Genetic and Biochemical Analysis of the Biotin Loci of *Escherichia coli* K-12

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Some 60 biotin auxotrophs of *Escherichia coli* K-12 were isolated and classified into four groups according to their cross-feeding patterns, excretion products, and their ability to show a growth response to various biotin vitamers. Since all the mutants could be transduced with λ dbio phages known to carry the entire *bioA* locus, it was concluded that all of the mutation sites were located in this locus. It was also possible to derive a gene order for the different mutant groups on the basis of transduction studies with various λ dbio phages that carry portions of the *bioA* locus. A possible biochemical pathway for the biosynthesis of biotin in *E. coli* K-12 is discussed.

The presence of biotin and biotin vitamers in the growth medium of various bacteria and fungi has been amply documented over the past two decades and especially by the recent extensive study of Ogata et al. (17). The identification of two of these vitamers as desthiobiotin and biotin-L-sulfoxide was achieved some years ago (22, 25), and only recently has the chemical nature of some of the other vitamers been established. However, over this period of time, there has been no concerted effort to determine the origin of these vitamers or their role in the cell metabolism.

There are now several lines of evidence available which suggest that desthiobiotin is directly on the pathway of biotin biosynthesis. This vitamer can support the growth of a variety of biotin-requiring organisms and also accumulates in the growth medium of certain biotin auxotrophs (15, 22). The formation of desthiobiotin can be enhanced by the addition to the growth medium of pimelic acid, which was shown to be a direct precursor of biotin (5, 14). Furthermore, the conversion of desthiobiotin to biotin has been demonstrated with a number of microorganisms and appears to be under the control of biotin itself (1, 4, 21).

Another vitamer, characterized by its avidin-uncombability (3, 6, 11), was recently proposed by Eisenberg (7) as a possible intermediate in the synthesis of biotin. Studies of the vitamer found in the filtrates of *Phycomyces blakesleeana* and *Penicillium chrysogenum* indicated that it possesses an open chain structure containing both an amino and a carboxyl group and is devoid of

sulfur (5-7). This same vitamer became labeled when the organism was grown in the presence of radioactive pimelic acid. When yeast cells were subsequently grown in the presence of the purified radioactive vitamer, it was found to be directly incorporated into the bound biotin (8, 9). As with desthiobiotin, the production of the unknown vitamer is under biotin control (9). The complete identification of the vitamer as 7-oxo-8-aminopelargonic acid became possible with its isolation in crystalline form (M. A. Eisenberg and R. Maseda, *in preparation*). An avidin-uncombable vitamer in culture filtrates of *Bacillus sphaericus* was similarly identified by chromatography in three solvent systems (14).

Two different schemes have been proposed for the biosynthesis of biotin. Okumura et al. (18), employing feeding experiments, compared the growth-promoting ability of a number of pelargonic acid derivatives with that of biotin and desthiobiotin for a variety of biotin-requiring organisms. As the result of this study, they proposed the following sequence for biotin synthesis: pimelic acid $\rightarrow$ 7,8-diketopelargonic acid $\rightarrow$ 7-oxo-8-aminopelargonic acid $\rightarrow$ 7,8-diaminopelargonic acid $\rightarrow$ desthiobiotin $\rightarrow$ biotin. In contrast to this scheme Lezius et al. (16), on the basis of isotope incorporation studies, proposed the formation of biotin from cysteine, pimelyl-coenzyme A (CoA), and carbamyl phosphate. The important difference between the two pathways was that the former scheme relegated the incorporation of sulfur to the final steps of the reaction sequence as first suggested by Tatum (22),

whereas the second hypothesis would introduce the sulfur at the first step, namely, the condensation of pimelic coenzyme A and cysteine.

In view of the contradiction between these hypotheses and the paucity of substantial evidence to support either one, it was felt that a more systematic approach to the problem was required. Accordingly, we have recently isolated a large number of biotin-requiring mutants of *Escherichia coli* K-12. A biochemical and genetic analysis of these mutants has thus far enabled us to classify them into four major groups which are readily distinguished by the number as well as the kind of the vitamins excreted into the growth medium. The vitamins have been identified by their chromatographic and electrophoretic properties. Information on the complexity of the chromosomal locus at which the mutations occur has also been obtained in a genetic analysis of the mutants with biotin-transducing λ phages.

