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Biochemical and Genetic Characterization of Dehydrobiotin Resistant Mutants of *Escherichia coli*

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Summary. Dehydrobiotin (DHB) resistant mutants were isolated from strains of *Escherichia coli* K-12 and were classified into two groups; *dhbA* and *dhbB*.

In *dhbB* mutants the structural genes for enzymes of the biotin pathway are expressed constitutively at a high rate. The *dhbB* gene is co-transducible with *argE* at a frequency of about 50% by P1 transduction and maps on the chromosome between *arg ECBH* and *rif*. The *dhbB*$^+$ gene is trans-dominant over the mutant allele indicating that the *dhbB*$^+$ gene controls the production of a diffusible substance such as a repressor molecule.

The *dhbA* mutants show biotin biosynthetic activity comparable to the wild type and are as sensitive to repression by biotin as the parent strain. The mutants appear to be deficient in DHB transport as suggested by the findings that the ability of the mutants to take up biotin is reduced significantly and that DHB, a competitive inhibitor of biotin uptake, is much less inhibitory to biotin uptake in the mutants than in the wild type.

Introduction

The biosynthesis of biotin in *Escherichia coli* involves the following reactions; pimelyl CoA → 7-oxo-8-aminopelargonic acid (7-KAP) → 7, 8-diaminopelargonic acid (DAP) → desthiobiotin (DTB) → biotin. These reactions are catalysed by enzymes, 7-KAP synthetase, 7-KAP-DAP aminotransferase, DTB synthetase, and biotin synthetase, respectively. A cluster of genes specifying these enzymes is located at 17 min on the genetic map (for a review, see Eisenberg, 1973). The expression of these genes is subject to repression by biotin (Pai and Lichstein, 1965, 1966; Eisenberg and Star, 1968; Pai, 1969, 1971; Guha *et al.*, 1971), but the molecular basis of this process remains unknown.

Various antimetabolites have been used to isolate regulatory mutants such as 5-methyltryptophan for *trpR* (Cohen and Jacob, 1959), L-canavanine for *argR* (Maas, 1961), and 1,2,4,-triazolealanine for histidine regulatory mutants (Roth *et al.*, 1966). Isolation of biotin regulatory mutants was a difficult problem because of lack of biotin analogues that could be used for the selection of such mutants. A mutant of *E. coli* constitutive for biotin synthesis was isolated using a biotin crossfeeding test (Pai, 1972), but the method was not too effective.

In this study we report biotin regulatory mutants of *E. coli* that were isolated by selection for α-dehydrobiotin (DHB)-resistance. DHB (Fig. 1) is a structural analogue of biotin produced by *Streptomyces lydicus* (Hanka, Bergy and Kelly, 1968; Hanka, Reineke and Martin, 1969). DHB inhibits growth of a variety of microorganisms and its antimicrobial activity is reversed by biotin. The mode of action of DHB is not known. Also reported here are DHB-resistant mutants that appear to be deficient in the transport of DHB.

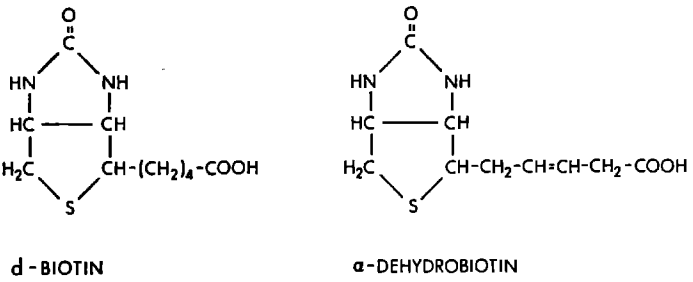

Fig. 1. Structure of d-biotin and α-dehydrobiotin. The structure of α-dehydrobiotin is according to Hanka *et al.* (1968, 1969)

Materials and Methods

Bacterial Strains

All bacteria used were strains of *Escherichia coli* K-12 and are listed in Table 1. The location of the markers used in this study, the origin of transfer of Hfr strains, and the portion of the chromosome carried by F-prime factor KLF10 are shown in Fig. 2.

