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Repression of Biotin Biosynthesis in *Escherichia coli* During Growth on Biotin Vitamers

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A strain of *Escherichia coli* in which the *lacZ* gene was fused to the *bioA* promoter was constructed. Colonies of this strain formed Lac^+ colonies on low-biotin agar (1.6 to 4.1 nM) and Lac^- colonies on high-biotin agar (41 nM). This *lac-bio* fusion strain was used to study the question of whether cells growing on the biotin vitamers D-biotin-*d*-sulfoxide (BDS) and dethiobiotin (DTB) generate enough biotin to give maximal repression of β-galactosidase synthesis. Repression by high concentrations (400 nM) of BDS was almost maximal (about 96%), whereas DTB repression reached a saturation level of about 80% with increasing DTB concentrations. The levels of repression obtained with both vitamers were sufficient to cause the colonies to appear Lac^-. When the *lac-bio* fusion was transduced into lines carrying mutations (*bis*) that prevent reduction of BDS to biotin, the transductants were not repressed by added BDS. Repression by BDS is unlikely to result from accumulation of extracellular biotin-related substances because (i) washed bis^+ cells were not detectably derepressed when transferred into medium containing BDS and (ii) washed *bis* cells were not detectably repressed when transferred into medium in which bis^+ cells had grown. Lactose agar plates containing high concentrations of DTB or BDS comprise an efficient selective medium for *bioB* or *bis* mutants and were used to isolate spontaneous mutations of these genes. This method should be adaptable to the selection of mutations in any biosynthetic pathway subject to end-product repression.

The enzymes catalyzing some of the terminal steps in the biosynthesis of biotin by *Escherichia coli* are coded by five adjacent genes (*bioABFCD*), whose transcription is coordinately repressed by high concentrations of extracellular biotin (reviewed in reference 14). The biotin operon resembles the *argECBH* and *malEFKlamB* operons of *E. coli* (17, 18) in that it is transcribed divergently (16). The *bioA* gene is transcribed leftward (counterclockwise on the standard *E. coli* map [2]), whereas *bioBFCD* are transcribed rightward. Both transcripts originate from a common control region (20). The final step in biotin biosynthesis is the conversion of dethiobiotin (DTB) into biotin. Although this conversion has not yet been demonstrated in cell-free extracts, the in vivo reaction requires the *bioB* gene and presumably is catalyzed by its protein product, called biotin synthetase.

Biotin can also be formed in *E. coli* by the enzymatic reduction of D-biotin-*d*-sulfoxide (BDS) to biotin. This reaction is not part of the normal biosynthetic sequence. Mutations in the *bioABFCD* genes render their bearers unable to form biotin from general carbon sources such as glucose, but do not interfere with sulfoxide reduction. Conversely, cells with mutations in the BDS (*bis*) genes do not require biotin for growth. Thus, BDS can serve as an alternative biotin source for *bio* mutants. The oxidation of biotin to BDS by molecular oxygen occurs spontaneously in solutions exposed to air. Hence, BDS reductase allows the cell to salvage biotin in the environment that has been inactivated by oxidation. A more important function of this reaction may be the reduction of intracellularly bound biotin which has become oxidized to BDS, thus putting a molecule of biotin carboxyl carrier protein (21) out of commission. The failure of added biotin to repress reductase synthesis (10) argues against the idea that salvaging external sulfoxide is its primary function. Although BDS reduction is superficially a single biochemical step, the integrity of four genes is needed for the production of active BDS reductase (13). Efficient reduction by extracts also requires a thioredoxin-like protein that is present in all *bis* mutants that have been tested and thus is probably not specific for the biotin pathway (A. del Campillo-Campbell, D. Dykhuizen, and P. P. Cleary, Methods Enzymol., in press). Substances like DTB and BDS that can sometimes replace biotin in promoting growth have been called vitamers. Figure 1 summarizes the

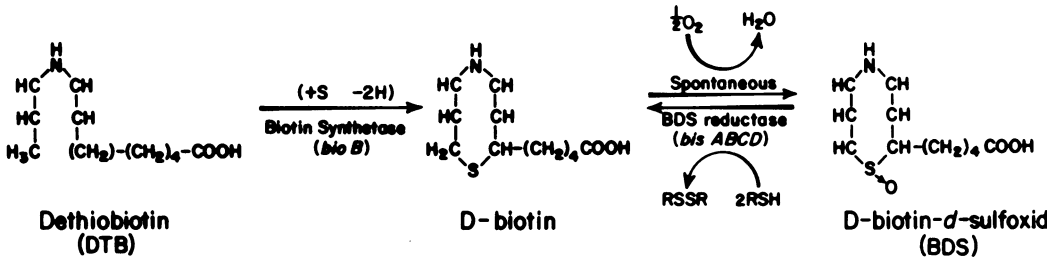

FIG. 1. *Relation of D-biotin to its biosynthetic precursor DTB and its oxidation product BDS. The last step of the biosynthetic pathway is the addition of a sulfur atom between two carbons of DTB to form a tetrahydrothiophene ring. This step, of unknown chemistry or enzymology, requires the bioB gene product biotin synthetase. D-Biotin can be spontaneously oxidized to BDS. Reduction of BDS to biotin requires an enzyme (BDS reductase) whose formation requires the integrity of four nonadjacent genes bisA, bisB, bisC, and bisD. (13). The immediate hydrogen donor in vitro is a thioredoxin-like protein (designated here as RSH).*

genetic requirements for biotin formation from its oxidation product BDS and from its biosynthetic precursor DTB.

