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The Distribution of Polysaccharide Production in *Aerobacter* and *Escherichia* Strains and its relation to Antigenic Character

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With a note on the Influence of Potassium Deficiency upon Production of Polysaccharide by *Aerobacter aerogenes*

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SUMMARY: Strains of *Aerobacter aerogenes*, *A. cloacae* and *Escherichia coli*, including mucoid capsulate, mucoid non-capsulate, smooth and rough variant forms, were grown on defined agar media. The polysaccharide content of the cultures was estimated by the anthrone method and related to the amount of growth as measured by the non-dialysable nitrogen content. Polysaccharide production, as measured by the polysaccharide:nitrogen ratio, was increased in all strains when growth was limited by a restricted supply of nitrogen source so that added carbohydrate remained in relative excess. A low incubation temperature, 15-20° instead of 35°, influenced polysaccharide production differently in the various strains and species; thus it greatly increased production in *E. coli* strains, but decreased it in *A. cloacae* strains grown on certain media. The various strains showed considerable differences in the amount of polysaccharide production and in the distribution of the polysaccharide between the different possible sites of accumulation: i.e. intracellular, capsular and as loose slime. The mucoid strains produced the largest amounts of polysaccharide, most of which was present extracellularly as capsules or slime; the smooth strains produced much smaller amounts, almost entirely intracellular; a rough strain produced least of all. The antigenic constitution of certain strains was examined, particularly that of a mucoid capsulate *A. aerogenes* and mucoid non-capsulate and non-mucoid smooth variants derived from it. The designation of such morphological and immunological variants is discussed.

The amount of polysaccharide produced varies greatly with different strains and species of Enterobacteriaceae. Among twelve strains of *Escherichia coli* grown at 13-17° on glucose medium, Morgan & Beckwith (1939) found that the amount of polysaccharide produced, as indicated by the degree of mucoidness, was large in five strains, moderate in two strains and small in the remainder; they found similar differences among salmonella strains. Mucoid, smooth and rough variants of the same strain may also show great differences in polysaccharide production. Boivin, Mesrobianu, Magheru & Magheru (1936) measured the amount of a polysaccharide-containing substance, the 'complete antigen', which was extracted with trichloroacetic acid from cultures of *E. coli*. Large amounts of this (0.41-0.98 mg./100 mg. moist culture) were found in all of six 'smooth' strains which gave smooth colonies and were not agglutinable by trypanflavine, while none was found in five of

six 'rough' strains which gave irregular granular colonies and were tryptaflavine-agglutinable. Certain tryptaflavine-agglutinable strains of intermediate colony type contained considerable amounts of the substance. Lankford, Hoyo & Lutteringer (1951), using the periodate-Schiff staining method, found that O-inagglutinable rough variants of salmonella and shigella strains lacked the stainable intracellular polysaccharide of the smooth parent strains; they therefore suggested that this polysaccharide corresponded to the hapten of the O antigen characteristic of the smooth form.

Duguid & Wilkinson (1953) studied the influence of different cultural conditions on the amount of polysaccharide produced by a single strain (A3) of *Aerobacter aerogenes*, distinguishing the polysaccharide situated intracellularly, in the capsules or in the loose extracellular slime. In the present investigation the same methods have been applied for examination of several different strains of *A. aerogenes*, *A. cloacae* and *Escherichia coli*. For each strain the effect was observed of variation in the sugar:nitrogen ratio of the medium and of variation in the temperature of incubation.

METHODS

Strains. The strains labelled 'A' were isolated in this laboratory from faeces or water. Those designated NCTC were obtained from the National Collection of Type Cultures, Colindale, London. The strains were identified by fermentation and other biochemical tests as described by Wilson (1935). Also, cultures on MacConkey's lactose peptone agar at 35° and at 15–20° were observed for colony form (mucoid, smooth or rough), and were examined by the wet-film indian ink method for the presence of capsules and loose slime (Duguid, 1951). The organisms were suspended in an aqueous solution of acriflavine (0.2 %, w/v) and sodium chloride (0.85 %, w/v) and were observed during several hours for the occurrence of spontaneous agglutination, indicative of the rough form.

The strains A1 and A3 of *Aerobacter aerogenes* corresponded with Wilson's *Bacterium aerogenes* type I. They had the following properties: capsulate and slime-forming, mucoid (Pl. 1, fig. 1), non-motile, methyl-red negative, Voges-Proskauer positive, utilized citrate, indole negative, Eijkman negative, did not liquefy gelatin, fermented glucose, lactose, sucrose, mannitol, inositol and glycerol, producing acid and gas. Two stable variants were derived from strain A3 which resembled the latter exactly in their biochemical reactions, but differed in their mode of polysaccharide production. Variant strain A3 (S1) originated on several occasions in growths of strain A3 in a defined liquid medium. It differed from A3 in being non-capsulate; it was slime-forming and gave mucoid growths which were unusually tough and sticky. Variant strain A3 (O) was obtained from strain A3 cultivated in broth containing homologous antiserum; it differed from A3 in being non-capsulate and non-slime-forming, and in giving smooth non-mucoid growths (Pl. 1, fig. 2). Strain D301 (R) (kindly supplied by Prof. E. M. Dunlop) was a non-capsulate and non-slime-forming rough colony variant (Pl. 1, fig. 3) of an *A. aerogenes* isolated from drinking water; it resembled strain A3 in most biochemical reactions and in being non-motile, but differed in fermenting inositol and

