JOURNAL OF BACTERIOLOGY, Nov. 1972, p. 830-839 Vol. 112, No. 2
Copyright © 1972 American Society for Microbiology
Printed in U.S.A.

Deletion and Complementation Analysis of the Biotin Gene Cluster of *Escherichia coli*

PAUL P. CLEARY[1] AND ALLAN CAMPBELL

Department of Biological Sciences, Stanford University, Stanford, California 94305

Received for publication 21 July 1972

Genetic deletions that terminate within the cluster of genes needed for biotin biosynthesis in *Escherichia coli* have been isolated and mapped by transduction with phages lambda and P1. These deletions order the point mutations in each of the five genes. Mutations causing biotin dependence were incorporated into λ*pbio* transducing phages. New *bio*$^-$ mutations were induced by exposure of λ*pbio* particles to ultraviolet light. Tests of complementation between such *bio*$^-$ *pbio* particles and *bio*$^-$ mutant cells divide the *bio*$^-$ mutations into five cistrons: *bioA*, *bioB*, *bioF*, *bioC*, and *bioD*. Certain *bioA* and *bioF* mutations exhibit intragenic complementation, suggesting that these genes determine enzymes composed of identical subunits.

Biotin, the coenzyme for many adenosine triphosphate-dependent carboxylases and transcarboxylases (12), is synthesized from pimelyl-coenzyme A (CoA), its first known specific precursor (5, 22, 28). Three intermediates in biotin synthesis have been identified, 7-keto-8-aminopelargonic acid (KAP), diaminopelargonic acid (DAP), and dethiobiotin; and three of the biosynthetic enzymes have been demonstrated. The syntheses of biotin and the known biosynthetic enzymes are repressed by biotin (13, 19, 28). Biotin-dependent mutants of *Escherichia coli* were classified into four groups (4). Group C mutants are blocked prior to KAP synthesis, group A mutants are blocked prior to DAP synthesis, group D mutants are blocked prior to dethiobiotin synthesis, group B mutants cannot transform dethiobiotin into biotin (4, 20, 22). A recent study (21) suggested that these mutants defined seven closely linked genes at 17 min on the *E. coli* map (31). An additional genetic locus at 65 min on the *E. coli* map (24, 31) is inferred from the Bio$^-$ phenotype of certain deletion mutants, whose biochemical block seems to be prior to KAP synthesis (22).

In this report we describe complementation tests using λ*pbio* phages carrying various mutations in the *bio* gene cluster, which define five genes. Two genes, *bioA* and *bioF*, exhibit intragenic complementation. We also report the isolation of deletion mutations that enter the *bio* gene cluster from either the *bioA* or the *bioD* end.

MATERIALS AND METHODS

Bacteria. Strains are listed in Table 1. Deletion mutations were isolated in either strain CA2441 or AM3100. Strain CR514 was used to select thermoresistant deletions broken in the *bio* cluster. CR514, a *chlD* deletion strain lysogenic for λ*cIts857 xis*, was constructed by D. Court. Biotin-requiring mutants of *E. coli* (4) were isolated by nitrosoguanidine mutagenesis or kindly supplied by Alice Campbell. New *bio*$^-$ mutations were mapped by λ*dbio* transduction (P. Cleary, thesis, Univ. of Rochester, 1971). Except for *bioE124*, *bioF110*, *bioG301* and *bioA309*, which were obtained from M. Eisenberg's laboratory, all *bio* mutations used in complementation studies were put in a common genetic background by transferring the biotin locus into SA291 (by P1 transduction with selection for Gal$^+$). Those *bio* mutants isolated during this study are not included in Table 1.

Phages. Amber mutants of phage λ (2) were from our collection. λ*cIts857 Nam7 nin5*, the strain used to select λ*pbio* phages, was constructed by D. Court (thesis, Univ. of Rochester, 1970). P1*kc* was obtained from Naomi Franklin.

Lambda lysates were prepared from single plaques on strain SA291, which carries a deletion of the *bio* cluster, and assayed on SA291 or C600. All P1 lysates were pregrown on a *bio*$^-$ deletion strain. Galactose-transducing variants λ*gal*$^+$, λ*galA*, λ*galE*, λ*galF*, and λ*galG* were kindly supplied by S. Adhya (25). The *pbioA0* lysate was constructed from λ*pbio21* (15)

Media. Synthetic broth, synthetic agar (SA), tryptone broth (TB), tryptone agar (TA), and eosin methylene blue agar containing galactose (EMBG)

[1] Present address: Department of Pediatrics, University of Minnesota, Minneapolis, Minn. 55455.

TABLE 1. *Bacterial strains*

Strain	Genotype	Source or reference
AB2492	*recA13*	M. Feiss
AB2463	*recA13 sup*	M. Feiss
AM3100	*str-r his*$^-$	S. Adhya
Br105	*bioB105*	B. Rolfe (21)
Br110	*bioF110*	B. Rolfe (21)
Br115	*bioG115*	B. Rolfe (21)
Br116	*bioD116*	B. Rolfe (21)
Br124	*bioE124*	B. Rolfe (21)
Br126	*bioA126*	B. Rolfe (21)
Br301	*bioG301*	B. Rolfe (21)
Br309	*bioA309*	B. Rolfe (21)
C173	*str-r his*$^-$Δ224	This report
C246	*str-r his*$^-$Δ246	This report
C268	*str-r his*$^-$Δ268	This report
C280-C282	*str-r his*$^-$Δ280-Δ282	This report
C600	*thr*$^-$*leu*$^-$*thi*$^-$*lac*$^-$ *tonA-r supE*	(2)
CA2441	HfrH*lac*(amber) *trp*(amber) *thi*$^-$	S. Adhya
CR514	*str-r his*$^-$*recA114* Δ(*chlD-pgl*) (λ*cIts857 xis1*)	D. Court
QR93	Δ(*gal-chlA*)	S. Adhya
QR94	Δ(*gal-chlA*) *recA*$^-$	S. Adhya
R865	*galK2 galT1* (λ*imm 434*)	Our collection
R871	*bioB2*	(4)
R872	*bioF3*	(4)
R873	*bioA4*	(4)
R874	*bioF12*	(4)
R875	*bioB17*	(4)
R876	*bioC18*	(4)
R877	*bioD19*	(4)
R878	*bioC23*	(4)
R879	*bioA24*	(4)
SA291	*str-r his*$^-$ Δ(*gal-chlA*)	S. Adhya
W602	*bioA0 leu*$^-$*thi*$^-$*gal*$^-$ *str-r*	(4)

have been described elsewhere (3). The following supplements were sterilized separately and added when appropriate: 1.5 mg of thiamine, 1.5 mg of nicotinic acid, 0.1 mg of D-biotin, 4.0 g of sugar, 20 mg of amino acid, 60 mg of 2,3,5-triphenyl-2H-tetrazolium chloride per liter.