We were curious whether the intracellular concentration and kinetic properties of the enzymes converting DTB and BDS allow the accumulation within the cell of repressing concentrations of biotin during steady-state growth. Investigation of these questions was facilitated by the use of a strain in which the *lacZ* gene had been connected to the *bioA* promoter by the operon fusion method of Casadaban (7, 8). A by-product of these studies was an efficient method for the selective isolation of new mutations in the *bis* and *bioB* genes.

MATERIALS AND METHODS

Media and general procedures. Methods for the cultivation of bacteria and the preparation of phage lysates from lysogens by induction with UV light and the compositions of tryptone broth and agar, eosin methylene blue agar, synthetic liquid medium, and tetrazolium agar have been described previously (4, 5, 19). On our tetrazolium agar (inorganic salts plus sugar and nutrilites), cells that ferment the sugar form red colonies, and nonfermenters are white. As strain BM1161 and its derivatives require thiamine, thiamine hydrochloride was added to all media at a concentration of 10 μg/ml.

Biotin and vitamers. D-Biotin was purchased from Nutritional Biochemicals Corp. (lot 3920). BDS, prepared by the method of Melville (22), was kindly given to us by P. Cleary. DTB was purchased as DL-desthiobiotin (K & K Pure Chemicals) from ICN Pharmaceuticals, Inc. Both vitamers contained less than 0.01% biotin, as judged by bioassay on SA291.

Enzyme assays. β-Galactosidase assays were performed as described by Miller (23), using chloroform-sodium dodecyl sulfate lysis. Specific activities were calculated from the equation given by Miller (reference 23, p. 354) as $(A_{420} - 1.74\ A_{550})/(t \times v \times A_{600})$, where t = time in minutes, v = volume of culture in 1.7 ml of reaction mixture, A_{600} = absorbance of the culture at 600 nm, and A_{420} and A_{550} are absorbances of the reaction mixture at 420 and 550 nm, respectively.

Extracts for the assay of BDS reductase were prepared from frozen washed cell pellets obtained by centrifugation of overnight cultures grown in tryptone broth. The frozen cells were thawed, suspended in 0.05 M potassium phosphate (pH 7.0)–3 mM 2-mercaptoethanol at 125 times their original concentration, and disrupted in a Bronson Sonifier (model W140). Cell debris was removed by centrifugation for 20 min at 23,500 × *g*, and the supernatant was dialyzed overnight against phosphate-mercaptoethanol buffer.

Specific activities of BDS reductase were calculated as micrograms of biotin formed per milligram of protein per 15 min. The assay was modified from that used by Cleary and Dykhuizen (10). The reaction mixture consisted of 25 μmol of ammonium acetate (pH 9.5), 6 μmol of dithiothreitol, 4.8 nmol of BDS, and cell extract containing 1.6 to 1.9 mg of protein, in a total volume of 0.25 ml. The reaction was started by adding BDS after preincubation of the other ingredients for 10 min at 37°C. It was stopped 15 min later by adding 0.2 mmol of trichloroacetic acid. The acid precipitate was removed by centrifugation, and 20 μl of supernatant was assayed for biotin by the disk microbiological method on the deletion strain SA291. Additional information relevant to the reductase assay is given by del Campillo-Campbell et al. (Methods Enzymol., in press).

Qualitative tests for nitrate reductase were performed as described previously (1). Tests were scored as positive if a dark-red color was produced and negative if they remained colorless. One of the strains scored as positive in our tests (S1138) gave an intermediate reaction and might have a partial defect in nitrate reductase.

Bacterial strains and bacteriophages. Table 1 lists the *E. coli* K-12 strains used in these investigations. The *lac-bio* fusion strain BM1161 was constructed as follows: strain MC4100 was mutagenized by infection with phage Mu (*cts*) and *bio*::Mu (*cts*) derivatives isolated after penicillin enrichment. Following the procedures developed by Casadaban (8), we lysogenized one of the strains which carried a Mu (*cts*) insertion in *bioA* with λp1(209). From the resulting lysogen, thermoresistant survivors able to form red colonies on minimal lactose agar containing 1.6 nM biotin were selected. BM1161 is one such survivor which failed to form colonies on lactose agar contain-