glycerol without gas production and in being agglutinable by acriflavine. Strain NCTC 5920 was a typical *A. cloacae* in being motile, mucoid, slime-forming and mainly non-capsulate; it resembled *A. aerogenes* (A3) in biochemical reactions, except that it liquefied gelatin and failed to produce gas in fermenting inositol and glycerol. Strain NCTC 5936 was supplied as '*Bacterium aerogenes* type I'; although not liquefying gelatin, it is treated here as *A. cloacae* which it resembled in being motile, slime-forming and non-capsulate, in failing to produce gas on fermentation of inositol and glycerol, and in its antigenic character.

The *Escherichia coli* strains corresponded to Wilson's *Bacterium coli* type I. They had the following properties: methyl-red positive, Voges-Proskauer negative, did not utilize citrate, indole positive, Eijkman positive, did not liquefy gelatin, fermented glucose, lactose, mannitol and glycerol producing acid and gas, but not inositol. Strains A55 and A102 were motile, and A49, A93 and A108 non-motile. Strain A93 was capsulate and mucoid, strains A49 and A102 were non-capsulate and, at 15–20°, slime-forming and mucoid, and strains A55 and A108 were non-capsulate and non-slime-forming, giving smooth non-mucoid colonies. Strain A49 (O) was a non-slime-forming, smooth colony variant derived from A49. Strain A108 (R) was a rough colony variant derived from A108; it was not agglutinable by acriflavine and was classed as a smooth-rough intermediate.

Culture methods. The strains were grown as described by Duguid & Wilkinson (1953) on cellophan disks over agar medium in open Petri dishes which were incubated in sealed tanks to ensure adequate aeration and constant humidity. Each strain was grown on a series of plates of 50 ml. medium containing different amounts of ammonium sulphate as the sole nitrogen source; each 50 ml. medium also contained: 250 mg. lactose; 500 mg. phosphate (Na_2HPO_4 , 3 parts by weight, KH_2PO_4 , 1 part; pH=7.3); 100 mg. NaCl; 50 mg. K_2SO_4 ; 10 mg. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 1 mg. $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$; 0.05 mg. FeSO_4 ; 1.0 g. agar powder (one batch of Japanese agar obtained pre-1939 was used for all media). Incubation was continued for 4 days at 35° or 14 days at 15–20°, these durations being found sufficient for completion of growth and full polysaccharide production.

Estimations. Measurements were made as described by Duguid & Wilkinson (1953) of the final pH value, the degree of mucoidness and the wet weight of the culture; the total cell count and average capsule diameter after suspension in saline solution; the dry weight, total nitrogen content and polysaccharide content after dialysis of the saline suspension. Polysaccharide estimations by the anthrone method were made separately for centrifuged deposits and supernatants; the deposits contained the intracellular and capsular polysaccharides together, and the supernatants the loose slime polysaccharide. A separate estimate of the intracellular polysaccharide (that in the cell but not capsule) was made according to the intensity of staining by the periodate-Schiff method of Hotchkiss (1948). The amount of polysaccharide production relative to the amount of bacterial protoplasm was estimated as the ratio of the polysaccharide content of the culture to the non-dialysable nitrogen

content (polysaccharide:nitrogen ratio). The degree of mucoidness was observed visually and was also measured as the ratio of the wet weight of the culture to its nitrogen content (wet weight:nitrogen ratio). The average length and width of the bacilli, not including the capsule, were calculated from measurements of 50–100 cells in a phase-contrast photomicrograph of an unstained wet film. The *total cell and capsule volume* of the culture suspended in saline solution was calculated as $(\frac{4}{3}\pi R^3 + \pi R^2(L-1)) \times \text{total cell count}$, and the *total cell volume* (excluding capsules) as $\pi r^2 L \times \text{total cell count}$, where R =half the 'average capsule diameter', r =average bacillus radius and L =average bacillus length (measurements in microns). For comparison, the *volume of the surface culture before suspension* was calculated as the wet weight converted from mg. to mm.³ (assuming specific gravity=1.1). This volume included the slime as well as the cells and capsules. In the surface culture the capsules are less hydrated and occupy a smaller volume than after suspension in saline solution (Duguid & Wilkinson, 1953).

Serological methods. Cultures were grown for 24 hr. at 35° on agar containing 0.5 g. lactose and 0.5 g. peptone/100 ml. Whole-culture suspensions (cells, capsules and slime) were made in saline solution to a density of about 10⁹ cells/ml. Rabbits were injected with such suspensions to prepare specific antisera for strains A3 and A3 (O). The antigens tested *in vitro* were, for strain A3 (O), the whole-culture suspension and, for strains A3 and A3 (SI), the separated cellular and slime fractions. The latter were obtained by high-speed centrifugation of the whole-culture suspension. The supernatant first separated was taken as a cell-free preparation of slime. The cellular deposit was washed by suspension in fresh saline and re-centrifugation, this being done 3 times for strain A3 and 6 times for the more viscous strain A3 (SI). Finally, a very dense suspension of the deposit was made in saline containing 0.08 g. sodium azide/100 ml. The A3 deposit provided 'washed capsulate cells' and the A3 (SI) deposit 'washed non-capsulate cells', in both cases slime-free.