MATERIALS AND METHODS

Bacterial strains. Biotin-requiring mutants were isolated from cultures of the *E. coli* K-12 strains Y10-1 (12) and HfrH (13). These parent strains, as well as two biotin-requiring deletion mutants of K-12 (T5-2 and T50-1), were kindly provided by C. Fuerst. The entire *bioA* locus is deleted from both of the biotin-requiring mutants (MacQuarrie and Fuerst, *personal communication*). Several deletion mutants of the *malA* locus which have a biotin requirement were kindly provided by M. Schwartz. Taylor and Trotter (23) recently referred to this biotin locus as *bioB*.

Phage strains. Preparation of λ^+ phage were obtained by ultraviolet (UV) induction of the lysogen Y10 (12). Defective biotin-transducing (λ *bio*) mutants of λ were obtained as phage stocks or lysogens from A Campbell and C. Fuerst. Two of the λ *bio* mutants, t75 and t103, carry the entire *bioA* locus (MacQuarrie and Fuerst, *personal communication*). The remaining λ *bio* transducing phages used in this investigation carry various amounts of *bioA*.

Media. Nutrient broth and phosphate-buffered saline (PBS) were prepared as described by Harris et al. (12).

Minimal medium contained (per liter of distilled water): KH_2PO_4 , 6.8 g; $(\text{NH}_4)_2\text{SO}_4$, 1.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g; $\text{Ca}(\text{NO}_3)_2$, 0.01 g; thiamine, 5.0 mg; glucose, 2.5 g; and L-leucine, 100.0 mg.

Minimal agar used in assay plates for biotin transduction assays contained the above ingredients plus 15 g of agar (Difco). In some cases, 1.0 ml of a 5% triphenyltetrazolium chloride was added per liter. Minimal agar used in overlays contained 10 g of agar per liter and all of the ingredients listed above.

Isolation of biotin-requiring mutants. The isolation of mutants was based on a limited enrichment technique in which growth of biotin-requiring mutants can occur, but at a reduced rate because of the suboptimal concentration of biotin employed in the plate agar. Reconstruction experiments with known mixtures of

bio⁻ and *bio*⁺ cells spread on minimal agar showed that the method was far more successful in discriminating between *bio*⁺ and *bio*⁻ colonies than isolation procedures based on replica-plating techniques. Supplementation of minimal agar with biotin (10^{-5} $\mu\text{g}/\text{ml}$) and incubation of the plates at 37 C for about 48 hr gave optimal contrast in colony size. Appreciable cross-feeding of *bio*⁻ colonies by *bio*⁺ colonies occurs if the plates are incubated for longer times. Biotin concentrations of 10^{-4} $\mu\text{g}/\text{ml}$ or greater minimize the size differences of *bio*⁻ and *bio*⁺ colonies.

Cultures of parental strains used for the isolation of mutants were grown overnight in nutrient broth and centrifuged. The cells were resuspended to their original concentration in PBS containing *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich Chemical Co.) at 25 $\mu\text{g}/\text{ml}$ and incubated for 30 min at 37 C. To minimize the possibility of isolation of sibling mutants, samples of each culture were dispensed into a large number of small tubes containing nutrient broth and were grown overnight at 37 C. These overnight cultures were diluted in PBS, and samples containing 100 to 150 viable cells were spread on minimal agar plates. The plates employed were supplemented with sub-optimal concentration of biotin (10^{-5} $\mu\text{g}/\text{ml}$); tetrazolium chloride was sometimes added to enhance the contrast between the colony types. The plates were then incubated at 37 C for 48 hr before their first examination. The location of each small colony was marked, and droplets of a biotin solution (1 $\mu\text{g}/\text{ml}$) were spotted onto the plates close to these marked colonies. The plates were then incubated at 37 C for another 18 to 24 hr. Those colonies which showed a marked increase in size over this period were picked with sterile toothpicks and tested for their biotin requirement. A high proportion of the colonies selected in this way were found to be biotin-requiring.