TABLE 1. *E. coli K-12 strains*

Strain	Genotype[a]	Origin/reference
W3350	F⁻ *galK2 galT1 lac*	5, 11
R875	*bioB17 lac*	Mutagenesis of Bio⁺ strain R881 (9, 12)
R876	*bioC18 bisA18 chlE lac*[a]	Mutagenesis of Bio⁺ strain R881 (12, 13)
R879	*bioA24 lac*	Mutagenesis of Bio⁺ strain R881 (12, 13)
DD34	*bioA24 bisC10 lac*	Mutagenesis of R879 (13)
DD38	*bioA24 bisD21 chlG lac*[a]	Mutagenesis of R879 (13)
DD130	*bioA24 bisA29 chlA lac*[a]	Mutagenesis of R879 (13)
SA291	F⁻ Δ(*gal-bisA*) *his str*[r]	S. Adhya (9)
MC4100	F⁻ *araD139 ΔlacU169 strA thi*	M. Casadaban (8)
BM1161	F⁻ *araD139 ΔlacU169 strA thi φ(bioA-lacZ)301*	Derived from MC4100 (this paper)
S1129	F⁻ *araD139 ΔlacU169 strA thi φ(bioA-lacZ)301 bis*[a]	Mutant of BM1161 (this paper)
S1130	F⁻ *araD139 ΔlacU169 strA thi φ(bioA-lacZ)301 bis*[a]	Mutant of BM1161 (this paper)
S1131	F⁻ *araD139 ΔlacU169 strA thi φ(bioA-lacZ)301 bis*[a]	Mutant of BM1161 (this paper)
S1133	F⁻ *araD139 ΔlacU169 strA thi φ(bioA-lacZ)301 bir*	Mutant of BM1161 (this paper)
S1134	F⁻ *araD139 ΔlacU169 strA thi φ(bioA-lacZ)301 bis*[a]	Mutant of GM1161 (this paper)
S1135	F⁻ *araD139 ΔlacU169 strA thi φ(bioA-lacZ)301* (Lac⁺ on biotin)[b]	Mutant of BM1161 (this paper)
S1136	F⁻ *araD139 ΔlacU169 strA thi φ(bioA-lacZ)301* (Lac⁺ on biotin)[b]	Mutant of BM1161 (this paper)
S1138	F⁻ *araD139 ΔlacU169 strA thi φ(bioA-lacZ)301 bis*	Mutant of BM1161 (this paper)
S1139	F⁻ *araD139 ΔlacU169 strA thi φ(bioA-lacZ)301 bis*[a]	Mutant of BM1161 (this paper)
S1142	F⁻ *araD139 ΔlacU169 strA thi φ(bioA-lacZ)301* (Lac⁺ on DTB)	Mutant of BM1161 (this paper)
S1143	F⁻ *araD139 ΔlacU169 strA thi φ(bioA-lacZ)301 bioB701*	Mutant of BM1161 (this paper)
S1144	F⁻ *araD139 ΔlacU169 strA thi φ(bioA-lacZ)301 bioB702*	Mutant of BM1161 (this paper)
S1148	F⁻ *araD139 ΔlacU169 strA thi φ(bioA-lacZ)301 bioB703*	Mutant of BM1161 (this paper)
S1150	F⁻ *araD139 ΔlacU169 strA thi φ(bioA-lacZ)301* (λ*bio1-imm434*)	Bis⁺ transductant of BM1161 (this paper)
S1152	*bioA24 bisC10* (λ*lac bio1*)[a,c]	Lac⁺ transductant of DD34 by S1150 (this paper)
S1153	*bioA24 bisD21 chlG* (λ*lacbio1*)[a,c]	Lac⁺ transductant of DD38 by S1150 (this paper)
S1154	*bioA24 bisA29 chlA* (λ*lacbio1*)[a,c]	Lac⁺ transductant of DD130 by S1150 (this paper)
S1156	*bioA24* (λ*lacbio1*)[c]	Lac⁺ transductant of R879 by S1150 (this paper)

[a] *bis*, Biotin sulfoxide reductase. Other gene symbols are as described in reference 2.

[b] Lac phenotypes refer to the color of colonies of lactose plus synthetic tetrazolium agar supplemented with 41 nM biotin or 470 nM DTB. BM1161 produces white colonies on these media, whereas colonies of S1135 are red. See text for more details.

[c] Strains S1152 to S1156 have not been checked for the number of prophage copies or their specificity of immunity. They are expected to carry a wild-type λ prophage as well as the *lacbiol* prophage. The *lacbiol* prophage should have the (*bioA-lacZ)301* fusion and the *bio*-λ novel joint of λ*bio1*.

ing 41 nM biotin. A more detailed description of these experiments will appear elsewhere.

In Table 1, the *bioA-lacZ* fusion in strain BM1161 is given the symbol φ(*bioA-lacZ)301*, consistent with previous designations of operon fusions prepared by this method (26). The complete history of the fusion strain involves several events, each of which creates one or more novel joints in the DNA. The disposition of the relevant parts of λ, *bio*, and *lac* in strain BM1161 is diagrammed in Fig. 2.

Phage stocks used in this study are listed in Table 2.

