription regulatory site with dyad symmetry was also identified immediately downstream from the putative promoter by homology to the regulatory sites (operators) of the *B. sphaericus* and *E. coli bio* operons. No additional sites that showed significant homology to a consensus vegetative promoter sequence or *bio* regulatory sequence were detected in the remainder of the *bio* operon.

To identify *bio*-specific RNA transcripts, *B. subtilis* prototroph PY79 (28) was grown in Spizizen's minimal salts medium (26) containing 0.04% Na glutamate in the presence or absence of biotin (100 μg/liter) to the early logarithmic stage. Total cellular RNA was isolated by disrupting cells in a mixture of glass beads (0.1 mm), phenol-isoamyl alcohol-chloroform, sodium dodecyl sulfate, and macaloid clay with a bead beater (Biospec Products) as described by Azevedo et al. (2) and analyzed by Northern blot hybridization (19). By using a probe homologous to the 5′ end of *bioW*, three steady-state transcripts of 7.2, 5.1, and 0.8 kb were detected in RNA from cells grown without biotin (Fig. 2, lane 1). The relative abundance of these transcripts was estimated to be 1 (7.2 kb) to 8 (5.1 kb) to 2 to 4 (0.8 kb). None of these three transcripts was detected in RNA from cells grown in the presence of biotin (lane 2), even when the filter was overexposed, suggesting that synthesis

* Corresponding author. Mailing address: OmniGene Bioproducts, Inc., 763D Concord Ave., Cambridge, MA 02138. Phone: (617) 576-1966, ext. 248. Fax: (617) 547-9256.

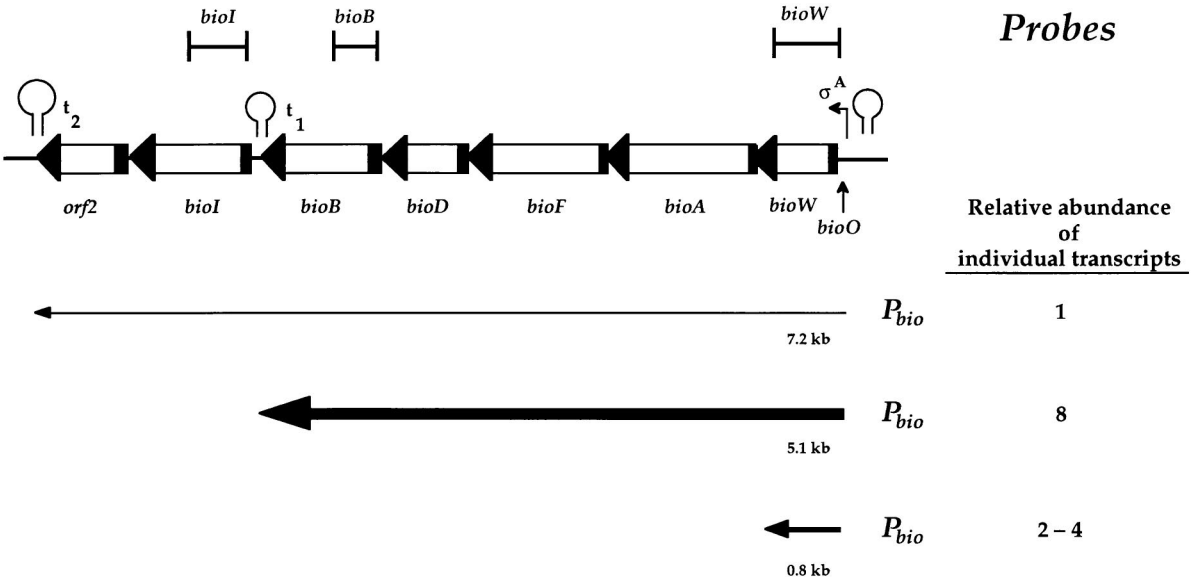

FIG. 1. Physical map of the *B. subtilis bio* operon and flanking DNA. The locations of *bio* structural genes, putative promoter and regulatory region, and transcription termination sites are shown in the upper diagram (7). Alignment of the *bio*-specific transcripts detected by Northern blotting shown in Fig. 2 and 3 is given below the diagram; the relative steady-state abundance of individual transcripts is shown on the right and represented by the thickness of the arrows. Probes used to detect *bio*-specific transcripts are indicated above the diagram. Symbols: ▭, open reading frames; ▮, *Bacillus* ribosome binding sites; ♌, putative *rho*-independent transcription termination sites $biot_1$ (t_1) and $biot_2$ (t_2); ↰, possible σ^A-recognized P_{bio} promoter; *bioO*, putative regulatory region.