Cross-feeding studies. Cross-feeding studies were carried out with cultures that had been grown in nutrient broth for 18 hr with continuous shaking at 37 C; they were then washed three times in PBS. Tubes containing 2.0 ml of minimal medium supplemented with biotin (10^{-4} $\mu\text{g}/\text{ml}$) were inoculated with the washed cells to a final concentration of 10^6 to 5×10^6 cells/ml and incubated for 18 to 24 hr. A control tube, without biotin, was inoculated with the same concentration of cells to check for contamination and revertants. Those cultures which showed growth in the control at the end of the incubation period were not used.

Two methods were employed for the cross-feeding studies. (i) Cultures of three different *bio*⁻ mutants were separately streaked on minimal agar plates. The streaking, in a side-by-side boomerang-like pattern, allows compounds excreted by one strain to diffuse toward another strain during incubation of the plate. By this procedure, a number of cross-feeding interactions may be scored on a single plate. (ii) Small metal cylinders (diameter and height of about 1 cm) were sterilized and pushed into the surface of minimal agar plates. Minimal overlay agar inoculated with cells of a single mutant strain was poured over the surface of the plate. Care was taken to ensure that each cylinder was pressed deep enough into the plate agar

to avoid contact of cells in the overlay with the interior of the cylinders. Washed suspensions of cells or solutions of biotin vitamers were added to the different cylinders on each plate. Cross-feeding under these conditions resulted from diffusion of the biotin vitamers excreted by the cell suspensions in the cylinders through the plate agar to the surrounding cells in the overlay agar.

Growth response experiments. The "total" biotin content of samples from the culture filtrates of each mutant was determined by the yeast disc assay procedure previously described (7). When biotin-requiring mutants of the various groups were used as the assay organism, 5×10^6 washed cells were added per milliliter of minimal agar medium.

Ascending paper chromatography was carried out on Whatman 3MM filter paper at 4 C with one of the following solvents: solvent 1, *n*-butanol-acetic acid-water (60:15:25); solvent 2, *n*-butanol-pyridine-water (1:1:1); solvent 3, *n*-butanol-formic acid-water (4:1:1). The chromatograms were air-dried and developed by placing the paper onto the surface of a minimal agar medium inoculated either with yeast cells or one of the *E. coli* mutants. When the *bio*⁻ mutants were used as the assay organism, tetrazolium dye was added to the medium to make the growth areas more prominent.

Combined chromatography and electrophoresis was carried out in the manner described elsewhere (6). Paper strip electrophoresis was carried out at 300 v for 3 hr in a Durrum cell (Spinco) with the following buffers: pH 2, 0.6 M acetic and 0.1 M formic acids; pH 3, 0.025 M sodium citrate; pH 5, 0.025 M sodium acetate; pH 7, 0.025 M sodium phosphate. The vitamers were demonstrated by bioautography as described above.

Reagents. *d*-Biotin and *dl*-desthiobiotin (DTB) were obtained from Mann Research Laboratories; *dl*-7,8-diaminopelargonic acid (DAP) was prepared from DTB by the method of duVigneaud et al. (24), and L-7-oxo-8-aminopelargonic acid (7-KAP) was synthesized by the method of T. Suyama and S. Kaneo (Japanese Patent 19,716, 1963). All other chemicals were reagent grade.

Terminology used. The term "total" biotin as used here refers to the biological equivalence of biotin vitamers in culture filtrates compared to biotin itself, as determined by growth of *Saccharomyces cerevisiae* in the disc assay procedure.

Mapping of mutation sites. A genetic analysis of the biotin-requiring mutants was carried out by transduction with λ *dbio* phages. Lysates of the λ *dbio* phages required for this purpose were prepared as follows. Stationary-phase cultures of each λ *dbio* lysogen were diluted 50-fold with fresh nutrient broth and incubated at 37 C until a cell concentration of about 2×10^8 /ml was obtained. The bacteria were centrifuged and resuspended to four times their original concentration in an 0.04 M MgSO₄ solution. The concentrated cell suspensions were irradiated with UV to induce phage development, and were immediately diluted fourfold with fresh, prewarmed nutrient broth containing 6×10^8 λ ⁺ particles/ml. The cell suspensions were incubated at 37 C until visible signs of lysis occurred (usually about 2 hr). The lysates were then sterilized with

chloroform, centrifuged, and stored at 4 C. The titer of the infective phage particles in these lysates was determined by plaque assay on Y10-1. Titration of λ *dbio* particles was performed by transduction assays with appropriate biotin-requiring mutants. All lysates containing λ *dbio* phage were diluted to a standard concentration of 2.0×10^9 infective phage particles/ml prior to use in mapping studies.