Complementation test for *bioB* function. To assay *bioB* function, the *bioB17* mutation (9) was crossed into the transducing phage λ*bio256cIts857* (25). Successive 10-fold dilutions of the lysate were spotted onto lawns of the strain under test (all lysogens immune to λ) on minimal glucose tetrazolium medium and incubated for 2 days at 37°C. Strains scored as positive showed heavy pink growth wherever 10^4 to 10^5 or more phage particles had been spotted. A λ*bio256cIts857* phage carrying the *bioA511* mutation (9) served as a negative control (because all our strains are *bioA*). A mixture of the λ*bio256bioB17cIts857* and λ*bio256bioA511cIts857* lysates was used as a positive control.

RESULTS

Repression by BDS. Figure 3 shows the specific activity of β-galactosidase as a function of BDS concentration in the growth medium. At the highest concentration used (390 nM), the degree of repression did not differ significantly from that seen at saturating concentrations of biotin.

BDS itself does not repress. The repression during growth on BDS could mean either that repressing concentrations of biotin were formed or that BDS itself repressed. These two possibilities can be distinguished by using mutants (*bis*) that are unable to convert BDS to biotin. To assay repression in *bis* mutants by measuring β-galactosidase, strains were needed that carried both the *lac-bio* fusion and *bis* mutations.

This was achieved by preparing a high-frequency-transducing lysate containing a derivative of λ*bio1* that includes the *lac-bio* fusion. First, strain BM1161 was transduced to Bio⁺ with λ*bio1imm434*. A possible structure for the transductant is diagrammed in Fig. 2. Induction

gave a mixture of phage types (Fig. 2, bottom line), one of which was a *lacbio*1 phage containing the *lac-bio* fusion. This lysate was used to transduce various recipient strains to Lac$^+$ (Table 3).

These Lac$^+$ transductants might result from insertion of the λ*lacbio*1 into either the *bio* operon or the *lac* operon of the recipient. Some of the latter might have the *lacZ* gene under control of the *lac* promoter rather than the *bio* promoter. As we desired transductants with *lacZ* under biotin control, we spotted a mixture of high-frequency-transducing lysate and a lysate of wild-type λ onto lawns of various recipients on eosin-methylene blue-lactose agar. The wild-type λ should facilitate the formation of double lysogens where the *lacbio*1 phage is inserted within the λ prophage. After 2 days of incubation at 37°C, these spots showed confluent Lac$^+$ growth. Pure lines of Lac$^+$ transductants were isolated from the growth in these spots by two successive restreakings on eosin-methylene blue-lactose agar. Cells from different lines were spotted onto lactose tetrazolium agar supplemented with (i) 0.41 nM biotin, (ii) 0.41 nM biotin plus 390 nM BDS, and (iii) 41 nM biotin.

A few transductants appeared Lac$^+$ on all media. These are presumably strains with *lacZ* under lactose control and were not studied further. Of 12 transductants that appeared repressible by biotin, the 7 transductants of the *bis* mutants DD34, DD38, DD130, and R876 also appeared Lac$^+$ on the BDS plates, whereas all 5 transductants of the control *bis*$^+$ strains R875 and R879 appeared Lac$^-$. The absence of BDS repression in these *bis* mutants was further verified by β-galactosidase assay (Table 3).

Qualitatively, the results of Table 3 show that BDS repression is decreased (S1152, S1153) or eliminated (S1154) in strains carrying *bis* mutations, compared with the *bis*$^+$ control. Quantitatively, strains S1153 and S1156 make more enzyme at 1.6 mM biotin than we have ever observed in the donor strain BM1161 (see Fig. 3 and 4 and Table 5). S1153 and S1156 may be multiple lysogens carrying two or more copies of the *lacZ* gene.

BDS repression is not due to extracellular corepressor. The fact that *bis* mutations prevented repression by added BDS strongly indicates that BDS itself does not repress. The simplest inference is that growth on BDS leads to the accumulation of a repressing concentration of biotin within the cell during steady-state growth in a medium devoid of biotin. A formal alternative, equally compatible with the results reported so far, is that, at the growth stage at which the cultures of Table 3 were assayed, sufficient sulfoxide reduction had taken place so that repressing concentrations of biotin (or perhaps of some unidentified intermediate or by-product of BDS reduction) have accumulated in the medium.

To test this alternative, cultures of the parent strain BM1161 and of a *bis* mutant (S1130) derived from it were grown in synthetic medium with 41 nM biotin, centrifuged, washed twice in cold water, and suspended at an A_{600} of 0.11 in biotin-free medium containing either (i) no BDS or (ii) 390 nM BDS. Figure 4 shows that the parent strain soon became derepressed on BDS-free medium, whereas no derepression was observed at the high BDS concentration. The mutant strain, on the other hand, became equally derepressed on both media.

After 2 h, the high-BDS culture of the parent strain was centrifuged. Washed cells of the mutant were suspended in the supernatant and incubated at 37°C. The course of derepression was the same as that seen for the mutant in unconditioned media (Fig. 4), indicating that the parent strain had not elaborated any extracellular product capable of repressing the mutant.

We conclude that the balance between biotin production and biotin efflux in a *bis*$^+$ strain growing in 390 nM BDS results in a steady-state concentration of intracellular biotin sufficiently high to repress the biotin operon maximally.

