MUCOID MUTANTS OF *ESCHERICHIA COLI*[1]

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The W strain of *Escherichia coli* has been used extensively in analyzing various biosynthetic pathways (Davis, 1952*a*) and also in studying drug resistance (Davis, 1952*b*). This strain is nonmucoid as ordinarily cultured on solid medium at 35 C. However, among mutants selected for various properties, i. e., auxotrophy, meiotrophy[4] and drug resistance, a few were observed to be mucoid. An investigation of a possible relation between the mucoid property and the associated biochemical mutation was undertaken in the hope of providing some information on the genetic and metabolic mechanisms controlling synthesis of capsular material. This problem seemed of particular interest because variations in capsule formation have been so important in studies, in other bacterial species, of genetic transformation by deoxyribonucleic acid (Ephrussi-Taylor, 1949; Leidy *et al.*, 1953). The results of the present study failed to show any connection between the mucoid and the other mutations.

In addition, the capsular polysaccharide formed by each of the mucoid strains was studied immunologically and shown to be the same as that formed by the wild type at low temperatures.

MATERIALS AND METHODS

The mutant strain were derived from *E. coli* strain W (ATCC 9637) following ultraviolet irradiation. Auxotrophic mutants were selected by means of the penicillin method (Davis, 1948, 1949; Lederberg and Zinder, 1948). Meiotrophic mutants, produced as reversions of auxotrophs, were selected by plating large inocula on suitable solid media. The same procedure was used for selection of drug-resistant mutants resistant to inhibition by D-serine (Maas and Davis, 1950).

Minimal medium A (Davis and Mingioli, 1950) was employed, supplemented or substituted as indicated. Unless otherwise specified the carbon source was 0.2 per cent glucose. Cultures were incubated at 35 C. The supplement used for auxotroph 161-51 was 40 μg/ml DL-α-aminobutyric acid plus 20 μg/ml L-tryptophan; that for 83-20 was 20 μg/ml L-tyrosine plus 40 μg/ml L-tryptophan. D-Serine resistant strain 6-4 tolerated 200 μg/ml of DL-serine, which inhibited growth of the parental strain and the other mutants.

Preparation of capsular material. Liquid cultures were grown for 18 hr with shaking and then clarified in a Sharples centrifuge. On adding to the supernatant 3 volumes of ethanol and a few crystals of sodium acetate, a flocculent precipitate was usually obtained. If none appeared within 24 hr, a few more crystals of sodium acetate were added. In the supernatants obtained from mucoid strains, this second addition always resulted in precipitation. The precipitate was separated by centrifugation, dissolved in distilled water and dialyzed against distilled water until the dialyzates were free of phosphate as determined by the method of Fiske and SubbaRow (1925). The capsular substance was reprecipitated as described above, washed with ethanol, and dried *in vacuo* over sodium hydroxide and phosphorus pentoxide. The dried powder was stored at room temperature. Solutions were prepared in 0.85 per cent saline.

Analysis of isolated capsular materials. Nitrogen was determined by the Markham method (1942). Glucose was determined by the Hagedorn-Jensen method (Bates *et al.*, 1942) after hydrolysis in 2 N HCl for 2 hr at 100 C. Methylpentose was determined according to Dische and Shettles (1948) and hexuronic acid by the carbazole method of Dische (1947).

[1] A preliminary report of this work has been published (Beiser and Davis, 1953).

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[4] This term has been proposed to denote mutations to decreased growth requirement (Englesberg, 1954–1955).

For determination of the constituent sugars about 50 mg of each of the isolated capsular materials was hydrolyzed for 2 hr at 100 C in 2 N HCl, and after evaporation to dryness the residue was dissolved in 0.5 ml of water. Ascending paper chromatograms of these hydrolyzed materials were prepared using phenol:water, butanol:acetic acid:water (4:1:5) (Partridge, 1950), and butanol:pyridine (Chargaff *et al.*, 1948) as solvents, and aniline hydrogen oxalate (Partridge, 1950) and *m*-phenylenediamine (Chargaff *et al.*, 1948) as indicators for reducing substances.