of these three transcripts was tightly regulated by biotin. Repeating the hybridization with an independent set of RNA preparations resulted in identical results (Fig. 3, lanes 1 and 2). In addition to the specific transcripts shown in Fig. 2 and 3, the probe hybridized nonspecifically to the 16S and 23S RNA. This nonspecific hybridization was reproducible, and these background bands were a reliable indicator of RNA loading differences. To determine if the *B. subtilis birA* gene product controlled synthesis of these transcripts, the hybridization was repeated with total RNA from a *birA* mutant, BI421, which is deregulated for biotin production (6). As shown in Fig. 3, transcripts from BI421 (lanes 3) were the same lengths and in the same proportions as those from PY79 (lane 1). Moreover, BI421 showed no significant difference in the transcripts

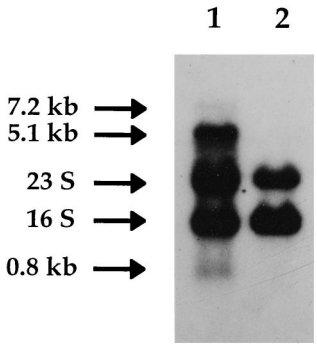

FIG. 2. Biotin-regulated synthesis of *bio*-specific transcripts. Total RNA was isolated from strains grown in minimal medium in the presence or absence of 100 μg of biotin per liter. Five micrograms of RNA was separated on a 1.2% agarose–formaldehyde gel and transferred to a nitrocellulose membrane filter, and the filter was incubated with PCR-synthesized *bioW* DNA labeled with [^{32}P]dCTP. Lanes: 1, PY79, no biotin; 2, PY79 with biotin. Unlabeled RNA markers, ranging from 0.36 to 9.49 kb (Promega), were used to estimate mRNA size. In addition to the specific transcripts shown, the probe hybridized nonspecifically to the 16S and 23S RNA as indicated in the photograph.

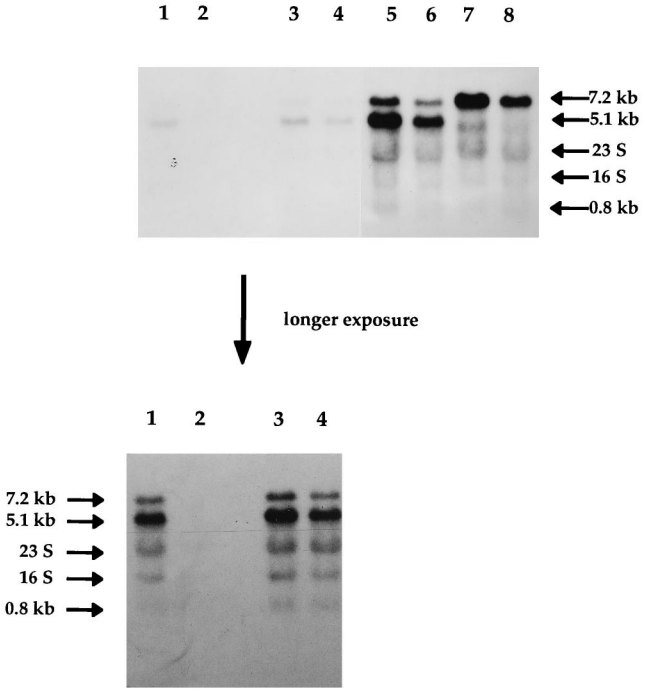

FIG. 3. Deregulated synthesis of *bio*-specific transcripts. RNA isolation and hybridization conditions are described in the legend to Fig. 2. Lanes were added with RNA as indicated: 1, PY79, no biotin, 5 μg; 2, PY79 with biotin, 1.5 μg; 3, BI421 (*birA*), no biotin, 5 μg; 4, BI421 (*birA*), with biotin, 5 μg; 5, BI294 (P_{15}*bio*), no biotin, 5 μg; 6, BI294 (P_{15}*bio*), with biotin, 5 μg; 7, BI303 [P_{15}*bioWAFDB*(Δt_1)*Iorf2*], no biotin, 5 μg; 8, BI303 [P_{15}*bioWAFDB*(Δt_1)*Iorf2*], with biotin, 5 μg. The exposure times were 20 min for lanes 1 to 8 shown at the top of the figure and 60 min for lanes 1 to 4 shown at the bottom.

present in cells grown in the absence (lane 3) or presence (lane 4) of 100 μg of biotin per liter. Similar results were also obtained with a second independent *birA* mutant, HB3 (6) (data not shown). This constitutive level of transcription is consistent with the role of *birA* as a negative regulator of biotin transcription in *B. subtilis*.