New *bis* mutants can be selected by their inability to be repressed by BDS. When the *lac-bio* fusion was transduced into a *bis* mutant, the resulting transductant was not repressed by added BDS (Table 3). A corollary to this finding is that medium containing lactose as carbon source plus a high concentration of BDS should be selective for *bis* mutants. We generally use a medium with 390 nM BDS plus a low (0.41 to 1.6 nM) concentration of biotin to supply the biotin requirement of *bis* mutants, although on the initial selection plates this precaution appears to be unnecessary.

Twelve mutant strains (S1129 to S1140, some of which are shown in Table 1) were obtained by purifying independent Lac$^+$ colonies arising on Lac$^-$ sulfoxide medium and further characterized by replicate plating and growth in liquid medium for (i) ability to use sulfoxide as the sole biotin source in glucose medium, (ii) growth on limiting biotin (0.41 nM) in glucose medium, and (iii) growth on lactose medium at high biotin (41 nM).

Five of the 12 isolates appeared Lac$^+$ on high biotin. Two of these (S1133, S1138) grew very poorly at low biotin concentrations. These mutants are presumably of the type (*bir*) previously characterized as having defects in biotin uptake and retention, as well as in repression (6; and R. Chang, D. Barker and A. Campbell, unpublished

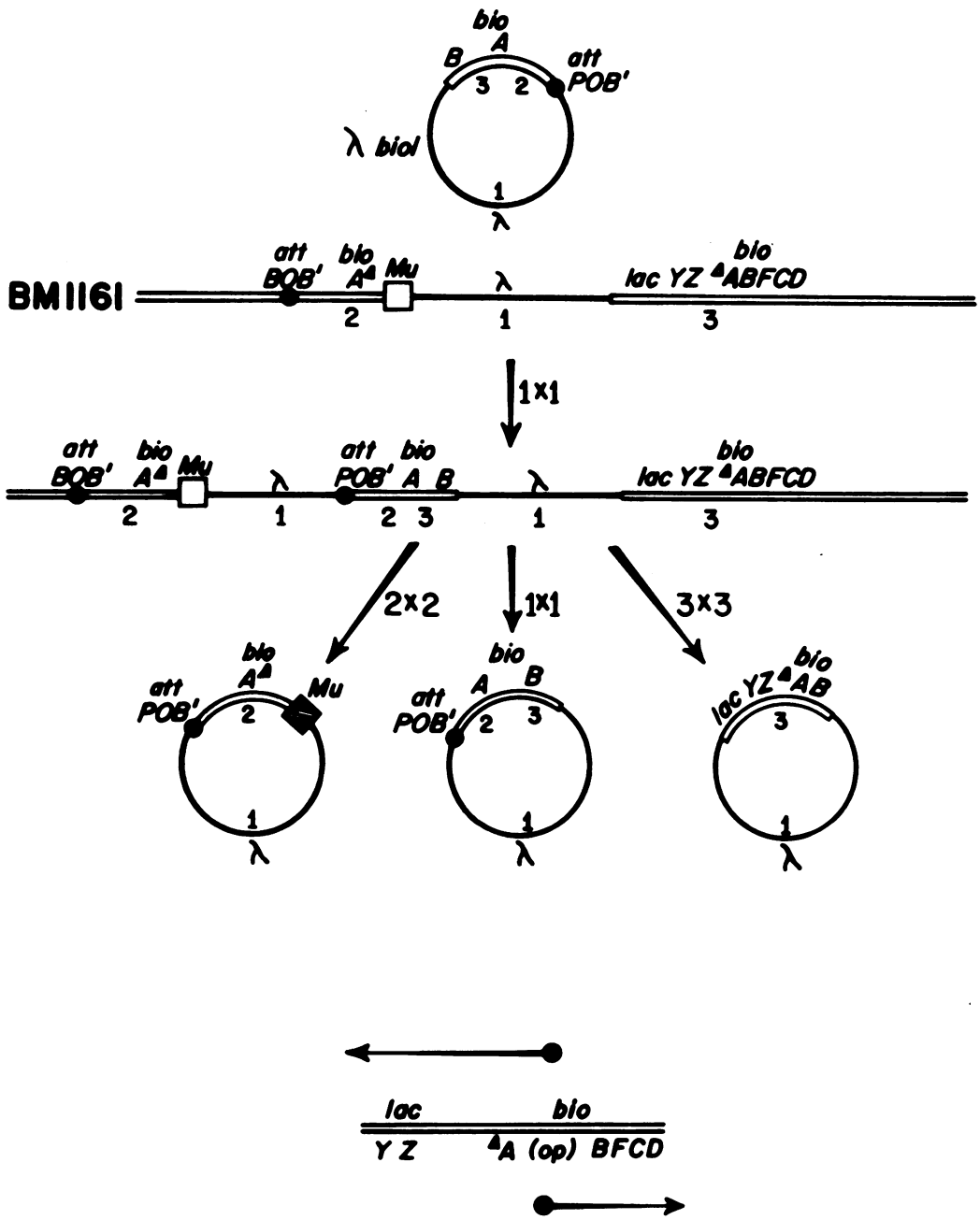

FIG. 2. *Production of high-frequency-transducing lysates that can transduce the lac-bio fusion. Phage λ bio1 imm434 (top circle) was used to transduce strain BM1161 to Bio⁺. Insertion could take place in any of the indicated regions (1 to 3) of homology between phage and host. It is shown here as taking place within λ DNA (region 1). After induction of this lysogen, excision can occur by recombination in any of the three homologous regions. This leads to a mixture of phage types, including the lac-bio phage shown at the right.*