Preparation of vaccines. Formaldehyde (final concentration 0.2 per cent) was added to an 18 hr culture of the appropriate strain. After standing overnight at room temperature the suspension was centrifuged, washed twice with 0.85 per cent saline, and resuspended. The nitrogen contents of the suspensions were determined and the vaccines were diluted before use to contain 0.1 mg of nitrogen per ml.

Preparation of antisera. Rabbits were immunized with vaccines of mucoid strains 2-1 and 6-4. Each rabbit received a total of 20 ml in 16 injections. These consisted of 4 injections each of 0.5 ml during the first week, and of 1.0, 1.5, and 2.0 ml, respectively, during the succeeding 3 weeks. The animals were bled by cardiac puncture 5 to 7 days after the final injection, the sera were separated and merthiolate (1:1,000) was added as preservative. Control bleedings were also taken from all rabbits prior to immunization.

All sera were adsorbed before use with a vaccine prepared from the nonmucoid wild type strain grown at 35 C. Ten ml of serum mixed with 0.1 ml of vaccine were incubated for 1 hr in a water bath at 37 C and then overnight at 4 C. After centrifugation the supernatant was decanted and 0.5 ml of 0.85 per cent saline was added to the residue, which was gently redispersed to reveal agglutination of the vaccine. The adsorption procedure was repeated with further additions of vaccine until no agglutination was observed. For some studies sera were further adsorbed with vaccines prepared from the various mucoid mutants.

Quantitative precipitin curves were carried out as described by Kabat and Mayer (1948) using a micro-Kjeldahl method (Markham, 1942) for nitrogen determinations.

RESULTS

Isolation of mucoid mutants. Among hundreds of mutants of the nonmucoid W strain of *E. coli* selected for other properties, a few were observed to be mucoid. Their genealogy is presented in figure 1. Each step indicates ultraviolet irradiation followed by an appropriate selective procedure.

It will be observed that two of these strains (161-51 and 83-20) are auxotrophs, one (2-1) is a reversion from lysine auxotroph 81-83, and the last (6-4) is resistant to inhibition by D-serine.

Effect of various environmental factors on capsule production. It is known that the production of capsular material by Enterobacteriaceae can be markedly affected by temperature, as well as by genetic changes. The wild type strain W of *E. coli* was found to be mucoid at 15 C on the minimal or the various supplemented media used, but not at 25 C or at 35 C. The exception, strain 83-20, was intermediate in its behavior; it was mucoid at 15 C and at 25 C, but not at 35 C.

Various chemical alterations in the medium did not result in changes in the character of growth. The wild type, a mucoid (6-4) and a nonmucoid D-serine resistant mutant, and a mucoid auxotroph (2-1) retained their mucoid or nonmucoid

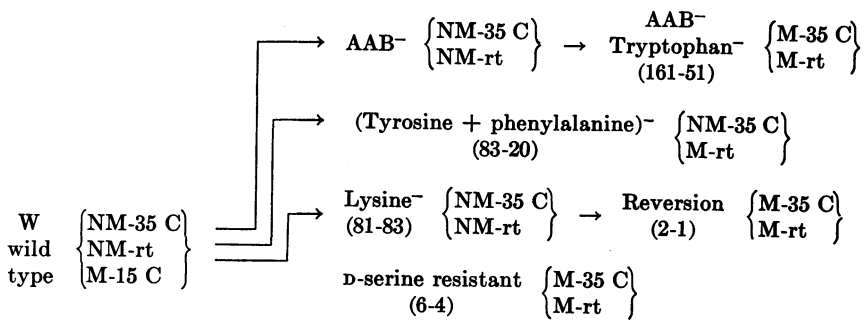

Figure 1. Preparation of mucoid strains

M = mucoid; NM = nonmucoid; rt = room temperature.

appearance at 35 C when the glucose concentration was varied from 0.05 to 5 per cent, when the pH was varied between 7 and 9, or when glucose (0.2 per cent) was replaced by the same concentration of various other carbon sources (galactose, mannose, xylose, fructose, mannitol, arabinose, succinate, lactate, or acetate).