On the basis of their ability to hybridize to a *bioW* probe, these transcripts all appeared to be initiated from the putative P_{bio} promoter and terminated at different sites within the *bio* operon. Since the largest transcript coincided with the size of the biotin operon, it appeared to represent the full-length *bio* message, ending at or near the potential *rho*-independent transcription termination site ($biot_2$) downstream from *orf2*. The 5.1-kb transcript appeared to comprise the first five biotin genes, *bioWAFDB*, ending at or near an internal transcription termination-like site between *bioB* and *bioI* ($biot_1$). The third transcript, 0.8 kb, appeared to end within the 3′ end of *bioW*; this region of the operon does not contain structures conforming to *rho*-independent transcription terminators. This alignment of transcripts to the biotin operon is diagrammed in Fig. 1 and was confirmed by repeating the hybridization with total RNA from PY79 grown without biotin and probes to the *bioB* and *bioI* genes (data not shown). The 5.1-kb transcript hybridized to a *bioB* probe but not to a *bioI* probe. The 7.2-kb transcript, however, hybridized to both probes, whereas the 0.8-kb transcript annealed to neither probe. In addition, the 0.8-kb transcript was not detected in a strain containing an in-frame deletion mutation of the *bioW* gene (data not shown).

Replacement of P_{bio} with a phage SP01 promoter. If the three *bio*-specific transcripts described above originated solely from the P_{bio} promoter, then replacing the native promoter-regulatory region with a stronger and constitutive promoter should result in increased and deregulated synthesis of the same three *bio*-specific transcripts. To test this possibility, the P_{bio} promoter and regulatory site were replaced in vivo with the constitutive phage SP01 promoter from the *Eco*RI* fragment 15 (SP01-15 [18]), and *bio*-specific transcripts synthesized from these engineered operons were analyzed by Northern blot hybridization.

A 952-bp region containing the P_{bio} promoter, the *bio* regulatory region, and the 5′ end of the *bioW* gene was synthesized by PCR and recloned into plasmid pBIO126A, resulting in plasmid pBIO144 (8). This PCR fragment included about 150 bp of sequence upstream of P_{bio} that contained the 3′ end of an open reading frame (*orf4*) and a putative *rho*-independent transcription termination sequence at the end of *orf4*. This terminator is ~50 bp upstream of the P_{bio} promoter. This PCR fragment was synthesized in three segments to install several unique restriction sites needed for promoter manipulation. The P_{bio} promoter and *bio* regulatory site in pBIO144 were replaced with a 200-bp SP01-15 promoter fragment synthesized by PCR from pNH202 (18), resulting in plasmid pBIO168. PCR primers were designed to include the AT-rich region upstream from the −35 site (which has been shown to enhance transcription [3, 20]), the phage transcriptional start site, and 24 bp of the phage mRNA leader. The 3′ SP01-15 primer also included an additional CTGT sequence to extend the potential stem-loop structure at the 5′ end of the SP01-15 *bio* mRNA. Such stem-loop structures have been found to stabilize mRNA transcripts in *E. coli* (13). pBIO168 was then used to replace the chromosomal P_{bio} promoter and *bio* regulatory site by DNA transformation. A 1.8-kb fragment containing native sequences upstream of the *bio* operon was synthesized by PCR and inserted upstream of the SP01-15 promoter to give plasmid pBIO180. Linearized plasmid DNA was used to transform strain PY79 ΔP_{bio}::*cat17*, a biotin auxotroph in which the promoter region of the *bio* operon has been replaced by the chloramphenicol resistance gene (*cat*) of pC194 (28). Selection of Bio^+ transformants resulted in the desired strain, BI294, in which the *cat* gene was replaced with the SP01-15 promoter. As expected, BI294 was chloramphenicol sensitive, and PCR analysis confirmed that the SP01-15 promoter was juxtaposed to the *bioW* gene.