Chlorate agar (25.5 g of Difco antibiotic medium no. 2, 50 mg of 2,3,5-triphenyltetrazolium chloride, 2 g of sugar, and 2 g of $KClO_3$ per liter) was used to select chlorate-resistant mutants of *E. coli*. Nitrate agar (1) was used to test nitrate reductase. Routine dilutions of phage and bacteria were made in 0.01 M $MgSO_4$.

Avidin solutions were prepared by dissolving 100 units of avidin (Nutritional Biochemicals) in 100 ml of distilled water. After thorough mixing, the undissolved residue was removed by filtration and the supernatant fluid was sterilized by membrane filtration (Millipore Corp.).

Biotin production by λ*pbio* plaques was tested on low biotin synthetic agar containing a growth-limiting concentration of biotin (10 ng/liter).

Chlorate-resistant mutants. Chlorate-resistant deletion mutants of strain CA2441 were selected as previously reported (1). All other deletions were isolated in the same manner except that the chlorate agar described above was used as the selective medium. This medium allowed detection of chlorate-resistant colonies which had simultaneously become deficient in galactose utilization. Colonies arising on chlorate plates were tested for biotin independence by replica plating onto SA plates with and without biotin. To prevent cross-feeding it was necessary to print only those plates with fewer than 100 colonies. To facilitate isolation of *gal*$^-$ *uvrB*$^+$ deletions, chlorate plates were exposed to 400 ergs of ultraviolet light (UV) per mm^2 before incubation.

Bacterial genes. To test UV sensitivity, a 10-fold dilution of a log-phase culture was spotted on TA and exposed to 400 ergs of UV/mm^2. Mutants that were *uvrB*$^-$ exhibited no growth on subsequent overnight incubation, but *uvrB*$^+$ strains grew confluently. Nutritional mutations were scored by replicating master plates onto appropriately supplemented synthetic agar. The Gal phenotype was determined by streaking on EMBG. Previously published methods were used to test for *att*λ (7), *chlA* and *chlD* (1), *aroG* (33), and *pgl* (31).

P1 transduction of bio mutants. A P1*kc* lysate which had been grown on the *bio*$^-$ point mutant in question was tested for ability to transduce a Bio$^-$ deletion to Bio$^+$. All crosses were first done by spot tests in which 10^8 cells were infected with 5×10^8 P1 particles in a total volume of 0.25 ml. After 20 min of adsorption 0.05 ml of the mixture and 0.05 unit of avidin were spotted together on SA plates. Presence of *bio*$^+$ recombinants was scored after 2 and 5 days of incubation. Appropriate control crosses were also performed. The Bio$^+$ character of the recombinants was verified by restreaking on minimal media. All negative or questionable crosses were repeated by a more sensitive method. P1 particles (10^9 to 2×10^9) were adsorbed to 10^8 cells as before, and then the entire contents plus 0.1 unit of avidin were overlayed on SA pour plates.

The end points of *bio*$^-$ deletions were mapped by crossing strains with λ*pbio*$^-$ transducing phages carrying known *bio*$^-$ mutations. A λ*pbio*$^-$ lysate (10^{10} plaque-forming units [PFU]/ml) was mixed 1:1 with a λ$^+$ lysate (10^{10} PFU/ml), and various dilutions of the mixture were tested for ability to transduce the *bio*$^-$ deletions to *bio*$^+$.

Construction of λpbio3100 and λpbio phages containing bio$^-$ mutations. Strain AM3100 and *bio*$^-$ mutants derived from it were lysogenized with λ*Nam7 cIts857 nin5* by spotting a high-titer lysate of this phage on a bacterial lawn and restreaking from the area of lysis to obtain pure lysogenic cultures. Low-yielding lysogens were those whose colonies produced isolated plaques when replicated onto AM3100 indicator plates and confluent lysis on R865 indicator plates. High-yielding lysogens produced confluent lysis on both indicators (D. Court, thesis, Univ. of Rochester, 1970).

Plaque-forming bio^- transducing phages were obtained from thermoinduced cultures of the bio^- low-yielding lysogens. TB cultures (2×10^8 cells/ml) were induced at 43 C for 20 min and then incubated at 37 C for 3 hr before the lysate was harvested. The lysates were plated on a strain carrying the 224 deletion (Fig. 1) on SA low biotin plates. Plaques with heavy growth around the perimeter contained λ*pbio*$^-$ phages which had incorporated the entire *bio* cluster. These were purified by replating on SA291 indicator plates. High-titer lysates were made by infection of SA291. Throughout this paper the terms bio^+ and bio^- plaques will refer to plaques which were or were not, respectively, surrounded by a zone of heavy bacterial growth indicating that the phage could cause biotin production in the bio^- bacteria on which it was growing.

An alternate procedure was followed to pick up *bioD* mutations in λ*pbio* phages. Plaques were stabbed into strains C541 (*bioC541*) and C224 (Δ224) overlayed on low biotin plates. Those able to feed the C541 and unable to feed C224 were purified and lysates were made. To insure that the λ*pbioD*$^-$ phage carried the entire *bio* cluster, lysates were spotted on C224, and bio^+ spontaneous revertants were detected around the periphery of the spot after 1 week of incubation.

Mutagenesis of λpbio3100. A lysate of λ*pbio3100* (which transduces the entire *bio* cluster) was mutagenized with UV by the method of Tomizawa et al. (32). Appropriate samples of the irradiated lysate were adsorbed to approximately 4×10^7 cells of C246 (which carries deletion 246, Fig. 1). The C246 cells had previously been washed once, resuspended in 0.01 M $MgSO_4$ at three times the original concentration, starved for 30 min by vigorous shaking at 37 C, and irradiated. Then an unwashed culture of strain SA291 (which carried a deletion of the entire *bio* cluster) was mixed with the infected culture. The mixture was plated on low biotin SA pour plates and incubated for 36 hr. The parent phage, λ*pbio3100*, fed the cells around the perimeter of the plaque, and the bio^- mutant phage did not.

To isolate $bioA^-$ mutants specifically, strains C246 and SA291 were replaced by strain C268 (which carries Δ268, Fig. 1).