Independence of the mucoid mutation from the other mutations. It will be shown below that the capsular polysaccharide formed by the wild type at low temperature is the same as that formed by the various mutants at higher temperatures. This fact suggested that the wild type possessed the genetic capacity to form the enzyme system required for capsule production, but that either the formation or the action of this system was prevented by the intracellular environment present in the wild type (but not by that in the mucoid mutants) at higher temperatures. It therefore seemed possible that capsule production in the mucoid mutants under investigation might be due not to a change in genes primarily concerned with polysaccharide production but rather to an indirect effect of the associated biochemical mutations.

To test this hypothesis, studies were carried out on the possibility of associated reversion of the mucoid property and the blocks in biosynthesis in strain 161-51, a double mutant requiring both α-aminobutyric acid (or isoleucine) and tryptophan. As diagrammed in figure 2, the mucoid property and each of the growth requirements reverted separately, both spontaneously and after exposure to ultraviolet irradiation. In no case did the mucoid character revert together with either of the growth requirements. The mutation to capsule formation therefore appears to be genetically distinct from the mutations to auxotrophy.

Further evidence for the independence of the mucoid mutation was provided by the results of efforts to isolate other mutants showing the same associated properties already observed. A number of D-serine resistant strains were obtained from the wild type. In contrast to strain 6-4 (figure 1), none proved to be mucoid at 35 C. A similar result was observed with a number of reversions obtained from lysine auxotroph 81-83.

Composition of capsular substance. Capsular substances were isolated from culture supernatants of mucoid strains 161-51 and 2-1 grown in liquid medium at 35 C, and from strain 83-20 grown at room temperature. In contrast, an identical procedure yielded no precipitate from the wild type strain or from 83-20 grown at 35 C.

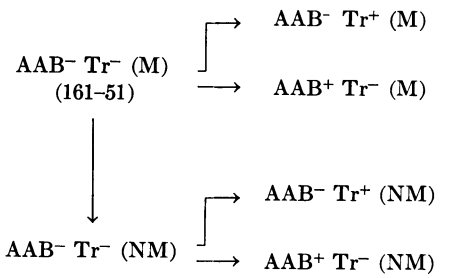

Figure 2. Reversion of mucoid strain

M = mucoid; NM = nonmucoid; AAB⁻ = requirement of α-aminobutyric acid; Tr⁻ = requirement for tryptophan.

TABLE 1

Analytical properties of isolated capsular substances

Material	N	Reducing Sugar (as Glucose)	Methyl-pentose	Hexuronic Acids
	%	%	%	%
2-1	0.4	65	29	25
83-20	0.3	63	28	25
161-51	0.3	64	25	23

TABLE 2

RF values of fucose, galactose, and components of concentrated hydrolyzates of capsular material from strains 161-51, 2-1, and 83-20

Solvent	Known		Hydrolyzate		
	Fucose	Galactose	Fucose	Galactose	X
Phenol:H_2O	0.65	0.43	0.66	0.44	0.15
Butanol:acetic acid:H_2O	0.30	0.15	0.30	0.15	0.08
Butanol:pyridine	0.53	0.34	0.53	0.33	

Quantitative analysis of the three isolated capsular preparations showed that they were identical in general composition (table 1). In addition, the reducing sugars in each were shown by paper chromatography with three different solvents to include galactose and fucose. Two of the solvents further revealed an unidentified slow moving third reducing component (table 2).

The three capsular preparations also appear to be immunochemically identical; they could not be distinguished from each other in their quan-

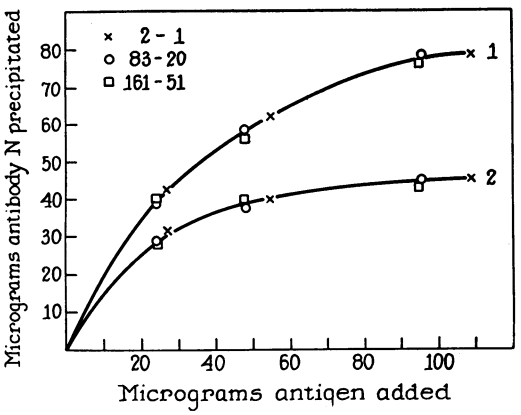

Figure 3. Reaction of isolated capsular materials with antisera. Curve 1 = reaction with antiserum to strain 2-1. Curve 2 = reaction with antiserum to strain 6-4.

titative reactions with antisera to two different mucoid strains (figure 3). Furthermore, when one of these sera was adsorbed with any one of the capsular preparations, addition of either of the other preparations did not yield any further precipitate.