When a *bioW*-specific DNA probe was used, transcripts from BI294 containing the single-copy *bio* operon driven by SP01-15 ($P_{15}bio$) appeared to be the same lengths and in the same proportions as they were for the wild-type operon (Fig. 3, lane 5). However, the steady-state levels of these transcripts were significantly increased, i.e., about 10-fold for the SP01-15-driven operon relative to the transcripts from the wild-type operon. In addition, transcription from the engineered operon was constitutive with respect to biotin (Fig. 3, lane 6). This is most likely due to the removal of the *bio* regulatory region from the engineered operon. We do note, however, that slightly fewer *bio*-specific transcripts were detected in RNA from strain BI294 grown with biotin than in RNA from the same strain grown without biotin. It is not known whether this result was simply caused by variation in RNA recovery or represents residual repression of transcription by exogenous biotin in these engineered strains.

Deletion of the $biot_1$ terminator between *bioB* and *bioI*. The greater abundance of the 5.1-kb *bio*-specific transcript relative to that of the full-length 7.2-kb transcript suggested that the terminator-like sequence between *bioB* and *bioI* is active. To test this possibility, the DNA sequence containing this putative terminator was deleted ($\Delta biot_1$) from the single-copy SP01-15-engineered *bio* operon and *bio*-specific transcripts were analyzed by Northern blot.

A 205-bp fragment containing a deletion of the $biot_1$ terminator was synthesized by PCR from pBIO289 (8) by use of an upstream primer covering the *Bsp*EI site 207 bp upstream of $biot_1$ and a downstream primer which anneals to the 3′ end of *bioB*, skips 51 bp, and reanneals to the ribosome binding site, start codon, and *Bbr*PI (isoschizomer of *Pml*I) site of *bioI*. The 205-bp PCR product was used to replace the *Bsp*EI-to-*Bbr*PI fragment of pBIO289 (7) to give pBIO181. To replace the chromosomal $biot_1$ terminator, linearized pBIO181 was used to transform strain BI300, a biotin-requiring derivative of BI294 containing a Δ*bioB*::*cat* mutation (7). Selection of Bio^+ transformants resulted in the desired strain, BI303, in which the *cat* gene was replaced with the remainder of the *bioB* gene and the $biot_1$ deletion by a double recombination event. Strain BI303 was chloramphenicol sensitive, and PCR analysis confirmed the deletion of the *bioB-bioI* terminator-like region.

We expected that deletion of this terminator-like region would alter the wild-type *bio* transcription pattern. In good agreement with this prediction, only the 7.2- and 0.8-kb *bio*-specific transcripts were detected in RNA from BI303 containing the single-copy $P_{15}bio$ operon with the $\Delta biot_1$ mutation (Fig. 3, lanes 7 and 8). Moreover, accumulation of the full-length 7.2-kb transcript from the $biot_1$-deleted operon was increased to a level similar to the 5.1-kb RNA levels observed with SP01-engineered operons with $biot_1$. These results suggest that the $biot_1$ terminator-like region is active and functions as a site to reduce transcription of the *bioI* and *orf2* genes (or alternately to promote processing of the mRNA). We note that loss of the 5.1-kb transcript in BI303 uncovered a new, minor, apparent steady-state RNA of between 4 and 5 kb (Fig. 3, lanes 7 and 8). It is not known whether this apparent RNA band represents a bona fide *bio*-specific message or a stable degradation product of the full-length *bio* transcript.

TABLE 1. Biotin production by strains containing single chromosomal copies of wild-type and engineered *bio* operons

Expt	Strain	Relevant genotype	Production	
			Biotin (μg/liter)[a]	Biotin + vitamers[b]
1	PY79[c]	*bio*$^+$	5	10
	DB16	*birA*	50	140
	BI294	P_{15}*bioWAFDBIorf2*	150	400
2	BI294	P_{15}*bioWAFDBIorf2*	220	500
	BI303	P_{15}*bioWAFDB*(Δt_1)*Iorf2*	250	810
	BI304	P_{15}*bioWAFDB*(ΩP_{15})*Iorf2*	180	3,000

[a] Biotin production was assayed with *L. plantarum.*
[b] Biotin plus vitamer production was assayed with *S. cerevisiae* and is expressed in dethiobiotin equivalents (micrograms per liter).
[c] Values for PY79 may represent biotin and vitamers remaining in the spent medium rather than de novo synthesis.