Media and Cultural Conditions

The minimal medium used was that of Davis and Mingioli (1950). For liquid culture of strain AB1157 and its derivatives the minimal medium was supplemented with Difco vitamin-free casamino acids (2 g/l) to increase cell yields. Complex media were also used: Difco tryptose blood agar base (TBAB), Difco antibiotic medium 3 (penassay broth) and L-broth (Miller, 1972). All culture were incubated at 37 C with shaking.

Enzyme Assay

Biotin Synthetase. Enzyme activity was assayed by measuring the amount of biotin synthesized from desthiobiotin by whole cells (Pai, 1972). Biotin was determined by microbiological assay using *Lactobacillus plantarum.*

Desthiobiotin Synthetase. The enzyme was assayed by measuring the amount of desthiobiotin synthesized from DAP using crude cell-free extracts. Procedures for the preparation of crude extracts and enzyme assays were described elsewhere (Cheeseman and Pai, 1970). DTB was measured by microbiological assay using *E. coli* K-12-1 (Pai, 1969).

Determination of Extracellular and Intracellular Free Biotin

Culture supernatant fluids were used for extracellular biotin determination. To extract intracellular free biotin, cells were washed once with chilled phosphate buffer (0.05 M, pH 6.8) and boiled in distilled water for 5 min. Biotin was assayed with *L. plantarum.*

Minimum Inhibitory Concentration (MIC) of Dehydrobiotin

Tubes (18 × 150 mm) containing 1.0 ml of minimal medium supplemented with required growth factors plus various amounts of dehydrobiotin were inoculated with washed cells of overnight cultures and were incubated on a tube shaker at 37 C. The lowest concentration of dehydrobiotin at which no growth was visible after 24 hr was taken as an MIC.

Uptake of Biotin

The uptake medium contained per ml: potassium phosphate (50 μmoles, pH 6.8), $MgSO_4$ (1.0 μmole), glucose (20 μmoles), chloramphenicol (50 μg) and ^{14}C-biotin. Washed cells from

Table 1. Bacterial strains

Strain	Relevant genotype[a]	Origin or reference[b]
AB1157	F⁻ *thr-1 leu-6 proA2 his-4 argE3 thi str-31*	
X407	HfrH *proB thi*	
K-12-20	*bioB20*	mutagenesis of K-12 (Pai, 1972)
P48-3	*bioR10*	mutagenesis of K-12 (Pai, 1973)
X341	HfrC *proC metB*	
KL16-99	HfrKL16 *recA thi*	
SB96	F⁻ *argH trpA36 rif*	E. Orias (Orias *et al.*, 1972)
AA6	F⁻ *proC purE trp metB str*	
342-167	F⁻ *thi-1 thr-1 leu-6 his-1 argC32 ppc-1 str*	CGSC strain No. 3594, originally from N. Glansdorff (1965)
P412	Same as AB1157 but *dhbA412*[c]	Mutagenesis of AB1157, Selection on 5 μg/ml of dehydrobiotin
P514	Same as AB1157 but *dhbB514*[c]	same as above
P493	Same as AB1157 but *dhbB493*	same as above
P493-1	Same as P493 but *leu*⁺ *metB*	Cross X341 × P493, Selection for Leu⁺ Sm^R
P514-1	Same as P514 but *leu*⁺ *arg*⁺ *metB*	Cross X341 × P514, Selection for Leu⁺ Sm^R
P493-1R	Same as P493-1 but *recA his*⁺	Cross KL16-99 × P493-1, Selection for His⁺ Sm^R
KLF10/JC1553	KLF10 (=F110) (*polA*⁺ *metB*⁺ *argE*⁺ *malB*⁺)/JC1553 *argG6 metB1 his-1 leu-6 recA1 str-104*	CGSC strain No. 4261, originally from K. B. Low
P601	Same as 342-167 but *rif*	Mutagenesis of 342-167, Selection on 100 μg/ml of rifampicin

[a] Only mutations relevant to this study are listed. AB1157, KLF10/JC1553, SB96, AA6, 342-167 and their derivatives have additional markers not shown here. The genetic symbols used are those of Taylor and Trotter (1972).
[b] The strains of which origin or reference is not mentioned were obtained from Dr. A. Ahmad of the University of Alberta. CGSC strains were obtained from Dr. Barbara Bachmann of *E. Coli* Genetic Stock Center, Yale University.
[c] For dehydrobiotin resistance.

overnight cultures were added at zero time at a concentration of 2 mg dry weight per ml and 1.0-ml samples were taken at various times and filtered through millipore filters (pore size, 0.45 μm; diameter, 25 mm). The filters were washed with 4 ml of chilled distilled water and dried. There was no significant loss of accumulated radioactivity with this washing procedure. The radioactivity was counted in a Nuclear Chicago Mark I liquid scintillation counter, using a toluene-based scintillation fluid.