For agglutination tests the whole-culture suspension or the washed cellular deposit was diluted to about 3×10^8 cells/ml. and mixed with equal volumes of antiserum in a range of doubling dilutions. The results were observed after incubation for 2 hr. at 37°, and again after standing overnight at 4°. For a capsulate strain such as A3, agglutination occurred only with low dilutions of antiserum and gave rise to compact floccules. For a non-capsulate strain such as A3 (O), agglutination usually occurred to a high titre and gave fine granules. 'Capsule swelling' tests were made by mixing a drop of diluted antiserum with the cell suspension and examining a wet film of the mixture microscopically. Precipitation tests were made to demonstrate the reaction of antiserum with the loose slime polysaccharide. Doubling dilutions of the antiserum in saline, from 1/2 to 1/32, were placed in narrow tubes and a 1/10 dilution of the culture supernatant was superimposed. The tubes were examined after 15 min. at 37°, when a positive reaction was indicated by the appearance of a white ring of precipitate at the interface.

For agglutinin-absorption and precipitin-absorption tests the absorbing reagent was added drop by drop to an equal volume of the antiserum, with

constant agitation, and the mixture was incubated for 30 min. at 37°. After removal of the precipitated cells or slime by centrifugation, the supernatant was tested with the appropriate antigen to show whether the 'capsule swelling', agglutinating or precipitating activity had been lost as a result of the absorption. In the 'capsule swelling' tests the minimum dilution tested was that of the absorbed serum (i.e. 1/2), while in the agglutination tests it was 1/10 for the A3 cells and 1/40 for A3 (Sl) and A3 (O) cells.

RESULTS

Polysaccharide production was observed in the various strains of *Aerobacter aerogenes*, *A. cloacae* and *Escherichia coli* grown on 50 ml. plates of medium containing 250 mg. lactose and different amounts of ammonium sulphate, from 2.5 to 250 mg. Representative results for the media with 75 and 7.5 mg. ammonium sulphate/50 ml. are shown in Table 1. For a given supply of ammonium sulphate all the strains gave nearly the same amount of growth as measured by the nitrogen content. In all cases the cultures with 75 mg. ammonium sulphate/50 ml. gave growth which was limited by exhaustion of the lactose and gave nearly minimal production of polysaccharide. This was shown by the finding that with 250 mg. ammonium sulphate/50 ml. the amount of growth was not greater, nor the polysaccharide:nitrogen ratio less. The cultures with 7.5 mg. ammonium sulphate/50 ml. gave growth which was limited by exhaustion of this sole nitrogen source, and gave maximal or nearly maximal production of polysaccharide. This was shown by the finding that cultures on 2.5 mg. ammonium sulphate/50 ml. gave a third the amount of growth, but hardly any further increase in the polysaccharide:nitrogen ratio in spite of their threefold greater excess of lactose. All the strains of *A. aerogenes*, *A. cloacae* and *E. coli* thus showed an upper limit to polysaccharide production in the same way as strain A3 (Duguid & Wilkinson, 1953); increase of the sugar:nitrogen ratio in the medium gave an increase of polysaccharide production only up to a maximal level and any further excess of sugar remained unconsumed.

While all strains showed this tendency to give increased polysaccharide production on media with a restricted supply of ammonium sulphate, they differed considerably in the amount of polysaccharide formed and in its distribution between the different possible sites of accumulation. Mucoid cultures were given by all the strains which formed capsules or loose slime in considerable amounts. The degree of mucoidness as observed visually and as measured by the wet weight:nitrogen ratio was increased in proportion to the amount of capsular and slime polysaccharide, but not according to the amount of intracellular polysaccharide. The degree of mucoidness corresponded to the extent whereby the volume of the surface culture (including capsules and slime) exceeded the total volume of the cells (not including capsules and slime). In the non-mucoid cultures of strains A3 (O), D301 (R), A55 and A102, the calculated total cell volume was found to be approximately equal to the surface culture volume as measured by wet weight. In the slightly mucoid cultures of strains A1, A3, A3 (Sl), NCTC 5920, NCTC 5936, A93 and A102

grown on the ammonia-rich media, the cells occupied from 13 to 44 % of the surface culture volume. In the highly mucoid cultures of these strains grown on the ammonia-poor media, the cells occupied only 1–5 % of the surface culture volume. Similarly, with increasing mucoidness the dry weight comprised a decreasing proportion of the wet weight of the culture; this proportion was 22–37 % in the non-mucoid cultures, 10–14 % in the slightly mucoid cultures and 3–6 % in the highly mucoid cultures.