Effect of increased gene transcription on biotin production. To measure the effect of replacing the putative wild-type promoter and operator with the SP01-15 promoter and deleting the $biot_1$ terminator between *bioB* and *bioI* on biotin production, strains containing these engineered operons were measured for biotin and vitamer (i.e., the last three intermediates in the biotin pathway, dethiobiotin, 7,8-diaminopelargonic acid, and 7-keto-8-aminopelargonic acid) production by a microbiological assay using *Lactobacillus plantarum* (27) and *Saccharomyces cerevisiae* ATCC 7754 (24), respectively. As shown in Table 1 experiment 1, BI294 containing the SP01-15 promoter produced more biotin and vitamers than a wild-type *B. subtilis* prototroph, PY79 (>30-fold), or a *B. subtilis* strain containing a mutation in the *birA* gene (3-fold) (6). Moreover, deletion of $biot_1$ in the SP01-15-driven operon resulted in a further increase (twofold) in biotin plus vitamer production but not in biotin production alone (Table 1, experiment 2). This increase in vitamer production appeared to coincide with the observed increase in transcription of the full-length *bio* message encoding the *bioI* and *orf2* gene products.

Introduction of a SP01 phage promoter upstream of *bioI*. To confirm the observation that higher expression of *bioI* leads to increased production of biotin vitamers, a second copy of the SP01-15 promoter was inserted within the P_{15}*bio* operon immediately upstream of *bioI*. A 224-bp fragment containing the SP01-15 promoter adjacent to the 5′ end of *bioI* was synthesized by PCR from pBIO168. This 224-bp PCR product was introduced into the unique *Bbr*PI site at the *bioI* start site of pBIO289, resulting in plasmid pBIO182. Linearized pBIO182 DNA was then used to introduce the SP01-15 promoter upstream of *bioI* in the P_{15}*bio* operon by transforming BI300 and selecting for Bio$^+$ transformants as described above. This transformation resulted in strain BI304. BI304 was chloramphenicol sensitive, and PCR confirmed that the SP01-15 promoter was juxtaposed to the *bioI* gene.

As shown in Table 1, experiment 2, introducing the SP01-15 promoter upstream from the *bioI* gene resulted in a fourfold increase in vitamer production relative to that in BI303 containing the P_{15}*bio* operon with the $\Delta biot_1$ deletion, without affecting biotin production. Moreover, this increase in vitamer production was in good agreement with Northern blots of total RNA from BI304 which showed an intense 2.3-kb steady-state message that comprised the *bioI* and *orf2* genes (data not shown). Taken together, these results support earlier studies that showed that *bioI* is involved in the early steps of biotin formation, possibly in the synthesis of pimelic acid (7).

Concluding remarks. We have identified and characterized the mRNA transcripts of the *B. subtilis* biotin biosynthetic operon. Three *bio*-specific transcripts were detected, the synthesis of which was regulated by biotin and the *B. subtilis birA* gene product. We assume that both the 7.2- and 5.1-kb polycistronic messages are translated to generate the biotin biosynthetic enzymes, although it is possible that they could be translated with different efficiencies. The function of the 0.8-kb transcript is unclear. We hypothesize that this RNA is a stable degradation product of one of the two polycistronic messages; however, it cannot be discounted that this small RNA represents a premature termination product caused by an unknown regulatory site in *bioW*.

The *B. subtilis bio* operon also contains a *rho*-independent transcription terminator or RNA processing site located between the *bioB* and *bioI* genes which appears to function independently of the biotin-BirA regulatory mechanism. Deletion of this region between *bioB* and *bioI*, or bypass of it by introduction of a promoter immediately upstream of *bioI*, results in higher levels of vitamers, but not biotin, suggesting that *bioI* expression is rate limiting for vitamer synthesis. Since bacteria normally require only trace amounts of biotin to grow, this regulatory feature may represent a mechanism by which *B. subtilis* limits production of intermediates in the biotin pathway.

ACKNOWLEDGMENTS

We thank V. Azevado and A. Sorokin for providing details on their macaloid clay RNA isolation protocol.

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