RESULTS

Deletion mapping. The *bio* cluster lies between two genes, *chlD* and *chlA* (Fig. 1), mutational inactivation of which renders cells resistant to chlorate. Mutations to chlorate resistance include deletions that terminate within the *bio* cluster.

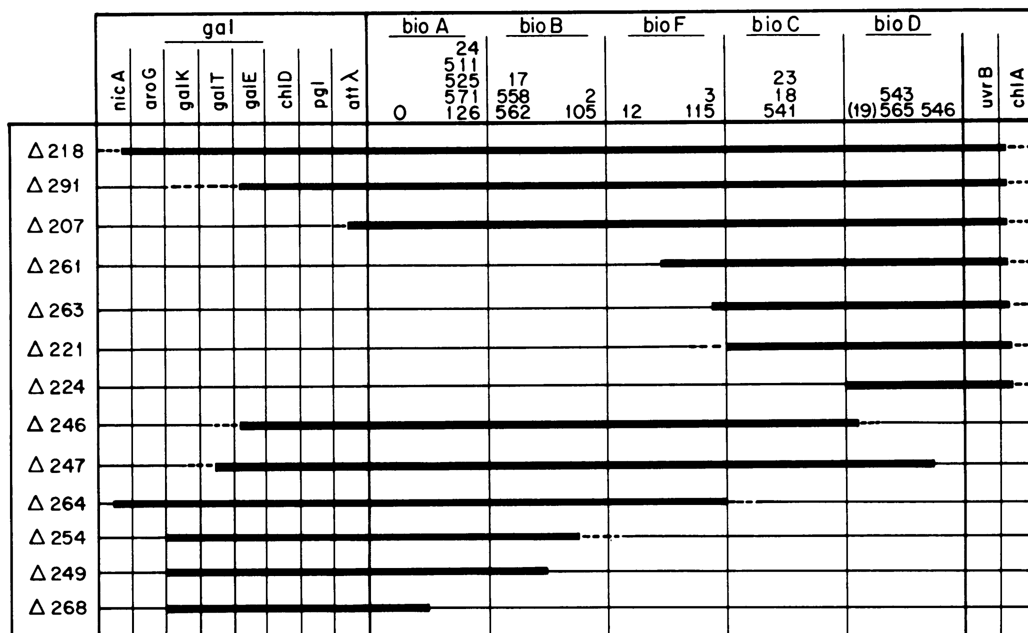

FIG. 1. *Deletion map of the bio gene cluster. Deletions Δ218, Δ207, Δ221 were isolated in strain CA2441. All other deletions were isolated in strain AM3100. Termini within the bio cluster were determined by P1 transduction. Mutation numbers are listed across the top of the figure. The dark solid lines represent deleted segments. Dashed lines indicate the regions, presence or absence of which cannot be or were not determined. Order of genes other than bio is taken from a previous deletion analysis (1, 31). Deletion Δ263 recombined with all bioF mutants but is known from complementation analysis to have deleted a portion of bioF (data not shown). The right terminus of Δ264 is tentative, because the sensitive P1 cross between it and the bioF mutants has not been done. The precise location of bioD19 is also uncertain (see text).*

Specific isolation of *chlD* deletions was facilitated by testing only Gal$^-$Chl^R colonies derived from a Gal$^+$Chl^R host. Tests of UV sensitivity indicated that most Gal$^-$Chl^R mutants bore deletions extending from *chlA* through *gal*. To eliminate these, in some experiments we irradiated the selection plates with UV. The surviving Chl^R colonies were then replicated onto synthetic agar with and without biotin to reveal deletions that entered the *bio* cluster.

P1 transduction between deletion mutants and various *bio*$^-$ point mutants generates the map shown in Fig. 1. A given *bio*$^-$ mutation is located within the region covered by a deletion if no Bio$^+$ transductants arise from the cross (P1 [*bio*$^-$] $\times$ *bio*$^-$ deletion).

Thirty-two deletions entering the *bio* cluster from the *chlA* side were isolated. Twenty-eight of these deletions (sixteen of which were obtained in an experiment where no precautions were taken to assure independence) were like Δ207 (Fig. 1). They had lost the entire *bio* cluster and terminated between *att*λ and *pgl*. Four mutants (Δ261, Δ263, Δ221, and Δ224) had lost all sites of the *bioD* gene for which we have genetic markers, yet retained all *bioA* and *bioB* markers. All four could utilize dethiobiotin in place of biotin, which requires an active *bioB* gene (4, 19, 22).

Among the Chl^{R}Gal$^-$ mutants tested, only six carried deletions that terminated within the *bio* cluster. Deletion Δ268 eliminated part of the *bioA* gene but did not interfere with growth on dethiobiotin. The other five deletions dissected the *bio* cluster at different points (Fig. 1). All five strains had lost part of the *bioB* gene and therefore did not respond to dethiobiotin. Deletion of the region between *bioA* and *bioB* (as in Δ249) severely inhibited the activity of all genes to the right of *bioB* (data to be published separately).

The order of the *bio* mutations determined by this analysis agreed with that based on transduction by λ*dbio* phages that penetrate the *bio* cluster to different extents (P. Cleary, thesis, Univ. of Rochester, 1971; 4). The one exception was the order of *bioD19* relative to other *bioD* mutations. We consider the position of *bioD19* within the *bioD* gene to be undetermined.

Deletion end points within the *gal* operon were detected by transduction with λ*dgal*$^+$ (carrying entire *gal* operon), λ*dgalE* (carrying the operator region and *galE*), λ*dgalF* (carrying *galE* and part of *galT*), and λ*dgalG* (carrying *galE*, *galT*, and part of *galK*). Only Δ246 and Δ247 terminated between the end points of λ*dgalE* and λ*dgalG*.

Specific isolation of deletions ending in bioA. Only one (Δ268) of the deletions shown in Fig. 1 terminated within the *bioA* gene. Since *bioA* mutants could not be ordered by λ*dbio* transduction, more such deletions were needed. To increase the number of deletions that could be screened, we needed a double selection that allowed survival only of deletions and not of point mutants. A lamdba prophage, defective in excision functions, provided this double selection.

A thermoinducible lambda prophage kills its host at high temperatures even if it cannot excise itself from the host chromosome. Either of two lambda-controlled functions suffices to kill. One of these is determined by a gene to the left of the *cI* gene. Derepression of genes controlling deoxyribonucleic acid (DNA) replication, which lie to the right of *cI*, also kills (17, 26). Therefore, a cell carrying as prophage λ*xis*$^-$*cIts857*, which is defective in excision (10) and forms a thermolabile repressor, can survive incubation at 43 C only when a double mutation or a deletion has inactivated the relevant prophage genes.