. data). Mutations (*bioP*) in the biotin transport system (15) might also be represented here, depending on whether BDS is taken up by the same system as biotin. The other three (S1135, S1136, S1139) grew well at low biotin and may be either operator mutations or regulatory mutations of the *bioR* type (15).

The other seven mutants all grew poorly or

not at all on glucose medium with 3.9 nM BDS, a concentration at which the parent strain and S1135, S1136, and S1139 grew well. Some of these mutants grew at higher concentrations of BDS (39 to 390 nM) or after prolonged incubation. These seven strains presumably carry *bis* mutations. Table 4 shows the growth properties of three of these seven strains and of one *bis*$^+$ control and also the amount of BDS reductase observed in extracts. The reductase activity of the three *bis* mutants varied from 1 to 5% of that seen in the *bis*$^+$ control. The control did not differ significantly from other K-12 strains such as R879, which has been extensively studied in our laboratory.

Dykhuizen (13) noted that the *bioD21* mutation in strain DD38 rendered the cell simultaneously Bis$^-$ and nitrate reductase deficient (Nit$^-$), as though the *bisD* gene and the nearby *chlG* gene belonged to the same operon. Consequently, we tested our 12 mutants for nitrate reductase. One of the seven *bis* mutants (S1134) was Nit$^-$.

The fact that all of those mutations that prevented repression by BDS but not by biotin were deficient in sulfoxide reduction reinforces the conclusion that BDS does not repress directly, but only by conversion to biotin.

Repression during growth on DTB. Our results with BDS encouraged us to investigate whether the same method could be applied to DTB, the immediate precursor of biotin in the biosynthetic pathway. The expectation for DTB differs from that for BDS, because the conversion of DTB to biotin is itself repressible by biotin. Given an appropriate K_m for BDS reductase and a sufficient amount of reductase per cell, a high BDS concentration can generate enough biotin to give maximal repression. With DTB, however, the biotin that accumulates represses the *bioB* gene, thus reducing the conversion of DTB to biotin. Whereas the exact shape of the repression curve depends on several kinetic parameters, the general expectation is that the approach to maximal repression with in-

TABLE 2. *Phage list*

Phage	Source/reference
λ	Our stocks (5)
λ*bio*1*imm*434	Recombination between λ*bio*1 and λ*imm*434 (this work)
λ*bio*256*bioB*17 *cIts*857	This work
λ*bio*256*bioA*511 *cIts*857	This work
λp1(209)	M. Casadaban (8)
Mu (*cts*)	M. Casadaban (8)

TABLE 3. *β-galactosidase assays in transductants of bis mutants*

Strain[a]	Relevant genotype	β-Galactosidase activity after growth in different media[b]		
		1.6 nM biotin	1.6 nM biotin–390 nM BDS	41 nM biotin
S1152	*bisC10*	53	22	2.9
S1153	*bisD21*	225	136	3.0
S1155	*bisA29*	96	92	2.3
S1156	*bis*$^+$	159	3.4	3.9

[a] See Table 1 for pedigrees of these strains.

[b] Bacteria were grown at 37°C with aeration in synthetic medium containing 0.2% glucose and 41 nM biotin, diluted 1:100 into synthetic medium with 0.2% glucose and the indicated supplements, grown to an A_{600} of 0.2 to 0.5, and assayed for β-galactosidase. Specific activity was calculated as described in the text.

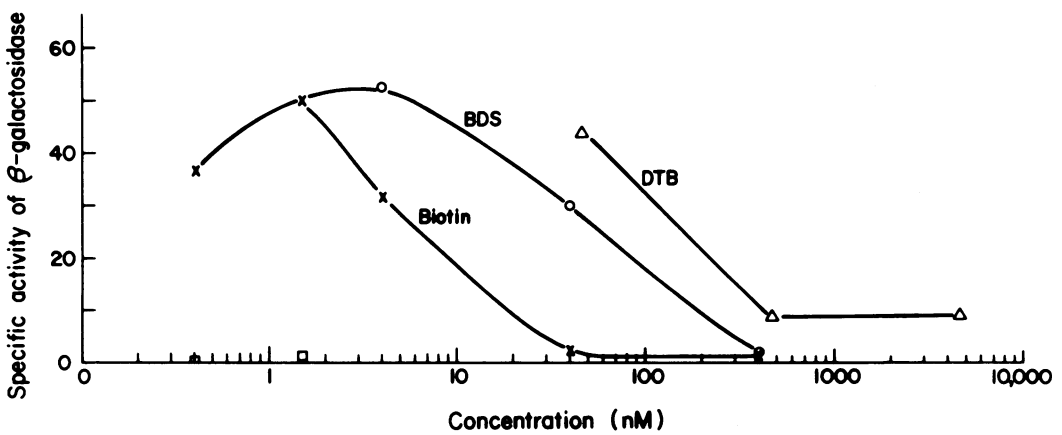

FIG. 3. *Specific activity of β-galactosidase in cells growing exponentially in synthetic medium with glucose as the carbon source and the indicated concentrations of biotin, BDS, or DTB or the indicated concentration of biotin plus 390 nM BDS* (□).