Deletion mapping of the biotin-requiring mutants by transduction analysis was carried out essentially as described by Del Campillo-Campbell et al. (2). Bacterial lawns of washed-cell suspensions of the different biotin-requiring mutants were prepared by pour-plating 2×10^8 cells/ml of overlay agar onto minimal agar plates (containing tetrazolium dye). Loopfuls of each transducing phage lysate were spotted onto these bacterial lawns, and the plates were then incubated at 37 C for 36 hr. The results of each spot test were scored as positive or negative depending on whether or not growth occurred. Growth, either confluent or as discrete colonies, was taken as evidence that the *bio* segment carried by the phage extended beyond the site of mutation in the bacterial mutant employed. Absence of growth showed that the *bio* segment in the phage did not extend as far as the point of mutation.

RESULTS

Sixty biotin auxotrophs of *E. coli* K-12 were isolated and their general growth characteristics examined. All the mutants were checked for their reversion frequencies, and those strains with high values were discarded. The selected mutants, 52 in all, showed only slight differences in growth rates when grown in either nutrient broth or liquid minimal medium supplemented with biotin (10^{-4} μ g/ml).

Detection of biotin vitamers. Pai and Lichstein (19) have shown that an exogenous biotin concentration of 10^{-4} μ g/ml does not cause repression of the biosynthesis of biotin. Filtrates of cultures grown in minimal medium supplemented with biotin at this concentration were therefore assayed (yeast disc) for the presence of biotin intermediates that might be expected to accumulate because of the biotin genetic block carried by the different mutants. These assays revealed marked differences among mutants, as shown by the fact that the levels of "total" biotin in the filtrate ranged from 4.0×10^{-1} μ g/ml to less than 2.0×10^{-5} μ g/ml. The latter value represents the lowest concentration of biotin that can be detected by the disc assay procedure.

A bioautographic analysis of these culture filtrates, with yeast as the assay organism, demonstrated that the mutants also differed as to the nature and number of biotin vitamers excreted, which provided a basis for their classification into four major groups. Table 1 shows the excretion pattern of the biotin vitamers for each of these groups. Group I mutants did not excrete any com-

TABLE 1. Vitamer excretion pattern of *Escherichia coli bioA* mutants

Group	R_F in solvent 1	Strain no.
I		110, 114, 115, 123, 124, 125, 128, 129, 138, 141, 301, 304, 315
II	0.62-0.67	104, 109, 121, 126, 130, 132, 134, 136, 137, 140, 309, 312, 314
III	0.62-0.67 0.36-0.45	113, 116, 117, 118, 119, 120, 127, 135, 139, 302, 303, 305, 306, 307, 308, 311, 317
IV	0.62-0.67 0.36-0.45 0.82-0.87	105, 107, 108, 112, 122, 133, 142, 310

ponents with vitamer activity. Group II mutants excreted a single vitamer with an R_F of 0.62 to 0.67. This same vitamer plus a new component (R_F of 0.36 to 0.45) was detected in filtrates of the group III mutants. The group IV mutants excreted the above two vitamers as well as a component with an R_F of 0.82 to 0.87.

The above chromatograms contained only 10 μ liters of culture filtrate, and frequently some of the vitamers were just barely visible. Since it was essential to determine whether minor components or less active vitamers were present in the filtrates of these four groups, the chromatography was repeated with 25 μ liters of filtrate. The vitamers localized in discrete areas (Fig. 1) which varied in size and density of growth, reflecting the concentration levels of each vitamer. A marked variation was observed in each vitamer concentration, not only among the four mutant groups but also among the different members of the same mutant group. No additional vitamers were found in groups I, II, and IV. However, a component with an R_F value of 0.24 to 0.26 (and barely visible) appeared in some of the mutant strains of group III. The parent strain also showed a very minor component with an R_F value of 0.45 (and barely visible). These minor components were not always detected in the culture filtrates and are at present under further investigation.