CR514, a lysogen of λ*xis*$^-$*cIts857*, yielded on induction only 10^4 PFU/ml, and 90% of these were λ*pgal* or λ*pbio* phages. Cells surviving induction were found at a frequency of approximately 10^{-6}. To select deletions that entered the *bioA* gene but did not affect *bioB*, samples of CR514 were spread on SA plates containing dethiobiotin and nicotinic acid. Only *bio*$^+$ cells and *bio*$^-$ cells able to utilize dethiobiotin can grow on these plates. Three out of 350 surviving colonies could not grow on nonsupplemented medium. Two strains, C280 and C281, required just biotin; one, C282, required nicotinic acid as well.

The parent strain CR514 already bears a deletion of the region *chlD-pgl* (S. Adhya, *unpublished data*). Strains C280 and C281 were both *gal*$^+$, and none of the markers, *Nam7*$^+$, *Nam53*$^+$, or *imm*λ, could be rescued from them by phage infection. As determined by the method of Gottesman and Yarmolinsky (7), neither strain C280 nor C281 was lysogenizable by λ. Therefore their deletions terminate between *att*λ and *gal*. Strain C282 was *nic*$^-$ and *gal*$^-$, indicating that its deletion extends into the *nicA* locus.

The right end points of Δ280, Δ281 and Δ282 were determined by transduction with the λ*pbio*$^-$ lysates constructed from known *bio*$^-$ bacterial mutants or isolated by UV mutagenesis (see Materials and Methods). None of the λ*pbio*$^-$ phages could lysogenize wild-

type *E. coli*. Therefore λ^+ helper phage was included in these transduction tests.

The *bioA* mutations were divided into four groups by Δ281, Δ280, Δ282, and Δ268 (Fig. 2).

Construction and characterization of λbio$^-$ transducing particles. For complementation and mapping studies, we wanted a λ*pbio* carrying the entire *bio* cluster. As starting material, λ*Nam7 cIts857 nin5* had the following desirable characteristics (D. Court, thesis, Univ. of Rochester, 1970). (i) Being defective in excision, induced single lysogens yielded 10^{-3} PFU/bacterium, of which 80 to 95% were transducing phages. (ii) The *nin* mutation permits deletion of phage genes through gene *N* without loss of plaque-forming ability. This allows incorporation of larger segments of bacterial DNA without exceeding the maximum amount of DNA that can be accommodated in the phage head. (iii) The *nin5* mutation is a deletion and therefore allows incorporation of a larger segment of foreign DNA.

Low-yielding lysogens of AM3100 strains with various bio^- mutations were isolated, purified, and induced. Phage particles able to make bio^+ plaques when plated on C173 must carry the entire biotin cluster. C173, when infected with such a λ*pbio* mutated in any gene other than *bioD*, could synthesize biotin, feeding uninfected cells around the plaque. No λ*pbio* made bio^+ plaques on its strain of origin. This procedure was not feasible for *bioD* mutants, because C173 lacks *bioD* function. λ*pbio* phages carrying *bioD116*, *bioD565*, and *bioD546* were isolated by stabbing plaques from TA plates into C173 and C541 (which carries the mutation *bioC541*). Transducing particles that made bio^- plaques on C173 and bio^+ plaques on C541 were selected.

In order to incorporate *bio* genes, the λ*pbio* must have lost genes from the left arm of the prophage chromosome (11). All of our λ*pbio* lysates failed to form plaques on AB2492 (rec^- su^-) or AB2464 (rec^-su^+), suggesting that the *red* and γ genes that allow growth on *recA* strains (15) had been replaced.

Mutagenesis of λpbio$^+$3100 as a means of obtaining λpbio$^-$ transducing particles. λ*pbio3100* was derived from a bio^+ lysogen of λ*Nam7 cIts857 nin5*. It carried the entire *bio* cluster and produced biotin when grown on SA291 (Δ291). We isolated new mutations in the *bio* genes carried by λ*pbio3100*.

A λ*pbio3100* lysate and the bacteria to which it was adsorbed were irradiated with UV (32). The host used was C246, which carries a deletion of the *bio* cluster but has an intact *uvrB* gene. The UV dose given to C246 increased phage survival 20-fold (to 10^{-5}). Phage adsorbed to irradiated C246 were plated onto SA291 for scoring. In two experiments, a total of 37/4,220, or 0.9%, of the plaques were bio^-.

This method allowed isolation of mutants in

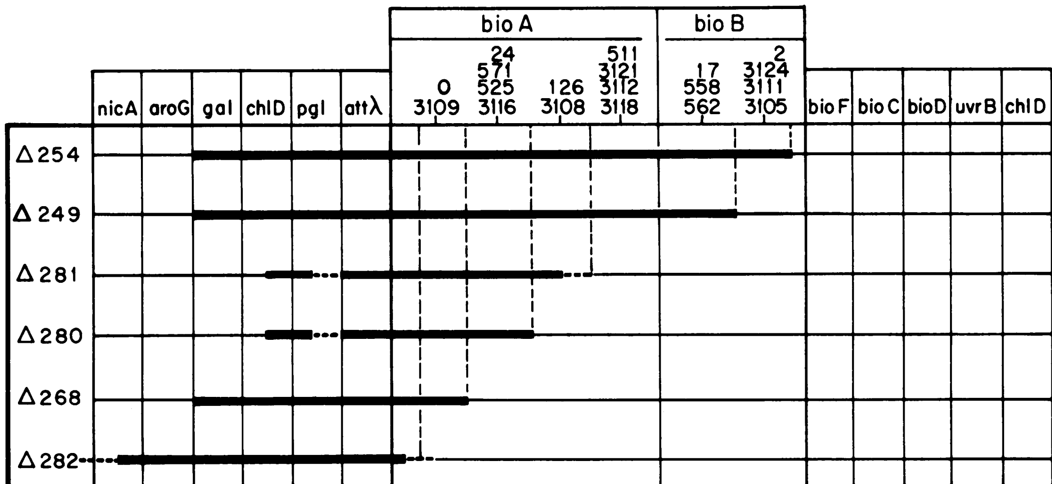

FIG. 2. *Deletion map of the bioA gene. Cells carrying deletions Δ254, Δ249 and Δ268 were isolated as chlorate-resistant mutants. The bioA and bioB genes are expanded to clarify the illustration, with λpbio phages carrying the bio$^-$ mutations listed along the top of the figure. Dark solid lines represent deleted segments. Broken lines indicate regions whose presence or absence was not determined. The location of bioAO relative to deletion Δ282 has not been determined. Transduction of Δ281 by λpbio126 gave variable results; we tentatively conclude that bioA126 is absent from Δ281.*

any prescribed gene. To obtain *bioA* mutants, specifically, strain C268, which carries a partial deletion of *bioA*, replaced both C246 and SA291 as adsorbing and plating bacteria. C268 complemented λ*pbio* particles with mutations in all genes except *bioA* (data not given).