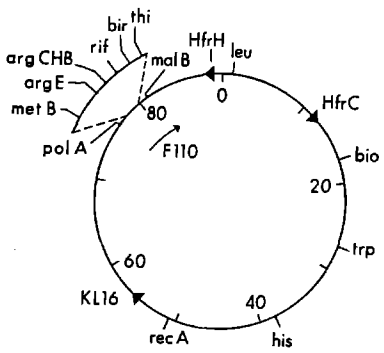

Fig. 2. *E. coli* genetic map. The location of the markers used in this study, the origin of transfer of strains X407 and X341, and the portion of the chromosome carried by F-prime factor KLF10 are shown (Low, 1972; Taylor and Trotter, 1972)

Genetic Studies

Procedures for generalized transduction by phage $P1_{vir}$ and conjugation were according to the methods of Miller (1972). All Hfr and F′ strains were purified before use by the methods of Low (1973).

Scoring of the Phenotype of dhbB mutants

Phenotype of *dhbB* mutation was determined by the ability to cross-feed a *bioB* mutant of *E. coli*. Agar plates were prepared consisting of two layers; the bottom layer, about 20 ml, in a standard-size petri dish, contained minimal agar with required growth factors, 2,3,5-triphenyltetrazolium chloride (50 μg/ml) and a washed cell suspension of *E. coli* K-12-20 (*bioB20*). After the bottom layer had solidified, 15 ml of the same medium but without *E. coli* cells were overlaid. When phenotypically cross-feeding positive strain was streaked on such a plate, the growth of the biotin auxotroph could be seen within 10 hr under and around the streak by the appearance of pink colonies.

Chemicals

d-Biotin-carbonyl-^{14}C with a specific activity of 31.5 mCi/mmole was purchased from Amersham/Searle Corp. α-Dehydrobiotin was obtained from Dr. W. E. Scott of Hoffmann-La Roche Inc., Nutley, N.J.

Results

Isolation of Dehydrobiotin (DHB) Resistant Mutants

Strains AB1157 and X407 were mutagenized with nitrosoguanidine (Adelberg *et al.*, 1965), and plated onto minimal agar containing DHB (5 μg/ml). After 2 days of incubation at 37° C, colonies were picked and purified by restreaking on TBAB plates.

Properties of DHB Resistant Mutants

Preliminary characterization of the DHB^R mutants indicated that they fall into two distinct categories. The first category comprises mutants (188 our of a total of 216) which overproduce biotin as indicated by a positive reaction on cross-feeding plates. The second category consists of mutants which are not

Table 2. Biotin biosynthetic activities of dehydrobiotin resistant mutants

Strain	Class	MIC[a] (μg/ml)	Cross-feeding[b]	Relative amount of[c] biotin produced		Relative specific activity[d]	
				Extra-cellular biotin	Intra-cellular free biotin	Desthio-biotin synthetase	biotin syn-thetase
AB1157	Wild type	0.78	—	1.0	1.0	1.0	1.0
P422	*dhbA*	25	—	2.5	1.0	2.1	1.8
P514	*dhbB*	>50	+	25	38	11	12

[a] Minimum inhibitory concentration of dehydrobiotin (see Materials and Methods).
[b] Ability to cross-feed *bioB* strain of *E. coli*.
[c] Cells were grown in minimal medium supplemented with Difco vitamin-free casamino acid (2g/l) for 16 hr, and the amount of biotin in culture supernatant fluid (extracellular biotin) and intracellular biotin that is extractable by boiling in distilled water (intracellular free biotin) were determined by *L. plantarum* assay. The actual values of AB1157 were 1.5×10^{-2} ng/ml for extracellular biotin and 0.2×10^{-1} ng/mg dry weight cells for intracellular free biotin.
[d] Cells were grown the same way as above, and crude extracts were used for desthiobiotin synthetase assay. Biotin synthetase was assayed with whole cells. The actual values of AB1157 were 0.55×10^{-2} ng d-biotin formed per min per mg dry weight cells for biotin synthetase and 0.7×10^{-6} μmoles d-desthiobiotin formed per min per mg protein for desthiobiotin synthetase.

distinguishable from the parent strain on crossfeeding plates. These two classes of mutants were designated *dhbB* and *dhbA*, respectively.