The identity of the vitamers was determined by a comparison of their chromatographic and electrophoretic properties with those of known compounds having vitamer activity. The results in Table 2 are confined to only three solvent systems and four buffer systems, but the same information was provided in other solvent systems and buffers at higher pH values. Under the conditions used, 25 μ liters was required to observe DAP in the electrophoretograms. Vitamer

1, the only vitamer excreted by group II mutants, behaved chromatographically and electrophoretically in a manner identical to 7-oxo-8-aminopelargonic acid. Vitamer 2, which was found along with vitamer 1 in the filtrates of group III mutants, behaved like 7,8-diaminopelargonic acid. Vitamer 3, which was present in addition to 7-KAP and DAP in the filtrates of both the group IV mutants and the parent strain, was identical to desthiobiotin. The presence of biotin in the parent strain could not be readily detected with *Lactobacillus plantarum* as the test organism. However, when the filtrate was concentrated about fivefold, it was readily observed.

On the basis of the foregoing results and with the knowledge of the precursor role of pimelic acid, one can formulate a reaction scheme for biotin biosynthesis with the underlying assumption that the vitamers appearing in the medium are derived directly from enzymatic reactions prior to the block.

To obtain additional evidence in support of this sequence of events, recourse was made to a series of feeding experiments. Mutants with genetic blocks that preclude growth on minimal medium should grow only on intermediates that are located beyond the genetic block, or on compounds that the cells are able to convert to these

FIG. 1. Chromatographic identification of biotin vitamers in filtrates of *E. coli* mutants. (1) Biotin; (2) group IV mutant; (3) desthiobiotin; (4) group III mutant; (5) 7,8, diaminopelargonic acid; (6) 7-oxo-8-aminopelargonic acid; (7) group II mutant.

TABLE 2. *Chromatographic and electrophoretic identification of biotin vitamers excreted by biotin-requiring mutants of E. coli*

Sample	Vitamin no.	R_F			Mobility (cm) ^a			
		Solvent 1	Solvent 2	Solvent 3	pH 2.0	pH 3.0	pH 5.0	pH 7.0
Group II (strain 34)	1	0.64	0.44	0.60	-8.8	-6.7	-1.1	+0.4
Group III (strain 307)	1	0.63	0.44	0.59	-8.8	-6.7	-0.9	+0.5
	2	0.37	0.18	0.31	-12.9	-9.6	-5.7	+2.8
Group IV	1	0.63	0.44	0.60	-8.8	-6.8	-0.7	+0.4
	2	0.39	0.16	0.32	-11.6 ^b	-9.2	+5.4	+2.5
	3	0.83	0.56	0.82	0.0	+0.7	+5.9	+7.5
Parent strain	1	0.64	0.43	0.59	-9.1	-6.9	-0.8	+0.5
	2	0.39	0.16	0.32	-12.1 ^b	-8.8	-5.5	+2.5
	3	0.83	0.56	0.80	-0.1	+0.2	+5.9	+7.2
7-KAP		0.63	0.45	0.60	-9.1	-7.3	-1.7	+0.4
DAP		0.38	0.20	0.30	-13.1 -12.3 ^b	-10.8 -9.3	-5.8 -5.8	+2.5 +2.6
DTB		0.83	0.56	0.81	0.0	+0.6	+5.6	+7.5
Biotin		0.77	0.54	0.74				
		0.52 ^c	0.42	0.52				

^a Migration toward the cathode is indicated by a minus; migration toward the anode is indicated by a plus. All values are corrected for electro-endosmosis.

^b Indicates that 25 μ liters of culture filtrate was used to observe DAP. All other values were obtained with 10 μ liters of filtrate.