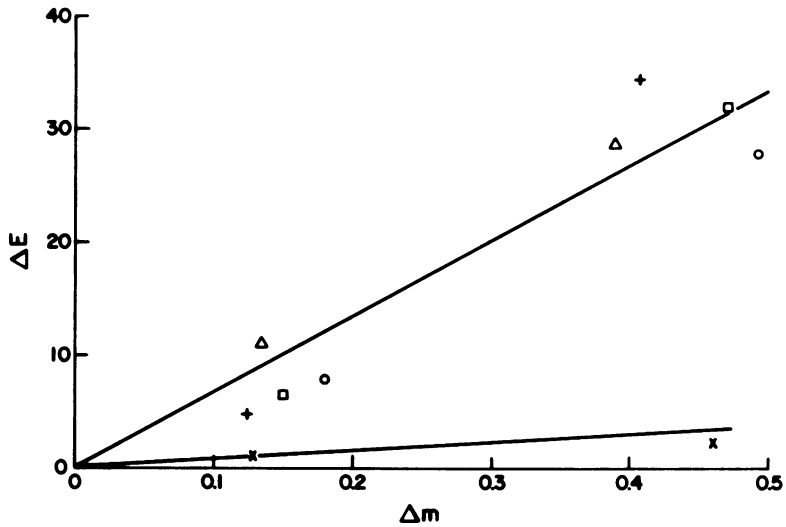

FIG. 4. *Effect of BDS on derepression of BM1161 (bis+) and S1130 (bis). Cultures of cells grown at a repressing concentration of biotin (41 nM) were centrifuged, washed twice, suspended at A_{600} of 0.12 in growth medium either with or without 390 nM BDS, and grown at 37°C with aeration. After 1 and 2 h of growth, the specific activity of β-galactosidase was measured. Results are plotted as ΔE versus Δm (24), where Δm = increment in A_{600} and ΔE = increment in total enzyme E, computed as E = specific activity × A_{600}. The initial specific activities were 1.6 to 1.9, whereas the specific activities after 2 h of growth were 43 to 69 for the upper curve and 3.6 for the lower one. BM1161, no BDS (○); BM1161, with BDS (×); S1130, no BDS (+); S1130, with BDS (□). After 2 h, the culture of BM1161 with BDS was centrifuged, cells of S1130 were suspended in the supernatant and aerated at 37°C, and enzyme assays were performed at 1 and 2 h (△).*

TABLE 4. *Properties of some mutants selected on lactose-BDS*

Strain	Klett reading at 48 h on glucose medium[a]					BDS reductase[b]
	Unsupplemented	3.9 nM BDS	39 nM BDS	390 nM BDS	41 nM biotin	
S1130[c]	0	0	0	0	93	0.0003
S1131[c]	2	2	0	0	84	0.00027
S1134[c]	1	0	98	97	91	0.001
S1135[d]	0	84	89	89	85	0.02
R879[e]	NT[f]	NT	NT	NT	NT	0.015

[a] Grown at 37°C without aeration.
[b] Specific activity of enzyme in extracts of cells grown in tryptone broth.
[c] Scored as *bis* mutants. (See Table 1 and text.)
[d] Scored as operator or *bioR* mutant. (See Table 1 and text.)
[e] Standard strain for preparation of reductase.
[f] NT, Not tested.

creasing concentrations of DTB will at best be much more gradual than that with BDS. In fact, the curve appears to level off at about 80% repression (Fig. 3).

Selection of new *bioB* mutants. The degree of repression by DTB at 470 nM, although incomplete, leads to the formation of Lac⁻ colonies on lactose tetrazolium agar. As with BDS, mutants insusceptible to repression on this medium can be picked as red papillae overgrowing white colonies. Eight mutants were purified and studied in more detail.

None of the eight mutants appeared Lac⁺ on high-biotin agar. Thus, this sample included no mutants completely defective in normal repression. However, only three of the mutants (S1143, S1146, S1148) showed any growth deficiency when replicated onto glucose plates containing 47 nM DTB, and two of these (S1146, S1148) grew well at 470 nM DTB. All three could be transduced to Bio⁺ by infection with λ*bio*1*imm*434, which carries the *bioAB* genes (20). This is consistent with a location of the DTB⁻ mutations within the *bioB* gene. Furthermore, the *bio*256*bioB*17cI857 phage showed a strong feeding reaction when spotted onto the parent strain BM1161, weaker feeding on S1146 and S1148, and almost none on S1143. Thus, the

mutations in these strain diminish their ability to complement for *bioB* function.