The *bio* markers in λ*pbio* phages were mapped by spotting the lysates on bacterial strains bearing deletions that enter the *bio* cluster (Fig. 2).

λpbio$^-$ complementation of bio$^-$ cells. All λ*pbio*$^-$ transducing particles, whether their *bio*$^-$ mutation came from a known *bio*$^-$ bacterium or was isolated directly in λ*pbio3100*, were tested for ability to complement various bacterial *bio*$^-$ mutants. If a *pbio*$^-$ phage, when spotted on a *bio*$^-$ bacterium, could supply the functions which were defective in that bacterium, then biotin was produced, feeding the λ-resistant cells within the plaque as well as the cells around the perimeter of the plaque.

Results of such tests (Table 2) divide the *bio*$^-$ mutations into five complementation groups. With the exception of group C mutants, these groups corresponded exactly to previous assignments based on feeding behavior (P. Cleary, thesis, Univ. of Rochester, 1971; 4). Mutants classified as group C by feeding tests represented two complementation groups, *bioF* and *bioC*.

Some mutant pairs which did not complement each other could generate *bio*$^+$ recombinants by either P1 transduction or λ*pbio*$^-$ transduction. On extended incubation (5 days), negative spots often gave rise to isolated areas of feeding at the periphery of the plaque and isolated λ-resistant colonies in the center of the plaque. This presumably resulted from recombinants generated within the plaque.

TABLE 2. *Complementation between λpbio$^-$ and bio$^-$ cells*[a]

Mutation carried by *bio*$^-$ cells[b]	Mutation carried by λ*pbio*$^-$[b]				
	bioA24 (10)	*bioB562* (4)	*bioF12* (3)	*bioC541* (3)	*bioD116* (2)
bioA24 (5)	−				
bioB562 (4)	+	−			
bioF12 (3)	+	+	−		
bioC541 (3)	+	+	+	−	
bioD19 (5)	+	+	+	+	−
bioE124 (1)	+	−	+	−	+

[a] See Table 3 for description of test and meaning of symbols.

[b] Numbers in parentheses are numbers of different mutations in the same gene that give identical complementation results.

bioC and bioF genes. Group C mutants are blocked early in the biosynthetic pathway (4, 22) and fall into two complementation groups, *bioF* and *bioC*. Both types of mutants complemented mutants in *bioA*, *bioB*, and *bioD* (Table 3).

Based on the transduction frequency of strains Br301 (*bioG301*) and Br110 (*bioF110*) with λ*dgal*$^-$*bio34* (which contained a UV-induced *bio*$^-$ mutation), B. Rolfe subdivided *bioF* into two units, *G* and *F* (21). We observed no complementation between *bioF110* and *bioG301* or between them and our *bioF* mutants. We observed one case of unidirectional complementation between *bioF* mutants. One mutant, λ*pbioF12*, complemented *bioF3* bacteria, but the reciprocal test did not result in biotin synthesis. A possible explanation for this asymmetry will be discussed later.

bioA gene. The 15 *bioA* mutations studied included some isolated by nitrosoguanidine mutagenesis of *E. coli* (4) and those obtained by UV reactivation of λ*pbio3100*. When both the bacterial strain and the λ*pbio*$^-$ phage bearing the same mutation were available, the reciprocal tests were done. However, those *bio*$^-$ mutations available only in λ*pbio3100* could not be tested against themselves or each other.

A series of presumptive *bioA* mutants, isolated as unable to complement strain C268 (in which part of *bioA* is deleted), were numbered

TABLE 3. *Complementation between λpbioF or λpbioC and bio$^-$ cells*[a]

Mutation carried by *bio*$^-$ cells	Mutation carried by λ*pbio*$^-$					
	bioF12	*bioF3*	*bioG115*[c]	*bioC18*	*bioC23*	*bioC541*
bioF12	−[b]	−	−	w$^+$	+	+
bioF3	+	−	−	N	+	+
bioG115[c]	−	−	−	+	+	+
bioF110	−	−	−	N	N	+
bioG301[c]	−	−	−	N	N	+
bioC18	+	+	+	−	−	−
bioC23	+	+	+	−	−	−
bioC541	+	+	+	−	−	−
bioA24	+	+	+	+	+	+
bioB562	+	+	+	+	+	+
bioD116	+	+	+	+	+	+

[a] λ*pbio*$^-$ lysates (2×10^8 PFU per drop) were spotted on synthetic agar overlay plates containing cells of the indicated genotype. Plates were scored after 2 days of incubation at 37 C.

[b] +, Feeding of cells around the perimeter of the plaque; w$^+$, just visible feeding; −, no feeding; N, not tested.

[c] These are mutations designated *bioG* by Rolfe (21). Our results place them in the *bioF* gene (see text).

bio3108 through *bio3130*. That these λ*pbio*⁻ mutants are indeed defective in *bioA* function was verified by complementation tests (Table 4). Except for λ*pbioA3117*, the λ*pbio3100* lysates could not complement known *bioA* mutants, but complemented *bioB*, *bioF*, *bioC*, and *bioD* mutants. λ*pbioA3117* weakly complemented the deletion SA291 (Fig. 1), indicating that *bioA3117* is leaky. Another exception, λ*pbio3123*, will be discussed later.

The *bioA* mutants fell into three complementation groups: two groups complemented each other, but neither complemented the third group. This pattern is typical of intragenic complementation.

Strain C126 (which bears the *bioA126* mutation) displayed a unique complementation pattern, being unable to supply either *bioA* or *bioB* function. λ*pbioB562* and λ*pbioB17* weakly complemented C126, but no complementation was detected in reciprocal tests. However, λ*pbioA126* complemented strains carrying the *bioB2* and *bioB105* mutations. We do not understand the nature of the *bioA126* mutation.

bioD gene. Mutations in the *bioD* gene all mapped at the right end of the *bio* gene cluster and blocked formation of the enzyme that converts DAP into dethiobiotin (4, 13, 22). Five *bioD* mutants tested behaved as one complementation unit. They did not complement each other but complemented mutations in all other genes (Table 5).