Capsulate Aerobacter aerogenes strains A1 and A3

Strain A3 gave results which were typical of the majority of *A. aerogenes* strains. Strain A1 differed from it in forming much larger capsules and in giving colonies of a tougher and stickier consistency. Comparison of the results given in Table 1 shows that the amount of growth and the total amount of polysaccharide production were similar in the two strains. However, strain A1 formed less polysaccharide intracellularly and more as loose slime. The average capsule diameter as measured after suspension in saline was twice as great in strain A1 as in strain A3. Correspondingly, the calculated total volume of cells and capsules in suspension was about eightfold greater for A1 than for A3. The larger capsular volume of strain A1 was not due to a greater production of capsular polysaccharide, but to a greater hydration and swelling of this material on suspension in water or saline solution. The great extent of this capsular expansion is shown by comparison of the volume of the surface culture before suspension (i.e. wet weight mg. converted to mm.³) with the calculated total volume of cells and capsules after suspension. The latter volume, although not including the loose slime, exceeded the former volume very greatly (about eightfold) in strain A1, but only slightly in strain A3 (Table 1). In spite of the great difference in physical character, the capsular polysaccharides of strains A1 and A3 were found to be identical antigenically; capsule-swelling (Pl. 1, fig. 4) and agglutination tests showed that both strains belonged to the klebsiella capsule type 54 of Edwards & Fife (1952), and cross-absorption tests failed to reveal any antigenic difference. Moreover, the O antigens of strains A1 and A3 were found to be identical by agglutinin-absorption tests on non-mucoid smooth variants of these strains. As reported previously (Duguid & Wilkinson, 1953), lowering of the incubation temperature from 35° to 15–20° gave little increase of polysaccharide production by strain A3: production was barely doubled in the ammonia-rich cultures and not increased at all in the ammonia-poor.

Aerobacter aerogenes strain A3, its slime-forming variant A3 (Sl), its 'smooth' variant A3 (O) and the 'rough' strain D301 (R)

The antigenic composition of strain A3 and its variants A3 (Sl) and A3 (O) was studied serologically. The results shown in Table 2 indicate the following facts. (1) The capsular and slime polysaccharides of strain A3 are antigenically similar or identical with each other and also with the slime polysaccharide of strain A3 (Sl). (2) The cells of strains A3, A3 (Sl) and A3 (O) all possess the same somatic ('O') antigen; this lies uncovered at the

TABLE 1. *Enterobacteriaceae* production by aerogenes, cloacae and coli strains grown on 50 ml. amounts of medium containing 250 mg. lactose, 500 mg. phosphate at pH 7.3 and different amounts of ammonium sulphate

Species	Strain	Ammonium sulphate in medium (mg.)	Degree of mucoidness	Capsule diameter (μ)	Int. poly. stain*	Cell count $\times 10^{-8}$	Calculated total volume (mm. ³)†			Wet weight (mg.)	Total nitrogen (mg.)	Polysaccharide (mg.)		Wet weight (mg./mg. nitrogen)	Poly-saccharide (mg./mg. nitrogen)
							Cells only	Cells and capsules	Cells and capsules			Deposit	Super-natant		
(a) Incubated for 4 days at 35°															
Aerogenes	A 1	7.5	+	3.3	+	430	155	8,600	1,170	8.3	10.8	7.9	140	2.3	
		7.5	+	9.8	+	40	32	21,300	2,350	1.6	16.1	24.6	1,500	26.0	
	A 3	7.5	+	1.6	+	520	270	1,580	930	7.4	10.6	2.7	130	1.8	
		7.5	+	4.3	+	54	45	2,630	2,410	1.5	22.5	20.8	1,600	29.0	
	A 3 (SI)	7.5	+	—	+	550	226	226	1,040	8.6	3.8	9.7	120	1.5	
		7.5	+	—	+	44	37	37	3,300	1.5	3.6	45.0	2,200	32.0	
	A 3 (O)	7.5	—	—	+	800	320	320	340	9.6	5.3	0.7	35	0.63	
		7.5	—	—	+	68	59	59	60	1.5	3.8	0.3	41	2.8	
	D 301 (R)	7.5	—	—	—	1000	300	300	340	9.9	1.8	0.3	34	0.21	
		7.5	—	—	—	94	57	57	70	1.4	0.25	0.34	49	0.41	
Cloacae	NCTC 5936	7.5	+	—	+	510	133	133	780	7.1	5.0	8.4	110	1.9	
		7.5	+	—	+	64	37	37	750	1.5	1.9	5.7	500	5.1	
	NCTC 5920	7.5	—	—	+	610	141	141	320	9.2	5.3	1.0	35	0.69	
		7.5	+	—	+	130	39	39	1,160	1.6	1.2	16.3	730	11.0	
Coli	A 93	7.5	+	2.5	+	460	161	3,960	1,270	9.3	12.3	1.8	140	1.5	
		7.5	+	3.3	+	53	33	1,190	1,230	1.5	6.9	5.4	810	8.1	
	A 102	7.5	—	—	+	620	286	286	440	9.2	5.4	0.4	48	0.64	
		7.5	—	—	+	76	44	44	70	1.4	1.8	0.3	49	1.5	
	A 55	7.5	—	—	+	800	408	408	450	9.3	6.2	0.8	48	0.75	
		7.5	—	—	+	79	46	46	50	1.4	1.2	0.1	35	0.90	
	(b) Incubated for 14 days at 15–20°														
	Coli	A 102	7.5	+	—	+	720	302	302	1,060	9.1	5.7	4.9	120	1.2
		7.5	+	—	+	55	32	32	990	1.6	1.7	8.4	640	6.5	
A 55		7.5	—	—	+	690	357	357	540	8.8	13.1	0.6	61	1.6	
		7.5	—	—	+	76	51	51	90	1.5	5.5	0.3	61	4.0	
A 49		7.5	+	—	+	68	.	.	970	1.5	1.4	8.7	640	6.7	
A 49 (O)		7.5	—	—	+	53	.	.	160	1.5	2.4	1.4	110	2.6	
	A 108	7.5	—	—	+	49	.	.	200	1.5	2.3	1.4	130	2.4	
	A 108 (R)	7.5	—	—	+	32	.	.	50	1.4	2.2	0.3	35	1.8	