^c Biotin-*d*-sulfoxide usually found on chromatography of biotin.

intermediates. Mutants blocked in an early step of the reaction sequence should therefore be fed only by other mutants blocked at a later step. Cross-feeding experiments, which are useful in ordering a biochemical reaction sequence, were therefore performed. The wild-type strain Y10-1 cross-fed all four mutant groups (Table 3). Group IV mutants fed the other three groups. Mutants of group II and group III fed only mutants in group I, which were unable to feed any other mutant group. These results are consistent with the reaction scheme shown above, except for the absence of cross-feeding between members of groups II and III.

When the mutants were tested for their growth response to various biotin vitamers, they reacted in the manner to be expected under the assumption that each group is blocked at a progressively later step in the biotin pathway (Table 4). A possible explanation for these divergent results was obtained when attempts were made to use a group I mutant as an assay organism. Bioautography of the group IV filtrates revealed only one vitamer, which had an R_F similar to desthiobiotin. As the group I organism will grow on both 7-KAP

TABLE 3. *Cross-feeding studies^a*

Mutant group being fed	Feeder				
	Y10-1	Mutant Group			
		I	II	III	IV
I	+	-	+	+	+
II	+	-	-	-	+
III	+	-	-	-	+
IV	+	-	-	-	-

^a The mutant strains, grown overnight in minimal medium supplemented with biotin (0.1 ng/ml), were washed twice and then separately streaked onto minimal medium plates. The cross-feeding interactions were then scored after 48 and 72 hr.

and DAP, one would have expected growth areas on the plate corresponding to these two vitamers, but none was observed. However, the addition of 10 ng of synthetic DAP, as a control, along with the filtrates also failed to produce a growth response, indicating a lower biological activity of

TABLE 4. Ability of biotin and biotin vitamers to support growth of the different mutant groups^a

Group	Biotin (4 ng)	DTB (8 ng)	DAP (2,000 ng)	7-KAP (50 ng)
I	+	+	+	+
II	+	+	+	—
III	+	+	—	—
IV	+	—	—	—

^a Each vitamer solution (0.2 ml, containing the above amounts) was placed on the minimal medium agar surface and spread uniformly. The plates were kept at room temperature for 1 hr prior to the addition of the cells from each mutant group. Only those cultures which showed no growth in the control were scored.

DAP for the mutant than for yeast. A calculation (based on the yeast disc assay) of the "total" biotin in the samples of the filtrates added indicated only 10 ng present; a previous yeast bioautogram of this material showed DTB as the main component.

To test the response of the group I mutant to the filtrates of the other mutant groups, a chromatogram was prepared with the filtrates of each group and various concentrations of DAP. DAP present in the group III and group IV filtrates failed to support the growth of the group I mutant (Table 5). At least 50 ng of DAP was required to obtain a barely discernible area of growth, and about 10 times this concentration (500 ng) was required to give an area of growth equivalent to that observed with the "total" biotin of the group IV filtrates, which is mainly DTB. It is apparent, then, that the amount of DAP present in 25 μ liters of filtrate from both group III and IV mutants is insufficient to support the growth of a group I mutant. On the other hand, the group II filtrate, containing 7-KAP, did give a growth response but only after 48 hr (and just barely visible). Since only 3.4 ng of "total" biotin was added to the chromatogram, this concentration must represent a minimal level of 7-KAP for supporting the growth of the group I mutant and would also explain the failure to obtain a response from the group III filtrate containing 7-KAP.

The poor response of the group I mutant to DAP and 7-KAP suggested the possibility of a similar response by the group II mutant to DAP, which would account for the negative results obtained in the cross-feeding experiment. By using two mutants from each group as the assay organism, a growth response curve was determined for each vitamer by the disc assay procedure. Table 6 shows the minimum concentration of each vitamer

TABLE 5. Bioautography of groups II, III, and IV culture filtrates and various concentrations of 7,8-diaminopelargonic acid with a group I mutant as assay organism

Sample	"Total" biotin in sample	Growth ^a	R _F
	ng	cm	
Group II.....	3.4	1.6	0.64
Group III.....	2.8	NG	
Group IV.....	10	4.9	0.83
DAP.....	50 ^b	1.3	0.38
DAP.....	100 ^b	2.2	0.38
DAP.....	200 ^b	3.2	0.38
DAP.....	500 ^b	4.4	0.38

^a Distance between the leading and trailing edges of growth in the direction of the solvent front. NG, no growth.