Two independent isolates of λ*pbioD116* complemented the highly polar mutation *bioB558* weakly in one case and undetectably in the other (Table 5). The basis of this difference was not studied further.

bioB gene. All *bioB* mutations blocked conversion of dethiobiotin to biotin and lay between *bioA* and *bioF* on the genetic map (4; P. Cleary, thesis, Univ. of Rochester, 1971). Complementation studies on these mutants were of special interest because we do not know whether insertion of the sulfur atom and closure of the thiophene ring require more than one enzymatic step specific to the biotin pathway.

All eight mutants tested appeared to represent one cistron, although interpretation was complicated by the weak response exhibited by some pairs (Table 6). *bioB562*, *bioB2*, and *bioB105* complemented mutants in all other genes. The *bioB2* mutant showed some activity by itself and some intragenic complementation. The *bioB558*, *bioB17*, *bioB3124*, *bioB3111*, and *bioB3105* mutants did not complement any *bioB* mutants, but complementation with *bioF*, *bioC*, or *bioD* was absent or reduced. All these mutants complemented *bioA24*. This apparent polarity was confirmed by another type of experiment.

Complementation between two mutations, both residing in λpbio particles. A second method was developed for studying complementation among *bio*⁻ mutants. Two λ*pbio*⁻ lysates were mixed together and spotted on low biotin from plates seeded with QR93 (*bio*⁻ deletion, *rec*⁺) and QR94 (*bio*⁻ deletion, *rec*⁻). If the two *bio*⁻ genomes complemented each other, then biotin was synthesized, feeding the background cells and the plaque.

Representative results from three experiments done in *rec*⁻ bacteria are illustrated in Table 7. The results agree with those obtained from the "λ*pbio*⁻ versus cell" tests.

In absence of recombination, the polarity of the *bioB558* mutation was more evident, as

TABLE 4. *Complementation between λpbioA and bio⁻ cells*[a]

Mutation carried by *bio*⁻ cells	Mutation carried by λ*pbio*															
	bio A3130	*bio A3113*	*bio A3109*	*bio A24*	*bio A525*	*bio A571*	*bio A3118*	*bio A3112*	*bio A3121*	*bio A3108*	*bio A3116*	*bio A126*	*bio A0*	*bio A511*	*bio A3117*	*bio B562*
bioA24	–	–	–	–	–	–	–	–	–	–	–	–	–	+	w^+	+
bioA525	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+
bioA571	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+
bioA309	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
bioA126	–	–	–	–	–	–	–	–	–	–	–	–	–	–	w^+	w^+
bioA0	w^+	+	w^+	w^+	+	+	–	–	–	–	–	–	–	–	w^+	+
bioA511	+	+	+	+	+	+	–	–	–	–	–	–	–	–	+	+
bioB558	+	+	+	+	+	+	+	+	+	+	+	–	w^+	w^+	+	–
bioB562	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	–
bioF12	+	+	+	+	+	+	+	+	+	+	+	+	N	+	+	+
bioC541	+	+	+	+	+	+	+	+	+	+	+	+	N	+	+	+
bioD116	+	+	+	+	+	+	+	+	+	+	+	+	N	+	+	+

[a] See Table 3 for description of test and meaning of symbols.

TABLE 5. *Complementation between λpbioD and bio$^-$ cells*[a]

Mutation carried by *bio*$^-$ cells	Mutation carried by λ*pbio*		
	bioD116[b]	*bioD116*[b]	*bioD565*
bioD116	–	–	–
bioD19	–	–	–
bioD543	–	–	–
bioD546	–	–	–
bioD565	–	–	–
bioA24	+	+	+
bioB558	–	w^+	w^+
bioB562	+	+	+
bioF115	+	+	+
bioC541	+	+	+

[a] See Table 3 for description of test and meaning of symbols.

[b] Two *bioD116* phages were isolated independently.

TABLE 6. *Complementation between λpbioB and bio$^-$ cells*[a]

Mutation carried by *bio*$^-$ cells	Mutation carried by λ*pbio*						
	bio B2	*bio B562*	*bio B17*	*bio B558*	*bio B3124*	*bio B3105*	*bio B3111*
bioB2	w^+	–	–	–	–	–	–
bioB562	w^+	–	–	–	–	–	–
bioB105	–	–	–	–	–	–	–
bioB17	w^+	–	–	–	N	N	N
bioB558	–	–	–	–	–	–	–
bioA126	+	w^+	w^+	–	–	–	–
bioA24	+	+	+	+	+	+	+
bioF115	+	+	w^+	w^+	+	w^+	w^+
bioC541	+	+	w^+	w^+	–	–	–
bioD19	+	+	w^+	w^+	–	–	w^+

[a] See Table 3 for description of tests and meaning of symbols.

many weak responses observed previously (Table 6) disappeared. The weak complementation between λ*pbioB558* and λ*pbioD116* might indicate leakiness of the *bioD116* mutation or decreased polarity of *bio558* on the *bioD* gene. λ*pbioB17* still weakly complemented mutations in genes to its right and is therefore less polar than *bioB558.*

DISCUSSION

Biotin is synthesized from pimelyl-CoA via at least four enzymatic reactions (4, 5, 22, 28). Of these enzymes, the KAP condensing enzyme (28), transaminase (M. Eisenberg, *personal communication*), and dethiobiotin synthetase (13, 18) have been demonstrated in cell-free extracts. Genetic analyses have revealed that mutations of the main biotin cluster define four feeding classes (21) and seven possible cistrons (21). To study this cluster further, we have isolated deletion mutants.

As in other systems (3, 27, 30), deletions in the *gal-bio* region have nonrandom end points. A large fraction of our *chlA* deletions terminate in or near the lambda attachment site. A "hot spot" was also found between *gal* and *bio* for similar deletions of *Klebsiella aerogenes.*

Deletion mapping. Deletions terminating within the *bio* cluster (Fig. 1 and 2) divide our *bio*$^-$ mutations into 13 groups and dissect the cluster within or between every *bio* gene.