* Intracellular polysaccharide staining intensity, —, $\perp$, + to + + + +.
 † I.e. when in suspension in saline solution.
 Note. In all cultures the final pH was in range 6.5–7.0, and mean bacillus length in range 1.3–1.9 μ .

surface of A3 (O) and A3 (SI) cells, but is entirely occluded by the capsule in A3 cells. (3) The A3 (O) whole cultures and the washed A3 (SI) cells are devoid of any A3 capsular or slime antigen.

Table 2. *Highest dilutions of antisera giving precipitation, agglutination and capsule-swelling reactions with various antigen preparations of Aerobacter aerogenes*

Antigen	Test*	A3 antiserum absorbed with						A3 (O) antiserum absorbed with	
		Nil	A3 washed			A3 (SI) washed		Nil	A3 (SI) washed cells
			A3 slime	cap- sulate cells	A3 (SI) slime	cells†	A3 (O) cells†		
A3 slime	P	16	0	0	0	8	16	0	0
A3 capsulate cells	A CS	20 8	0 0	0 0	0 0	10 8	20 8	0 0	0 0
A3 (SI) slime	P	16	0	0	0	8	16	0	0
A3 (SI) washed cells†	A	640	640	640	640	0	0	20,480	320
A3 (O) cells† (whole culture)	A	640	640	640	640	0	0	10,240	160

* P=precipitation; A=agglutination; CS=capsule-swelling. † I.e. non-capsulate.

Capsule-swelling titres are initial dilutions before mixing with antigen; agglutination titres are final dilutions.

Aerobacter aerogenes strains A3 and A3 (SI)

As compared with the parent strain A3, strain A3 (SI) differed in being non-capsulate and in giving colonies which were much tougher and stickier in consistency, though of similar mucoid appearance. While the total amount of polysaccharide produced was almost the same in the two strains, its distribution was quite different. Strain A3 (SI) gave over twice as much polysaccharide in the loose slime fraction and correspondingly less in the cellular deposit; presumably this was the consequence of its failure to retain extracellular polysaccharide as capsules. The amount of intracellular polysaccharide revealed by periodate-Schiff staining was about the same in the two strains. It is thus assumed that the intracellular polysaccharide content of the A3 cultures was equal to the whole of the deposit polysaccharide content of the A3 (SI) cultures. On this basis it is estimated that in strain A3 the proportion of the deposit polysaccharide occurring in the capsules was about 65% in the ammonia-rich cultures and 85% in the ammonia-poor, the remainder occurring intracellularly. Although A3 (SI) slime was identical antigenically with A3 slime, it differed physically in being much more viscous in solutions of equal concentration and in failing to increase the surface charge on red cells so as to inhibit viral haemagglutination (Macpherson, Wilkinson & Swain, 1953).

Aerobacter aerogenes strain A3 (O)

As compared with the parent strain A3, strain A3 (O) differed in being non-mucoid, smooth in colony form, non-capsulate and non-slime-forming. It gave a similar or slightly greater amount of growth than A3, but a much smaller total production of polysaccharide. Most of its polysaccharide (about 90%) was found in the deposit fraction, and, since capsules were absent, all of

this must have been situated intracellularly. Indeed, periodate-Schiff staining showed that the intracellular polysaccharide content of strain A3 (O) was slightly greater than that of strain A3. If, as suggested by Lankford *et al.* (1951), this 'intracellular' polysaccharide corresponds to the 'O' somatic antigen, some of it must permeate the cell wall and lie exposed on the surface. The small amount of free extracellular polysaccharide found in the supernatant fraction of A3 (O) cultures may have been some liberated 'O' polysaccharide; at least it did not correspond to the capsular and slime polysaccharide antigens of strain A3, since serological tests showed that A3 (O) cultures were devoid of these. The polysaccharide:nitrogen ratio of strain A3 (O) was increased about fivefold in ammonia-poor cultures as compared with ammonia-rich (compare 15- to 20-fold increases for A3 and A3 (Sl)). This increase was not accompanied by any increase in the degree of mucoidness, presumably because it is only extracellular polysaccharide which confers the mucoid character. That A3 (O) cultures did not contain any appreciable volume of extracellular material was confirmed by the close approximation of the calculated total cell volume with the surface culture volume (wet weight).