The biotin biosynthetic activities of representative strains of *dhbA* and *dhbB* mutants were compared with those of the parent strain. As shown in Table 2, strain P514 (*dhbB*) accumulated a large amount of biotin in the culture supernatant fluid and in the intracellular free pool with significantly elevated levels of biotin synthetase and desthiobiotin synthetase. Strain P422 (*dhbA*), although almost as resistant to DHB as strain P514, was similar to the parent strain (AB1157) with regard to the amount of biotin produced, biotin present in intracellular free pool, and the levels of the biotin biosynthetic enzymes. Furthermore, the biotin biosynthetic enzymes of *dhbB* mutants were not repressed by 10 ng per ml of biotin whereas those of *dhbA* mutants were repressed as much as in the wild type under the same conditions (Table 3). The amount of biotin precursors that accumulated in the culture supernatant fluid was also examined by microbiological assay using *Saccharomyces cerevisiae* (data not shown). *S. cerevisiae* responds not only to biotin, but also to its precursors including DTB, DAP and 7-KAP. In *dhbB* mutants as much biotin precursors (presence of 7-KAP and DTB was confirmed by bioautographic technique described by Pai, 1969, 1971) were accumulated in biotin excess medium as in biotin-free medium, but in *dhbA* mutants and in the parent strain accumulation was significantly reduced in the presence of exogenous biotin. These results suggested that the mechanisms of resistance to DHB are entirely different in these two classes of mutants. In *dhbB* mutants inhibitory effects of DHB appear to be overcome by acquiring a defective system of regulation that results in constitutive synthesis of the biotin biosynthetic enzymes, but in *dhbA* mutants the mechanism of DHB resistance was not apparent from the experimental results.

Table 3. Effect of exogenous biotin on levels of biotin biosynthetic enzymes[a]

Strain	Biotin[b] concentration (ng/ml)	Specific activity[c]	
		Desthiobiotin synthetase	Biotin synthetase
AB1157 (*dhb*$^+$)	0	1.0	1.0
	10	0.09	0.18
P422 (*dhbA*)	0	1.0	1.0
	10	0.12	0.2
P514 (*dhbB*)	0	1.0	1.0
	10	0.96	1.2

[a] Cells were grown in minimal medium containing 2 g per liter of Difco vitamin-free casamino acids for 16 hr.
[b] Indicated amount of biotin was added at zero time.
[c] Expressed as a fraction of value obtained with corresponding controls without added biotin.

Uptake of Biotin by dhbA Mutants

Since no significant difference was found in the biotin biosynthetic activities between *dhbA* mutants and the wild type, the possibility of impaired uptake of DHB by the mutants was investigated. Because neither radioactive DHB nor a reliable microbiological assay of the analogue (C. H. Pai, unpublished ovservation) were available the problem was studied by comparing biotin uptake and its inhibition by DHB in the wild type and the *dhbA* mutants. It was reasoned that, because of their structural similarity, both biotin and DHB should share the same transport mechanism and hence any change in observed biotin uptake could be interpreted as indicating a change in DHB transport.

Uptake of ^{14}C-biotin by a *dhbA* mutant was compared with the parent strain (Figs. 3A and B). The initial rate of uptake as well as the amount of biotin accumulated was much reduced in strain P422 (*dhbA*) as compared with AB1157 (the parent strain). The K_m values for the parent and mutant strains were 3.3×10^{-8} M (Fig. 4) and 9.2×10^{-8} M (data not shown), respectively. Biotin uptake in the wild type was competitively inhibited by DHB (Fig. 4). The parent strain was much more sensitive to DHB than was the mutant in its inhibition of biotin uptake (Fig. 5). These results suggested that the biotin transport system of the *dhbA* mutants was deficient for uptake of biotin and also for DHB, a structural analogue of biotin.