Terminal deletions removing part of the first structural gene of the histidine, tryptophan, or lactose operons are extremely polar on the residual genes of these operons (6, 9, 16). This effect was not observed in the biotin gene cluster. Deletions Δ268 and Δ224, which remove a portion of the *bioA* and *bioD* gene,

TABLE 7. *Complementation between λpbio$^-$ phages carrying different mutations*[a]

Mutation carried by λ*pbio*$^-$(II)	Mutation carried by λ*pbio*$^-$(I)									
	bioA24	*bioA0*	*bio558*	*bioB17*	*bioB562*	*bioF3*	*bioF115*	*bioF12*	*bioC18*	*bioD116*
bioA24	–[b]									
bioA0	+	–								
bioB558	+	+	–							
bioB17	+	+	–	–						
bioB562	+	+	–	–	–					
bioF3	+	+	–	w^+	+	–				
bioF115	+	+	–	w^+	+	–	–			
bioF12	+	+	–	w^+	+	+	–	–		
bioC18	+	+	–	w^+	+	+	+	+	–	
bioD116	+	+	w^+	w^+	+	+	+	+	+	–

[a] Two λ*pbio* lysates (2×10^8 PFU per drop) were spotted together on synthetic agar overlay plates seeded with QR94 bacteria. Plates were incubated for 2 days at 37 C.

[b] Symbols as in Table 3.

respectively, do not prevent expression of the *bioB* gene. This suggests that the biotin cluster does not constitute a single operon. In fact, studies on dethiobiotin synthetase levels in deletion and polar mutants (Cleary, Campbell, and Chang, Proc. Nat. Acad. Sci. U.S.A., *in press*) indicate, in agreement with the conclusion of Guha et al. (8), that the four genes *bioB*, *bioF*, *bioC*, and *bioD* are transcribed rightward from an internal promoter located between *bioA* and *bioB*.

Complementation analysis. The development of a reliable complementation test serves two purposes. (i) It allows us to group the *bio*$^-$ point mutants into cistrons (genes); (ii) it supplies a qualitative index of gene activity.

The existence of at least five closely linked genes in the biotin cluster is confirmed by two kinds of complementation tests. Our collection contains fifteen *bioA* mutants, seven *bioB* mutants, three *bioF* mutants, three *bioC* mutants, and five *bioD* mutants.

Two genes, *bioA* and *bioF*, exhibit intragenic complementation. Seven of the 16 *bioA* mutants shown in Table 1 fail to complement any other nine mutants, two (*bioA0*, *bioA511*) comtions are scattered throughout the gene. Of the other nine mutants, two *bioA0*, *bioA511*) complement the other seven, but not each other. The distribution of these mutations along the genetic map does not correlate in any simple way with their complementation behavior. The *bioA0* and *bioA511* mutations lie at opposite ends of the genetic map, and complementing and noncomplementing mutants are interspersed. This indicates that *bioA* comprises a single gene.

Intragenic complementation can result from interaction between heterologous monomers in an enzyme normally composed of identical subunits. The enzymes determined by *bioA* and *bioF* could have such a structure.

Mutants defective in *bioB* function cannot utilize dethiobiotin in place of biotin (18, 20, 22). Five out of eight *bioB* mutants examined are also deficient in *bioF*, *bioC*, and *bioD* function. The precise location of these five mutations is unknown. They all lie between *bioA* and *bioF*. Some lie within the deletion Δ249, whereas others map outside of it. One of these mutants, *bioB558*, can further mutate to a state where dethiobiotin can be utilized but the strain is still *bio*$^-$ (P. Cleary, thesis, Univ. of Rochester, 1971). These partial revertants are not completely understood. All five polar mutations were nonsense mutations (Cleary, Campbell and Chang, Proc. Nat. Acad. Sci. U.S.A., *in press*).

Mutants in cross-feeding group C (4, 22; P. Cleary, thesis) are divisible into two complementation groups, *bioF* and *bioC* (reference 21 and this paper). Star et al. (28) found that *bioC* mutants have the enzyme KAP synthetase, which is absent in *bioF* mutants. Mutants in *bioC* are presumably blocked before synthesis of pimelyl-CoA, the substrate for KAP synthetase (22).

Rolfe (21) classified *bioF* mutants into two groups, *G* and *F*. Even though two *G* mutants and one *F* mutant from his collection were used in our studies, our results do not indicate the existence of a separate *bioG* gene. Different methods were employed in the two studies.

Rolfe found that λ*dgal*$^-$*bio34*, carrying a UV-induced "*G*" mutation, could transduce *bioC* and some *bioF* mutants, yet transduced other mutants (classified as *G* mutants) at only a low frequency (21). He could not, however, test other mutants in *F* and *G* groups against each other. His *G* and *F* mutations were located in adjacent but non-overlapping segments of the genetic map.

Our tests reveal unidirectional complementation between λ*pbioF12*, which lies in the *F* region, and *bioF3*, which lies in the *G* region. However, neither λ*pbioF12* nor λ*pbioF3* complements *bio115*, *bio301* (both *G* mutants) or *bio110* (an *F* mutant) (Table 3). We conclude that the observed complementation is intragenic.

The unidirectional complementation between *bioF3* and *bioF12* (Table 3) indicates an effect of gene dosage, understandable if KAP synthetase consists of more than two subunits. Negative complementation by *bioF3* is unlikely because λ*pbioF3* forms *bio*$^+$ plaques on wild-type hosts and on hosts carrying mutations in other *bio* genes. A more plausible hypothesis is that a multimer containing mainly *bioF12* subunits is active, but one containing mainly *bioF3* units is not. A pure *bioF12* multimer must of course be inactive; otherwise the mutant would not have a Bio$^-$ phenotype.

The five *bioD* mutants complement all *bioA*, *bioB*, *bioF*, and *bioC* mutants (except the polar *bioB* mutants) and fail to complement each other. The *bioD* gene determines the enzyme dethiobiotin synthetase (13). This enzyme, which has been purified 200-fold, fixes CO_2 into the ureido ring of biotin (14).

The mutant *bioE124* excretes no intermediates, is fed only by biotin or by *bio*$^+$ cells, and does not revert spontaneously (21; P. Cleary, thesis, Univ. of Rochester, 1971). Our complementation data (Table 2) indicate that this

mutant forms reduced amounts of the *bioB* and *bioC* gene products. Also, the specific activity of KAP synthetase (the *bioF* gene product) in a *bioE124* strain is only one-fifth that in wild-type *E. coli* (28). These pleiotropic effects suggest that *bioE124* is either a polar mutation in the *bioB* gene or a double mutation, one of whose components is polar on other genes of the operon. We do not think that the unique properties of this mutant justify the inference (21, 31) that a "*bioE*" gene lies between *bioB* and *bioF*.

ACKNOWLEDGMENTS

We thank Donald Court for many suggestions and ideas and Barry Rolfe for access to unpublished data and bacterial strains.