Aerobacter aerogenes strain D301 (R)

The rough *Aerobacter aerogenes* strain D301 (R) was also non-capsulate and non-slime-forming. It differed from strain A3 (O), not only in the rough character of its colonies, but also in its much smaller production of intracellular polysaccharide as shown by the virtual absence of staining by the periodate-Schiff method. This rough strain produced less polysaccharide than any smooth or mucoid strain, and it exhibited a minimal, barely twofold, increase of the polysaccharide:nitrogen ratio on restriction of the ammonium sulphate supply.

Aerobacter cloacae strains NCTC 5920 and 5936

The two strains of *Aerobacter cloacae* gave generally similar results. They formed slime and intracellular polysaccharide, but not capsules. (In NCTC 5920 cultures at 15–20° a proportion of the cells showed soft, indefinitely demarcated capsules which slowly disintegrated and dispersed in wet indian ink films (see Pl. 1, fig. 5)). Limitation of the ammonium sulphate supply increased the amount of polysaccharide production in both strains, though to a lesser extent in NCTC 5936. The maximal production was not as great as in the mucoid *A. aerogenes* strains. As compared with the 35° cultures (Table 1), those at 15–20° gave similar amounts of growth, but differed in the amount of polysaccharide production. At the lower temperature the polysaccharide:nitrogen ratio of NCTC 5936 cultures, ammonia-poor and ammonia-rich, was decreased to about a third; that of NCTC 5920 cultures was decreased only slightly if ammonia-poor, and increased about threefold if ammonia-rich. When tested with an antiserum prepared for the typical gelatin-liquefying *A. cloacae* strain U8 (described by Edmunds, 1954) the slimes of strains NCTC 5920 and 5936 both gave precipitation to the significant titre (1/16). In precipitin absorption tests, the U8 antibodies were absorbed entirely by NCTC 5936 slime, though not entirely by NCTC 5920 slime.

Escherichia coli strains

Tests were made with representative strains of the three varieties of *Escherichia coli* distinguished by Duguid (1951), the rare mucoid capsulate variety (A 93), the fairly common mucoid slime-forming non-capsulate variety (A 49 and A 102), and the very common non-mucoid variety, which is non-capsulate and not markedly slime-forming (A 49 (O), A 55 and A 108). The capsulate *E. coli* strain A 93 gave results similar to those of the capsulate *Aerobacter aerogenes* strain A 3, forming capsules and slime almost as abundantly at 35° as at 15–20°. The increase of polysaccharide:nitrogen ratio obtained on limitation of the ammonium sulphate supply was, however, only a third as great as in strain A 3.

The *Escherichia coli* strains A 49 and A 102, when grown at 15–20°, gave mucoid growths with abundant slime formation; their cultures then resembled those of the slime-forming non-capsulate *Aerobacter cloacae* strains. When grown at 35°, however, these *E. coli* strains produced very much less slime polysaccharide, so that their cultures appeared quite non-mucoid. With strain A 102 the lowering of incubation temperature from 35° to 15–20° increased the total polysaccharide production by twofold for ammonia-rich cultures and fourfold for ammonia-poor. Similar large increases were obtained with several other slime-forming *E. coli* strains. These strains resembled the strains found by Morgan & Beckwith (1939) and Beiser & Davis (1953) to give mucoid growths only at low temperatures. The small amount of slime polysaccharide formed in the non-mucoid growths at 35° appeared to be identical antigenically with that formed in large amounts in the mucoid growths at 15–20°. A rabbit was immunized by injection of a non-mucoid A 102 culture grown at 35° on the ammonia-rich medium; it showed abundant production of precipitating antibodies for the slime of a mucoid A 102 culture grown at 15–20° on the ammonia-poor medium.

The *Escherichia coli* strains A 49 (O), A 55 and A 108 gave results which were generally similar to those of the non-mucoid *Aerobacter aerogenes* strain A 3 (O). While incubation at 15–20° did not induce formation of slime in appreciable amount, it increased the production of intracellular polysaccharide. Decrease of the incubation temperature and limitation of the ammonium sulphate supply increased the total polysaccharide production almost as much in strain A 55 as in strain A 102. In A 55, however, the main increase occurred in the intracellular (deposit) fraction, and in A 102 in the slime (supernatant) fraction. The small intracellular polysaccharide content of A 55 grown at 35° on ammonia-rich medium is shown in Pl. 1, fig. 6, the moderate content of the growth at 35° on ammonia-poor medium in Pl. 1, fig. 7, and the large content of the growth at 15–20° on ammonia-poor medium on Pl. 1, fig. 8. As compared with its slime-forming parent strain A 49, non-slime-forming variant A 49 (O) showed an increase of intracellular polysaccharide. Loss of the power to form polysaccharide extracellularly led to increased storage of polysaccharide intracellularly.