Immunological observations with intact cells were carried out with all four mutants and with the mucoid growth of the wild type obtained at 15 C. Adsorption of either of the antisera with vaccines prepared from any of these strains completely removed the agglutinins for all the others. However, adsorption with vaccines prepared from various nonmucoid strains failed to remove agglutinins for the mucoid mutants.

DISCUSSION

Mucoid variants of *E. coli* strain W are not very common. Since they were encountered in our studies among certain biochemical mutants, it seemed possible that the mucoid character and the property selected for might both result from a single mutation. However, it was observed that the mucoid property and the requirements for various growth factors not only could revert independently, but failed to revert simultaneously. It therefore appears probable that the two observed inheritable changes arose from two different mutations in a single cell. The possibility has not been excluded, however, that in an ultraviolet treated population the appearance of a mucoid mutation is favored in the cells that are undergoing another kind of mutation (auxotrophic, meiotrophic, drug resistant) (Ryan, 1954; Bryson and Davidson, 1951).

Since genes controlling capsule production have proved particularly convenient to follow in studies on bacterial transformation (Ephrussi-Taylor, 1949; Leidy *et al.*, 1953), it is of interest to note that in the strain of *E. coli* studied here mucoid growth can be caused not only by mutation, but also by lowering the temperature. A similar effect of temperature has previously been observed with certain strains of Salmonella and Escherichia (Morgan and Beckwith, 1939). Alkalinity has also been reported to cause mucoid growth in a strain of *Bacterium coli-mutabile* (Zamenhof, 1946), but with the strains investigated here several pH values between 7 and 9 were without effect. Growth on media poor in nitrogen, phosphate, or sulfate has also resulted in greatly increased polysaccharide production by various Aerobacter and Escherichia strains (Duguid and Wilkinson, 1953; Wilkinson *et al.*, 1954). In the present study variations in the concentration or nature of the carbon source did not produce such an effect.

Chemical and serological tests indicated that the several mucoid variants studied all elaborate the same capsular material. Furthermore, at low temperature this substance is also formed by the wild type. These findings emphasize the possibility that a mutation or transformation resulting in mucoid growth might involve modification of an already existing enzyme system rather than provision of a new enzymatic reaction.

Boivin (1947) reported that the capsular material of one of his strains, S_1, was approximately one-third uronic acids and two-thirds neutral sugars by weight, and did not contain any hexosamines. The present results indicate a similar composition for the capsule of *E. coli* strain W. Different strains of *E. coli*, of course, may produce different capsular substances (Morgan and Beckwith, 1939; Boivin, 1947).

SUMMARY

Among many mutants of *Escherichia coli* strain W selected for various properties (auxotrophy, meiotrophy, drug resistance) a few were found to be mucoid when grown at the usual temperature of incubation (37 C), and one at 25 C but not at 37 C. Furthermore, at temperatures as low as 15 C even the wild type exhibited mucoid growth.

By serological cross adsorption studies, all mucoid strains tested (including the wild type)

were found to produce the same capsular material. This substance has been isolated and partially characterized.

Since the change to mucoid growth at 37 C appeared simultaneously with certain biochemical mutations, and since the parental strain already had the genetic potential for such growth (seen at 15 C), it seemed possible that the mucoid property might be a consequence of the altered intracellular environment resulting from the auxotrophic or other biochemical mutation. However, tests on one mucoid auxotroph showed that the mucoid property and the growth requirement reverted independently and did not revert simultaneously. It therefore appears that the two inheritable changes arose from two distinct mutations.

REFERENCES

BATES, F. J., AND ASSOCIATES. 1942 *Polarimetry, saccharimetry, and the sugars*. U. S. Government Printing Office, Washington, D. C.

BEISER, S. M. AND DAVIS, B. D. 1953 Mucoid mutants of *Escherichia coli*. Federation Proc., **12**, 436.

BOIVIN, A. 1947 Directed mutation in colon bacilli, by an inducing principle of desoxyribonucleic nature: its meaning for the general biochemistry of heredity. Cold Spring Harbor Symposia Quant. Biol., **12**, 7–17.