^b Absolute concentration of synthetic DAP (ng/25 μ liters).

necessary to give a barely discernible area of growth after 22 hr of incubation. The minimum amount of DAP which could be detected by group I and II mutants was 625 times greater than that for biotin, assuming only one active isomer. In contrast to these findings with DAP, the amounts required for DTB and 7-KAP were, respectively 2.5 and 10 times greater than for biotin. Thus, it is apparent that the failure to show cross-feeding between mutants of groups II and III is probably due to an insufficient concentration of DAP in situ to support growth of group II strains.

Biotin mutants. A recent report by Schwartz (20) demonstrated that some *E. coli* mutants of the *malA* locus show a nutritional requirement for biotin. The biotin locus defined by these mutants has since been called *bioB* (23). These "biotin" mutants isolated by Schwartz were tested for their growth response to the different vitamers used in the present study; they were found to grow in the presence of biotin, DTB, DAP, and 7-KAP at concentrations similar to those used in the studies of our biotin-requiring strains.

Location of the mutation sites in *bioA*. The *bioA* locus maps next to the *gal att* λ region of the bacterial chromosome (23). The recent isolation of λ *dbio* transducing phages (2, 10) has made it possible to carry out genetic studies of the *bioA* locus by transduction analysis. Since these transducing phages can carry the entire as well as varying amounts of the *bioA* locus, it is possible to establish whether the individual biotin-requiring mutants map at the *bioA* locus or not, and to position them relative to the end points of the biotin information carried by the various λ *dbio* transducing phage particles.

The initial screening of the mutants was carried out by using the biotin-transducing phages t75 and t103, which carry the entire *bioA* locus. All of our biotin-requiring mutants were transduced by these two phages and must therefore be mutant in *bioA*. With the aid of other λ dbio transducing phages, which carried different amounts of the *bioA* locus, it was possible to determine the chromosomal sequence of the mutant sites in the members of the four biotin-requiring groups. This analysis (Table 7) clearly establishes that the mutant groups are located relative to the λ attachment site on the bacterial chromosome in the order att λ II IV I III. A more refined genetic analysis of the mutant groups will be presented in another paper.

DISCUSSION

We have described an analysis of the synthetic pathway of biotin based on a biochemical and genetic study of a large number of biotin-requiring mutants of *E. coli* K-12. The biochemical analysis of the excretion products in culture filtrates of

the biotin-requiring mutants has provided a basis for the classification of the mutants into four groups. The excretion products of each group detected in bioassays employing yeast are: no detectable vitamers (group I); 7-KAP (group II); 7-KAP and DAP (group III); and 7-KAP, DAP, and DTB (group IV).

On the basis of the analysis reported here, as well as other studies still in progress, we envisage the reaction sequence shown in Fig. 2 for the biosynthesis of biotin. The initial step appears to be a condensation of pimelyl-CoA and alanine to form 7-oxo-8-aminopelargonic acid. Evidence for this step has been obtained in cell-free extracts (M. A. Eisenberg and C. Star, *in preparation*). Although the mechanism of conversion of 7-KAP to DAP is as yet uncertain, we assume that it occurs via a transaminase reaction. The group II mutants are probably bacteria with mutations in the cistron coding for this transaminase enzyme. Conversion of DAP to desthiobiotin could involve the formation of the ureido ring through the incorporation of CO₂. Evidence has already been found by Lezius et al. (16) that, in *Achromobacter*, the carbonyl group of the ureido ring can arise from CO₂. Furthermore, they suggested that the carbonyl group and one N group might be supplied by carbamyl phosphate in the biosynthesis of biotin. Whether CO₂ is fixed directly into DAP or is supplied by some CO₂-transferring reaction is presently under investigation. Mutants of group III probably represent bacteria with a genetic block in the CO₂ fixation reaction. It is also interesting to note that the *pyrA* mutants of *E. coli*, which appear to be blocked in carbamyl phosphate synthesis, do not show a biotin requirement (W. Maas, *personal communication*). Finally, the group IV mutants, which can grow only on biotin, probably lack the enzymatic apparatus for introducing the sulfur atom to form the tetrahydrothiophene ring.