Mapping of dhbB Gene

The constitutive synthesis of the *bio* enzymes can be caused by mutations leading to the synthesis of a defective repressor molecule or a defective operator. Since *bio* is located at min 17 on the chromosome, and a mutation near the *arg ECBH* region at min 79 was reported to cause a derepression of the *bio* enzymes (Pai, 1972), all mutants that are cross-feeding positive (CF$^+$) were examined to see if any of the mutant genes were located at one of these two sites. All CF$^+$ mutants isolated from AB1157 (F$^-$ *leu argE met*$^+$ *str*) were crossed with X341 (HfrC *leu*$^+$ *arg*$^+$ *metB str*$^+$) by conjugation selecting for Leu$^+$ Sm^R. The recombi-

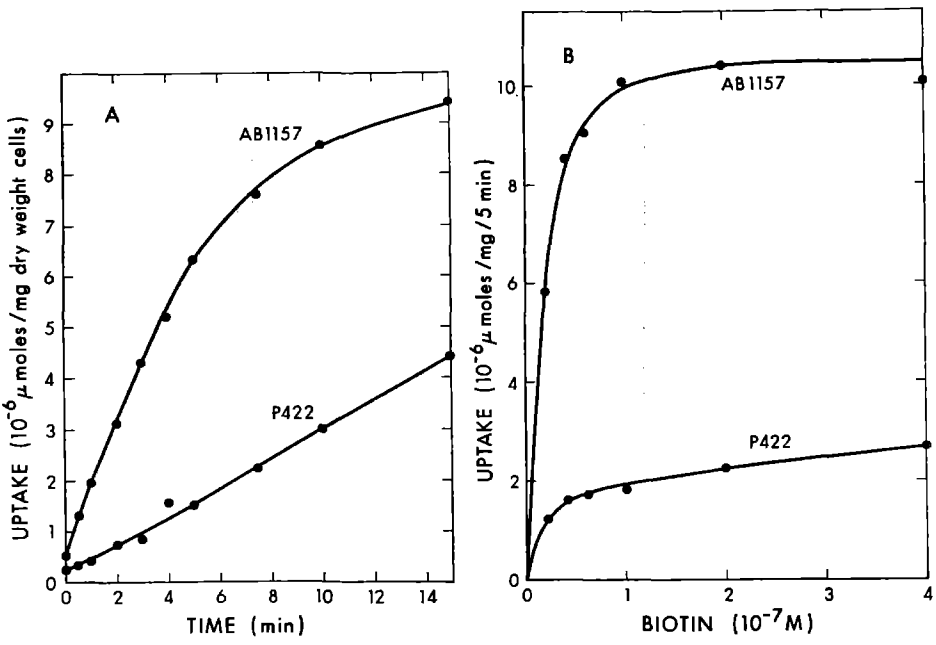

Fig. 3 A and B. Comparison of biotin uptake by a *dhbA* mutant (strain 422) with wild type (strain AB1157). Cells were grown for 16 hr in minimal medium supplemented with Difco vitamin-free casamino-acids (2 g/l) and washed with warm distilled water. Tubes containing ^{14}C-biotin were equilibrated at 37° C for 5 min before cells were added and incubated for the indicated duration. Samples were filtered through millipore filters, which were then washed with chilled distilled water and dried. The radioactivity was counted in a toluene-based scintillation fluid. (A) Uptake versus time. Concentration of ^{14}C-biotin used was 2×10^{-7} M. (B) Uptake versus concentration of biotin. Samples were taken at zero and five min and zero time values were subtracted from those of five min

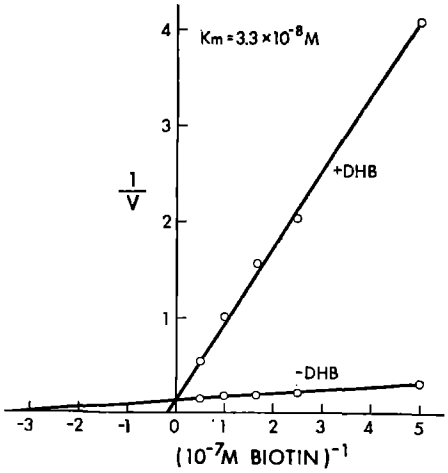

Fig. 4. Effect of DHB on biotin uptake in the wild type (strain AB1157). Experimental conditions were as described in Fig. 3. DHB when added was at a concentration of 10^{-6} M. Velocity was expressed in 10^{-6} μmoles of biotin taken up per 5 min