Our research was supported by Public Health Service research grant AI 8573 from the National Institute of Allergy and Infectious Diseases. P.C. held a Public Health Service predoctoral traineeship from the Department of Biology, University of Rochester.

LITERATURE CITED

1. Adhya, S., P. Cleary, and A. Campbell. 1968. A deletion analysis of prophage lambda and adjacent genetic regions. Proc. Nat. Acad. Sci. U.S.A. **61**:956-962.
2. Campbell, A. 1961. Sensitive mutants of bacteriophage λ. Virology **14**:22-32.
3. Curtiss, R. III. 1965. Chromosomal aberrations associated with mutations to bacteriophage resistance in *Escherichia coli*. J. Bacteriol. **89**:28-40.
4. Del Campillo-Campbell, A., G. Kayajanian, A. Campbell, and S. Adhya. 1967. Biotin-requiring mutants of *Escherichia coli* K-12. J. Bacteriol. **94**:2065-2066.
5. Eisenberg, M., and R. Maseda. 1966. An early intermediate in the biosynthesis of biotin. Biochem. J. **101**: 601-606.
6. Fink, G. R., T. Klopotowski, and B. Ames. 1967. Histidine regulatory mutants in *Salmonella typhimurium*. IV. A positive selection for polar histidine-requiring mutants from histidine operator constitutive mutants. J. Mol. Biol **30**:81-95.
7. Gottesman, M., and M. Yarmolinsky. 1968. Integration-negative mutants of bacteriophage lambda. J. Mol. Biol. **31**:487-505.
8. Guha, A., Y. Saturen, and W. Szybalski. 1971. Divergent orientation of transcription from the biotin locus of *Escherichia coli*. J. Mol. Biol. **56**:53-62.
9. Ippen, K., J. Miller, J. Scaife, and J. Beckwith. 1968. New controlling element in the lac operon of *E. coli*. Nature (London) **217**:825-827.
10. Kaiser, A. D., and T. Masuda. 1970. Evidence for a prophage excision gene in λ. J. Mol. Biol. **47**:557-564.
11. Kayajanian, G. 1968. Studies on the genetics of biotin-transducing, defective variants of bacteriophage λ. Virology **36**:30-41.
12. Knappe, J. 1970. Mechanism of biotin action. Annu. Rev. Biochem. **39**:757-766.
13. Krell, K., and M. Eisenberg. 1969. Desthiobiotin synthesis from 7,8-diaminopelargonic acid in cell-free extracts of a biotin auxotroph of *E. coli*. J. Biol. Chem. **244**:5503-5509.
14. Krell, K., and M. Eisenberg. 1970. The purification and properties of desthiobiotin synthetase. J. Biol. Chem. **245**:6558-6566.
15. Manly, K., E. Signer, and C. Radding. 1969. Nonessential functions of bacteriophage λ. Virology **37**:177-188.
16. Matsushiro, A., S. Kida, J. Ito, K. Sato, and F. Imamoto. 1962. The regulatory mechanism of enzyme synthesis in the tryptophan biosynthetic pathway of *Escherichia coli* K-12. Biochem. Biophys. Res. Commun. **9**:204-207.
17. Neubauer, Z., and E. Calef. 1970. Immunity phase-shift in defective lysogens: non-mutational hereditary change of early regulation of λ prophage. J. Mol. Biol. **51**:11-13.
18. Pai, C. H. 1969. Biosynthesis of desthiobiotin in cell-free extracts of *Escherichia coli*. J. Bacteriol. **99**:696-701.
19. Pai, C. H., and H. Lichstein. 1965. The biosynthesis of biotin in microorganisms. II. Mechanisms of the regulation of biotin synthesis in *Escherichia coli*. Biochim. Biophys. Acta **100**:28-35.
20. Pai, C. H., and H. C. Lichstein. 1967. Biosynthesis of biotin in microorganisms. VI. Further evidence for desthiobiotin as a precursor in *Escherichia coli*. J. Bateriol. **94**:1930-1933.
21. Rolfe, B. 1970. Lambda phage transduction of the *bioA* locus of *Escherichia coli*. Virology **42**:643-661.
22. Rolfe, B., and M. A. Eisenberg. 1968. Genetic and biochemical analysis of the biotin loci in *Escherichia coli* K-12. J. Bacteriol. **96**:515-524.
23. Sato, K., and A. Campbell. 1970. Specialized transduction of galactose by lambda phage from a deletion lysogen. Virology **41**:474-487.
24. Schwartz, M., D. Hatfield, and M. Hofnung. 1969. Genetic analysis of the maltose *A* region in *Escherichia coli*. J. Bacteriol. **98**:559-567.
25. Shapiro, J. A., and S. Adhya. 1969. The galactose operon of *E. coli* K-12. II. A deletion analysis of operon structure and polarity. Genetics **62**:249-264.
26. Sly, W. S., H. Eisen, and L. Siminovitch. 1968. Host survival following infection with or induction of bacteriophage lambda mutants. Virology **34**:112-127.
27. Spudich, J. A., V. Horn, and C. Yanofsky. 1970. On the production of deletions in the chromosome of *Escherichia coli*. J. Mol. Biol. **53**:49-67.
28. Star, C., and M. A. Eisenberg. 1968. Synthesis of 7-oxo-8-aminopelargonic acid, a biotin vitamer, in cell-free extracts of *Escherichia coli* biotin auxotrophs. J. Bacteriol. **96**:1291-1297.
29. Stouthamer, A. H., and K. Pietersma. 1970. Deletion-mapping of resistance against chlorate in *Klebsiella aerogenes*. Mol. Gen. Genet. **106**:174-179.
30. Streisinger, G., Y. Okada, J. Emrich, J. Newton, A. Tsugita, E. Terzaghi, and M. Inouye. 1966. Frameshift mutations and the genetic code. Cold Spring Harbor Symp. Quant. Biol. **31**:71-84.
31. Taylor, A. L. 1970. Current linkage map of *Escherichia coli*. Bacteriol. Rev. **34**:155-175.
32. Tomizawa, J., K. Shimada, and H. Ogawa. 1968. Studies on radiation-sensitive mutants of *E. coli*. I. Mutants defective in the repair synthesis. Mol. Gen. Genet. **101**:227-244.
33. Wallace, B., and J. Pittard. 1967. Genetic and biochemical analysis of the isozymes concerned in the first reaction of aromatic biosynthesis in *Escherichia coli*. J. Bacteriol. **93**:237-244.