The *Escherichia coli* smooth strain A 108 and its rough variant A 108 (R) did not differ in the same manner as the *Aerobacter aerogenes* smooth strain A 3 (O) and the rough strain D 301 (R). Strain A 108 (R) differed from A 108 only in

forming less slime polysaccharide. Unlike D301 (R) it formed a considerable amount of intracellular polysaccharide and was not agglutinable by acriflavine. It thus appeared that A108 (R) was a smooth-rough intermediate and not a true rough strain as D301 (R).

DISCUSSION

The present findings show that polysaccharide production is greatly increased under conditions of nitrogen limitation and carbohydrate excess, not only with mucoid capsulate *Aerobacter aerogenes* strains as found by Duguid (1948) and Duguid & Wilkinson (1953), but also in mucoid non-capsulate, non-mucoid smooth and non-mucoid rough strains of *A. aerogenes*, and in strains of *A. cloacae* and *Escherichia coli*. However, these latter strains differed markedly in the amount of polysaccharide produced and in the proportions accumulated as capsules, as loose slime and intracellularly. For each strain there were lower and upper limits to polysaccharide production: a minimum which was still produced when growth was limited by exhaustion of carbohydrate in the medium and a maximum which was not exceeded even when unconsumed carbohydrate remained available after cessation of growth due to nitrogen starvation. The difference between the maximal and minimal productions (polysaccharide:nitrogen ratios) was greater in the mucoid strains (e.g. 5–20-fold) than in the non-mucoid smooth and rough strains (e.g. 2–4-fold).

The mucoid strains produced the largest amounts of polysaccharide, forming much of this extracellularly as capsules or slime (50–90 %); the non-mucoid smooth strains produced smaller amounts, mostly intracellularly; the one completely rough strain produced the smallest amount, lacking even an appreciable intracellular content. The amount of polysaccharide which could be formed extracellularly was much greater than that which could be formed intracellularly. For the *Aerobacter aerogenes*, *A. cloacae* and *Escherichia coli* strains the maximal intracellular polysaccharide yields were respectively 2.5, 1.3 and 3.7 mg./mg. culture nitrogen, while the maximal extracellular polysaccharide yields were 30, 10 and 6 mg./mg. nitrogen.

Colony form, whether mucoid (M), smooth (S) or rough (R), may serve as an indication of the classes of antigens, capsular (K), somatic 'smooth' (O) and 'rough' (R), which the bacterium is genetically conditioned to produce. However, the relationship is not invariable since colony form is partly determined by cultural conditions. Confusion can thus arise when bacterial variants differing in antigen production are named according to colony form. The difficulties in the case of pneumococci have recently been emphasized by Austrian (1953). He points out that capsulate strains which produce the K polysaccharide in large amounts give mucoid colonies, while those producing K polysaccharide in small amounts give smooth colonies indistinguishable in appearance from the colonies of non-capsulate K-free strains. Accordingly, capsulate and non-capsulate pneumococci have been classified respectively as 'smooth' and 'rough' by some authors and as 'mucoid' and 'smooth' by others. The same difficulty is encountered in *Aerobacter aerogenes*, *A. cloacae* and *Escherichia coli* strains. In the experiments shown in Table 1, the capsulate and slime-forming strains yielded mucoid growths on the ammonia-poor media

which gave a large amount of polysaccharide, but non-mucoid smooth growths on the ammonia-rich media which gave a small amount. In the case of *E. coli* strain A102, serological tests confirmed that the non-mucoid culture grown at 35° contained small amounts of the same antigen (K) as was present abundantly in the slime of the mucoid culture grown at 15–20°.

The value of colony appearance as an indicator of antigen production is thus limited. If a culture is obviously mucoid under favourable cultural conditions it may be assumed to form K antigen, but if it is non-mucoid it may or may not form K antigen and this must be decided by serological tests. As suggested by Kauffmann (1949) the antigenic variants should be designated according to the classes of antigens which they produce rather than according to their colony form. In addition to the capsular (K) antigens, Kauffmann proposed a second class of extracellular antigens, the 'mucoid' (M) antigens which are non-capsular and occur as loose slime not firmly occluding the O antigen. He thus distinguished eight variant forms: MKO, KO, MO, O, MKR, KR, MR and R. The loose slime polysaccharides observed in the present series of strains apparently correspond to Kauffmann's M antigens. The slime of strain A3 (Sl), for instance, was shown to differ from the K antigens in failing to render the unheated bacilli inagglutinable by O antibody (A3 (O) antiserum). We suggest, however, that the M antigens should not be distinguished in a major class apart from the K antigens which they resemble very closely. As defined by Kauffmann (1947), the K class already includes morphologically different varieties of antigen, L, A and B; the L and B antigens, although occluding the O antigens, are not associated with demonstrable capsules like the A antigens. Pickett & Cabelli (1953) also described a further K antigen called Sm, obtained after prolonged immunization, which is common to all mucoid forms of Friedländer's bacillus, being present in addition to their main type-specific K antigen. The K class might conveniently be widened further to include the slime antigens and haptens. Although differing morphologically, the slime antigens resemble the capsular antigens chemically and serologically. For instance, the similarity in antigenic specificity of slime and capsules was demonstrated for a klebsiella strain by Edwards & Fife (1952) and for strains A3 and A3 (Sl) in the present investigation. We therefore suggest that the antigenic variants be classified immunologically in four groups only: 'K' forms which produce capsular or slime antigens (e.g. type-specific M, L, A or B antigens) and are distinguished as 'KO' or 'KR' according to whether or not the group-specific O antigen is also produced; 'O' forms producing O antigen but not K antigen; 'R' forms not producing either O or K antigens. Thus strains A1, A3 and A3 (Sl) are KO forms, strain A3 (O) is an O form, and D.301 (R) is an R form.