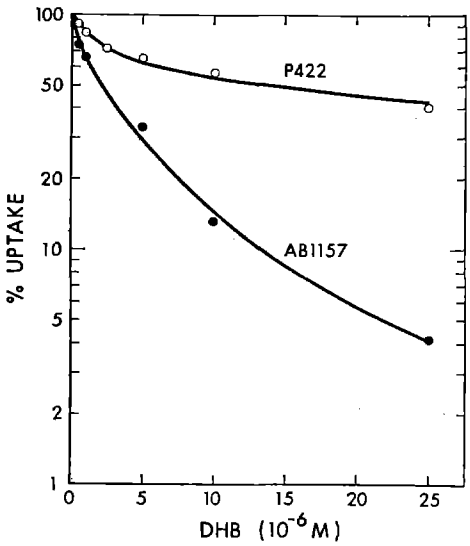

Fig. 5. Inhibition of biotin uptake by DHB in a *dhbA* mutant (strain 422) and the parent strain (AB1157). Experimental conditions were as described in Fig. 3. Uptake medium contained 4×10^{-8} M biotin and varying concentrations of DHB. The amount of ^{14}C-biotin accumulated in the absence of DHB in the first 6 min was taken as 100%

nants were then scored for CF, Arg, and Met phenotypes. In all mutants the mutant gene(s) causing CF+ phenotype was linked tightly with *argE* and *metB*. Also, all CF+ mutants isolated from X407 (HfrH [*proC thi*) were crossed with AA6 (F− *proC purE trp metB str*)] selecting for Trp+ Sm^R. None of Trp+ recombinants were CF+.

Having established the approximate location of *dhbB* gene, its orientation with respect to *metB*, *argE*, and *rif* was determined by phage P1 transduction.

The results of 3-factor crosses shown in Table 4 suggested the order *metB arg(ECBH) dhbB rif* clockwise. A summary of gneetic studies of *dhbB* gene is shown in Fig. 6.

A mutation that causes constitutive synthesis of the *bio* enzyme was reported from this laboratory (Pai, 1972, 1973). Since the mutant gene (*bioR*) was found to be cotransducible with *argE* also, genetic properties of the *bioR* strain (P48-3) was compared with those of *dhbB* mutants. Both strains are resistant to DHB and cross-feeding positive. The *bioR* mutation is also located between *argH* and *rif* (Table 5). It seems plausible that all presently isolated DHB-resistant cross-feeding positive mutants (*dhbB*) and the *bioR* mutants are altered in the same gene.

Dominance

To determine the dominance relationship of *dhbB*, a merodiploid strain was constructed. A *recA* derivative of strain P493-1 (*F− argE metB his dhbB493 leu+ str*) was obtained by crossing it with strain KL16-99 (HfrKL16 *recA his+ str+*), selecting for His+ Sm^R. A UV-sensitive His+ Arg− Met− CF+ recombinant (strain

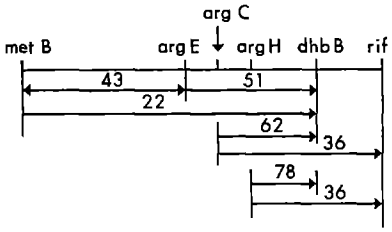

Fig. 6. Position of *dhbB* on the genetic map of *E. coli*. The figures represent co-transduction percentages, and the arrowheads indicate the unselected markers. Where reciprocal transduction have been done (double arrowhead), the co-transduction frequencies have been averaged. The relative location of *metB argECBH* and *rif* is according to Taylor and Trotter (1972)

Table 4. Three-factor transduction crosses ordering *dhbB*

a) Donor: AB1157 (met$^+$ *argE3 dhb*$^+$).
Recipient: P514-1 (*metB arg*$^+$ *dhbB514*).
Selected phenotype: Met$^+$.