Among our mutants that were derepressed on BDS media, all of those that were Bis$^+$ were derepressed on high biotin. The five mutants that grew on DTB glucose yet were still subject to biotin repression thus have no analogs in the BDS experiments. The difference probably arises from the fact that DTB repression is incomplete in the parent strain, so mutations causing small quantitative changes in repressibility or promoter activity may elevate the rate of transcription at high DTB enough to generate a Lac$^+$ phenotype on DTB.

Table 5 shows the results of β-galactosidase assays of the parental strain, the three DTB$^-$ mutants, and one of the DTB$^+$ mutants (S1142) as a function of biotin and DTB concentration. All of the mutants were derepressed on 1.6 nM biotin–470 nM DTB, whereas the parent strain was 80% repressed under these conditions. The DTB$^+$ mutant S1142, although susceptible to biotin repression, showed about twofold-greater activity at high biotin concentrations than the DTB$^-$ or parental strains. This behavior is consistent with a minor lesion in biotin regulation not specifically related to DTB utilization.

DISCUSSION

We have shown that repression of the biotin operon in the presence of two potential precursors, DTB and BDS, results from conversion of these compounds to biotin. That repression is caused only by the end product of the pathway accords with the results of comparable studies on other biosynthetic operons. In the case of DTB, a previous study (3) indicated that prior growth in medium containing 200×10^{-4} μg of DTB (about 100 nM) per ml had little effect on the ability of *E. coli* cells of the Crookes strain to elaborate biotin or its vitamers. Comparison with Fig. 3 suggests that this concentration of DTB is too low to have much effect. Whether a still higher concentration of added DTB can repress a *bio*$^+$ strain is uncertain. The maximal repression by DTB (80%) seen in these experiments is comparable to the degree of autorepression of the *bioD* gene by endogenous biotin previously observed in *bio*$^+$ strains (6). The autorepression of *lac* calculated by comparing S1150 with BM1161 appears to be somewhat lower (about 45%; data not shown).

One reason for caution in the interpretation of experiments employing very high concentrations of vitamers is the possible presence of chemical impurities. Our BDS and DTB are vitamin-free by bioassay. Moreover, the fact that selection for derepression yields mutants unable to convert these compounds to biotin strongly argues that repression is caused by biotin produced by metabolism of the vitamers.

Our work exploited the properties of a *lac-bio* fusion strain constructed by the Casadaban (7, 8) method. Use of such strains is convenient because the β-galactosidase assay is simple and highly sensitive. However, in principle, all our results on operon activities under various conditions could have been obtained without use of the fusion strain, by either enzyme assay or messenger hybridization, techniques that have furnished most of our present information on biotin regulation (reviewed in reference 14).

One dividend that results from use of the fusion strain is the possibility of directly selecting mutations that prevent repression by inactivating steps in the biosynthesis of corepressor. For many purposes it is useful to have a direct and efficient method for selecting auxotrophic mutations in a pathway under study. Spontaneous mutations, in particular, are hard to obtain by classical methods such as penicillin selection. As the Casadaban method can be adapted to any operon, this approach should be

TABLE 5. *Repressibility of some mutants selected on lactose-DTB*

Strain[a]	Growth properties		β-Galactosidase activity of cultures grown on glucose media[b]					
	Glucose–47 nM DTB	Lactose–470 nM DTB–1.6 nM biotin	1.6 nM biotin	4.1 nM biotin	41 nM biotin	410 nM biotin	470 nM DTB	470 nM DTB–1.6 nM biotin
BM1161	+[c]	Lac$^-$[d]	50	31	2.3	2.3	13	10
S1142	+	Lac$^+$	103	73	6.8	3.5	144	85
S1143	–	Lac$^+$	116	55	1.9	1.4	NT[e]	66
S1146	–	Lac$^+$	47	29	3.1	2.1	NT	50
S1148	–	Lac$^+$	81	49	2.4	1.3	NT	47

[a] The last four strains were selected as mutants of BM1161 that were Lac$^+$ on 470 nM DTB–1.6 nM biotin.
[b] Growth conditions as in Table 3. Specific activity was calculated as described in the text.
[c] +, Visible growth on synthetic solid medium after 1 to 2 days of incubation at 37°C; –, little or no growth.
[d] Lac$^-$, Tiny white colonies on lactose tetrazolium synthetic agar; Lac$^+$, large red colonies.
[e] NT, Not tested (because DTB$^-$ cultures do not grow without biotin).

generally applicable, although modifications may be necessary when the concentration of end product generated from intermediates of the pathway itself does not repress strongly enough to generate a Lac$^-$ phenotype.

A specific application, illustrated in the present paper, is the possibility of analyzing spontaneous *bis* mutations for their effects on nitrate reductase. Obviously, our observation that one of seven of the *bis* mutants were Nit$^-$ proves nothing by itself. In conjunction with previous data (13), the result suggests that a single genetic change can affect both functions.

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