The compounds 7-KAP and DTB of the above reaction sequence have already been implicated in biotin biosynthesis in previous studies of a variety of bacteria and fungi (8, 14, 19, 22), but the role of DAP has only been inferred as a result of experiments in which various pelargonic acid derivatives were fed to biotin-requiring mutants of fungi (18). The presence of DAP has not been reported previously in culture filtrates of bacteria, although its presence was indicated in investigations of filtrates from *Penicillium chrysogenum* (9). The previous failures to detect DAP in bacterial cultures were probably due to both the low concentrations of the vitamer and its low biological activity compared with biotin. That detection of DAP can be a problem is made very

TABLE 6. Minimum amounts of biotin vitamers required to support growth of the different mutant groups

Group tested	Biotin (ng/disc)	Biotin vitamer (ng/disc)		
		DTB	DAP	7-KAP
I	0.02	0.05	25	0.2
II	0.02	0.10	25	
III	0.02	0.10		

TABLE 7. Transduction of biotin mutants by λ dbio phages^a

Transducing phage	Mutant group			
	II	IV	I	III
t75	+	+	+	+
t103	+	+	+	+
53 (3-29) ^b	+	+	+	+
55 (18-14)	+	+	+	0
38 (46-2)	+	+	+	0
8 (42-2)	+	+	+	0
16 (37-1)	+	+	0	0
26 (33-1)	+	0	0	0
34 (19-5)	+	0	0	0

^a Symbols: + = transductants; 0 = no transductants.

^b The numbers in parentheses are those originally assigned by A. Kayajanian to these λ dbio phages. The numbers preceding the parentheses were suggested by A. Campbell for simplification (*personal communication*).

FIG. 2. Proposed mechanism for biotin biosynthesis.

evident by the quantitative growth studies of *E. coli* mutants described herein.

Transduction experiments by deletion mapping confirm the classification of the mutants into four different groups on the basis of their biochemical properties. The genetic studies show that the group order on the K-12 chromosome is *attλ II IV I III*. Our feeding data indicate that the biochemical reaction sequence is

Our groups are presumably equivalent to those of Del Campillo-Campbell et al. (2), which are arranged in the sequence *attλ ABCD*. If so, there is a point of contradiction in the results obtained in the two laboratories. Their feeding experiments are consistent with the reaction sequence.

Thus, according to these two schemes, our group II should correspond to their group A and our group III should correspond to their group D. Mutants of group II should therefore feed mutants of group III. Yet, our feeding data show that it is the group II mutants which have the earlier block in biotin synthesis. Del Campillo-Campbell et al. pointed out that their order for "the two middle steps (A and D) was less certain and was based on rather weak feeding between one mutant pair" (2). The inconsistency in the conclusions drawn in the two laboratories is probably due to difficulties encountered in cross-feeding experiments because of the low concentrations of DAP excreted into the medium by the biotin-requiring mutants, and also the low biological activity of DAP as compared with biotin. All our group II mutant strains responded to the addition

of synthetic DAP, whereas the same and even greater quantities of DAP failed to support growth of the group III mutants.

The group I mutants have been considered here as an all-inclusive group to cover those biochemical reactions prior to 7-KAP. Recent evidence (B. Rolfe, *in preparation*) has indicated that this group can be further subdivided into four complementation groups by transduction analysis with biotin-transducing mutants of λ . In addition, it has been shown that two of these complementation groups code for the enzyme condensing pimelyl-CoA and alanine to form 7-KAP (M. A. Eisenberg, and C. Star, *in preparation*). The other two cistrons probably code for enzymes responsible for the formation of pimelic acid and pimelyl-CoA.

The *malA* mutants of *E. coli*, which require biotin for growth, were also shown to grow in the presence of DTB, DAP, and 7-KAP. The fact that these mutants will grow on 7-KAP indicates that the *bioB* locus is involved in an early step in the biosynthesis of biotin before the condensation of pimelyl-CoA and alanine to form 7-KAP.

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