metB	*argE*	*dhbB*	No. of recombinants	Order implied by results
1[a]	1	1	35	
1	1	0	33	*metB-argE-dhbB*
1	0	1	5	
1	0	0	116	

b) Donor: P514-1 (*arg*$^+$ *dhbB514 rif*$^+$).
Recipient: P601 (*argC32 dhb*$^+$ *rif*).
Selected phenotype: Arg$^+$

argC	*dhbB*	*rif*	No. of recombinants	Order implied by results
1[a]	1	1	47	
1	1	0	69	*argC-dhbB-rif*
1	0	1	2	
1	0	0	70	

c) Donor: P514-1 (*arg*$^+$ *dhbB514 rif*$^+$).
Recipient: SB96 (*argH dhb*$^+$ *rif*).
Selected phenotype: Arg$^+$.

argH	*dhbB*	*rif*	No. of recombinants	Order implied by results
1	1	1	97	
1	1	0	138	*argH-dhbB-rif*
1	0	1	8	
1	0	0	57	

[a] "1" and "0" refer to donor and recipient phenotypes, respectively.

Table 5. Analysis of three-factor transduction cross involving *argH*, *bioR*, and *rif*
Donor: P48-3 (*arg*$^+$ *bioR10 rif*$^+$).
Recipient: SB96 (*argH bioR*$^+$ *rif*).
Selected phenotype: Arg$^+$.

argH	*bioR*	*rif*	No. of recombinants	Order implied by results
1[a]	1	1	95	
1	1	0	46	*argH-bioR-rif*
1	0	1	4	
1	0	0	72	

[a] "1" and "0" refer to donor and recipient phenotypes, respectively.

Table 6. Repressibility of desthiobiotin synthetase in haploid and merodiploid *dhbB* mutants[a]

Genotype[b]	Desthiobiotin synthetase[d]	
	without biotin	with biotin
dhbB$^+$	1.0	0.12
dhbB$^-$	9.5	9.8
dhbB$^-$/F′*dhbB*$^+$	1.2	0.11
dhbB$^-$[c]	10.5	9.9

[a] Enzyme assays were performed after overnight growth in minimal medium plus Difco vitamin-free casamino acids (2 g/l) with or without biotin (10 ng/ml).
[b] All strains were *recA* derivatives of strain AB1157. The *dhbB*$^-$/F′*dhbB*$^+$ strain was constructed by crossing KLF10/JC1553 with P493-1-R.
[c] A segregant from *dhbB*$^-$/F′*dhbB*$^+$ strain.
[d] Expressed as a function of the specific activity of *dhbB*$^+$ strain grown without biotin.

P493-1-R) was then crossed with strain KLF10/JC1553 (*argG his leu metB*) that carries F′ factor KLF10 selecting for Leu$^+$ His$^+$ Arg$^+$ Met$^+$. Sixty-six colonies were picked and all were found to be CF$^-$. Two of these strains were plated out on TBAB plates and 30 colonies from each strain were tested again for Arg, Met, and CF phenotypes. They contained a mixture of Arg$^-$ Met$^-$ CF$^+$ and Arg$^+$ Met$^+$ CF$^-$ colonies, indicating that the isolates from the cross between strains P493-1-R and KLF10/FC1553 are CF$^-$ phenotypically and heterozygous for *dhbB*. The repressibility of the biotin pathway was examined in haploid and merodiploid *dhbB* mutants by measuring the levels of desthiobiotin synthetase under repressed and non-repressed conditions (Table 6). Since the *dhbB*$^-$ and dhbB$^-$/F′dhbB$^+$ strains are both *recA*, strain AB1157 (*dhbB*$^+$) was made *recA* and used as a control. This precaution was taken because *recA* strains grow more slowly than their *recA*$^+$ parents (Willetts and Mount, 1969). In the *dhbB*$^-$/*dhbB*$^+$ merodiploid desthiobiotin synthetase was as much repressible as in the *dhbB*$^+$ haploid, and a *dhbB*$^-$ segregant from the merodiploid produced the enzyme constitutively. The *dhbB*$^+$ gene is therefore dominant over the mutant allele.

Discussion

The DHB resistant mutants isolated in this study were grouped into two classes, *dhbA* and *dhbB*.