BRYSON, V. AND DAVIDSON, H. 1951 Spontaneous and ultra-violet-induced mutations to phage resistance in *Escherichia coli*. Proc. Natl. Acad. Sci., U. S., **37**, 784–791.

CHARGAFF, E., LEVINE, C., AND GREEN, C. 1948 Techniques for the demonstration by chromatography of nitrogenous lipide constituents, sulfur-containing amino acids, and reducing sugars. J. Biol. Chem., **175**, 67–71.

DAVIS, B. D. 1948 Isolation of biochemically deficient mutants of bacteria by penicillin. J. Am. Chem. Soc., **70**, 4267.

DAVIS, B. D. 1949 The isolation of biochemically deficient mutants of bacteria by means of penicillin. Proc. Natl. Acad. Sci., U. S., **35**, 1–10.

DAVIS, B. D. 1952*a* Intermediates in the biosynthesis of proline, ornithine, histidine, lysine, and the aromatic amino acids. Symposium on Microbiol. Metabolism 2nd Intern. Congr. Biochem., Paris, **5**, 32–40.

DAVIS, B. D. 1952*b* Analysis of the biochemical mechanism of drug resistance in certain biochemical mutants. Proc. Natl. Acad. Sci., U. S., **38**, 775–785.

DAVIS, B. D. AND MINGIOLI, E. 1950 Mutants of *Escherichia coli* requiring methionine and vitamin B_{12}. J. Bacteriol., **60**, 17–28.

DISCHE, Z. 1947 A new specific color reaction of hexuronic acids. J. Biol. Chem., **167**, 189–198.

DISCHE, Z. AND SHETTLES, L. B. 1948 A specific color reaction of methylpentoses and a spectrophotometric micromethod for their determination. J. Biol. Chem., **175**, 595–603.

DUGUID, J. P. AND WILKINSON, J. F. 1953 The influence of cultural conditions on polysaccharide production by *Aerobacter aerogenes*. J. Gen. Microbiol., **11**, 59–71.

ENGLESBERG, E. 1954–1955 Gain mutation in microorganisms. Ann. Rept.. Biol. Lab., Cold Spring Harbor, New York, pp. 23–28.

EPHRUSSI-TAYLOR, H. 1949 Additive effects of certain transforming agents from some variants of pneumococcus. J. Exptl. Med., **97**, 467–482.

FISKE, C. H. AND SUBBAROW, Y. 1925 The colorimetric determination of phosphorus. J. Biol. Chem., **66**, 375–400.

KABAT, E. A. AND MAYER, M. M. 1948 *Experimental immunochemistry*. Charles C Thomas, Springfield, Ill.

LEIDY, F., HAHN, E., AND ALEXANDER, H. E. 1953 In vitro production of new types of *Hemophilus influenzae*. J. Exptl. Med., **97**, 467–482.

LEDERBERG, J. AND ZINDER, N. 1948 Concentration of biochemical mutants of bacteria with penicillin. J. Am. Chem. Soc., **70**, 4267.

MAAS, W. K. AND DAVIS, B. D. 1950 Pantothenate studies. I. Interference by D-serine and L-aspartic acid with pantothenate synthesis in *Escherichia coli*. J. Bacteriol. **60**, 17–28.

MARKHAM, R. 1942 A steam distillation apparatus suitable for micro-Kjeldahl analysis. Biochem. J. (London), **36**, 790–791.

MORGAN, H. R. AND BECKWITH, T. D. 1939 Mucoid dissociation in the colon-typhoid-Salmonella group. J. Infectious Diseases, **65**, 113–124.

PARTRIDGE, S. M. 1950 Partition chromatography and its application to carbohydrate studies. Biochem. Soc. Symposia, **3**, 52-61.

RYAN, F. J. 1954 The delayed appearance of mutants in bacterial cultures. Proc. Natl. Acad. Sci., U. S., **40**, 178–186.

WILKINSON, J. F., DUGUID, J. P., AND EDMUNDS, P. N. 1954 The distribution of polysaccharide production in Aerobacter and Escherichia strains and its relation to antigenic character. J. Gen. Microbiol., **11**, 59–71.

ZAMENHOF, S. 1946 Unstable strains of the Colon bacillus. J. Heredity, **37**, 273–275.