In the *dhbA* mutants, biotin biosynthetic activity is normal in that the amount of biotin and its precursors excreted in the culture supernatant fluids and the levels of two biosynthetic enzymes tested are similar to the wild type strain, and that the biotin biosynthetic pathway is subject to repression by biotin. The results of biotin uptake and its inhibition by DHB are suggestive of a defective transport of the antimetabolite in these mutants. This hypothesis is based on the following observations: (*a*) uptake of biotin in *E. coli* is inhibited competitively by DHB (Fig. 4) suggesting that biotin and DHB are transported by the same system; (b) uptake of biotin is greatly reduced in *dhbA* mutants (Fig. 3); (c) biotin uptake in *dhbA mutants* is not as much inhibited by DHB as in the wild type (Fig. 5). A mutation that causes an alteration of the biotin transport system could result in a loss of affinity for biotin, and even to a greater degree, for DHB. Mutants of *E. coli* with a simultaneous reduction in the transport of an amino acid and its analogue have been found among L-canavanine (Schwartz *et al.*, 1959) and DL-*p*-fluorophenylalanine (Ames, 1964) resistant mutants. In canavanine resistant mutant, for example, the uptake of L-arginine is reduced and, furthermore, arginine uptake which is inhibited significantly by canavanine in the wild type is only slightly affected in the mutant.

The *dhbA* locus has not been mapped in the present study.

The *dhbB* mutants appear to be resistant to DHB as a result of a mutation in the system that regulates the synthesis of the *bio* enzymes. The mutants accumulate a large amount of biotin and its precursors in culture supernatant fluids with elevated levels of the biotin biosynthetic enzymes. Furthermore, the biotin pathway is not repressed by biotin. The location of the *dhbB* gene far from the cluster of structural genes of the biotin pathway and a trans-dominant relationship of the $dhbB^+$ gene over the mutant allele suggested that it controls the production of a diffusible substance. Such a relationship has been established as a genetic criterion in defining a mutation in the repressor molecule for a series of regulatory systems, such as β-galactosidase (Jacob and Monod, 1961) and the arginine pathway of *E. coli* (Maas and Clark, 1964). Investigation is in progress to identify the nature of the *dhbB* product.

Mutants of *E. coli* with constitutive synthesis of the *bio* enzymes were reported by Campbell *et al.* (1972) and by Pai (1972, 1973). Campbell's mutant (*bir*) was isolated as a high biotin requirer and appears to be deficient in biotin transport and retention causing a derepression of the biotin pathway as a secondary effect. The *bir* gene was located between *argE* and *thi*, but its orientation with respect to *rif* was not determined. Pai's mutant (*bioR*) was isolated as a high biotin excreter and was different from the *bir* mutant in a number of biochemical characteristics (Pai, 1972). The *bioR* mutant was identical with the *dhbB* mutant with respect to phenotypic properties and the location of the mutant genes. This suggests that *bioR* and *dhbB* are the same gene, although the *bioR* mutant was selected for biotin overproduction and the *dhbB* mutant for DHB-resistance.

The mode of action of DHB is not known at the present time. A competitive inhibition of biotin uptake by DHB (Fig. 4) could account for the inhibition by the analogue of growth of biotin-requiring microorganisms, such as *Lactobacillus plantarum* and *Saccaromyces cerevisiae* (Hanka *et al.*, 1969). DHB may also inter-

fere with the utilization or biosynthesis of biotin. Lane *et al.* (1964) found that the enzymatic synthesis of holotranscarboxylase from apotranscarboxylase and (+)-biotin in *Propionibacterium shermanii* is inhibited competitively by a number of biotin analogues, such as (+)-homobiotin, dl-o-heterobiotin. Although DHB was not tested in their studies, if one assumes that the synthesis of biotin holoenzymes is similarly inhibited by DHB, overproduction of biotin as a result of derepression would overcome an inhibition of growth by DHB. The possibility of DHB interfering with the biosynthesis of biotin was reported by Vrancic and Guha (1973). The *bio* genes are arranged in two divergently transcribed gene clusters with the promotor-operator region located among the structural genes (Guha *et al.*, 1971; Clearly *et al.*, 1972). Vrancic and Guha (1973) found that transcription of both *l* and *r* transcribing gene clusters is repressed by biotin, and DHB mimics the action of biotin by repressing the *l* transcribing cistron although the *r* transcribing cistrons are not affected. A mutation in the repressor molecule, a combining site for DHB, could result in constitutive synthesis of the *bio* enzymes and, at the same time, resistance to DHB.

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