The morphological classification of variants should be stated quite separately, defining the colony form as mucoid, smooth or rough, and the cell form as capsulate and slime-forming, non-capsulate and slime-forming or non-capsulate and non-slime-forming. The cultures for these observations should be made on media with a high carbohydrate:nitrogen ratio, both at 35° and at 15–20° (Duguid, 1951; Duguid & Wilkinson, 1953).

Note on the Influence of Potassium Deficiency upon Production of Polysaccharide by *Aerobacter aerogenes*

BY J. P. DUGUID and J. F. WILKINSON

In a previous investigation (Duguid & Wilkinson, 1953) it was found that polysaccharide production by *Aerobacter aerogenes* strain A3 was greatly increased when growth in a lactose-containing medium was limited by deficiency of the nitrogen, phosphorus or sulphur sources. Observations have now been made on cultures where growth was limited by deficiency of potassium. The cultures were grown aerobically for 4 days at 35° on 50 ml. agar medium containing 250 mg. lactose, 250 mg. ammonium sulphate, 500 mg. phosphate (Na_2HPO_4 , 3 parts by weight; NaH_2PO_4 , 1 part), 100 mg. NaCl, 10 mg. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 mg. $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 0.05 mg. FeSO_4 and different amounts of potassium chloride as sole source of potassium. The results may be compared with those shown in the third row of Table 1 (strain A3 on 75 mg. ammonium sulphate) for a culture containing potassium in great excess, and limited by the lactose supply. Similar cultures, giving maximal growth and minimal polysaccharide production, were obtained with potassium supplies down to 1.5 mg. KCl/50 ml. When the amount of potassium was less than this, the amount of growth was decreased in proportion and the amount of polysaccharide production slightly increased. Thus, the results for a medium containing about 0.25 mg. KCl/50 ml. were as follows: final pH 6.5, mucoidness +, average capsule diameter 1.8μ ., intracellular polysaccharide staining +, cell count 58×10^9 , wet weight 310 mg., total nitrogen 1.5 mg., deposit polysaccharide 4.8 mg., supernatant polysaccharide 1.1 mg., wet weight:nitrogen ratio 207, polysaccharide:nitrogen ratio 3.7. Further decrease in the potassium supply diminished the amount of growth without increasing the polysaccharide:nitrogen ratio. Thus, potassium deprivation, by limiting growth and preserving the carbohydrate supply, led to an increase of polysaccharide production, but this increase (twofold) was much smaller than the increases (10- to 20-fold) given by equivalent deprivations of the nitrogen, phosphorus or sulphur sources. This suggests that a deficiency of potassium is specifically inhibitory to polysaccharide synthesis in *A. aerogenes* and agrees with the findings of Bernheimer (1953) for washed suspensions of pneumococci.

From the results of this and the previous investigation, the main nutritional requirements of *Aerobacter aerogenes* strain A3 are calculated to be as follows. Growth to give a non-dialysable nitrogen content of 1 mg. (i.e. $30\text{--}60 \times 10^9$ cells) requires as a minimum the nitrogen in 5 mg. ammonium sulphate, the phosphorus in 0.45 mg. phosphate (Na_2HPO_4 , 3 parts by weight; KH_2PO_4 , 1 part), the sulphur in 0.13 mg. sodium sulphate, the potassium in 0.18 mg. potassium chloride, and the carbon and energy of 25 mg. lactose in aerobic conditions.

REFERENCES

- AUSTRIAN, R. (1953). Morphologic variation in pneumococcus. I. An analysis of the bases for morphologic variation in pneumococcus and description of a hitherto undefined morphologic variant. *J. exp. Med.* **98**, 21.

- BEISER, S. M. & DAVIS, B. D. (1953). Mucoid mutants of *Escherichia coli*. *Fed. Proc.* **12**, 436.
- BERNHEIMER, A. W. (1953). Synthesis of type III pneumococcus polysaccharide by suspensions of resting cells. *J. exp. Med.* **97**, 591.
- BOIVIN, A., MESROBEANU, L., MAGHERU, G. & MAGHERU, A. (1936). Sur les rapports existants entre l'aspect des colonies données par les colibacilles et la présence ou l'absence d'antigène somatique 'complet' dans ces bactéries. *C.R. Soc. Biol., Paris*, **121**, 169.
- DUGUID, J. P. (1948). The influence of cultural conditions on the morphology of *Bacterium aerogenes* with reference to nuclear bodies and capsule size. *J. Path. Bact.* **60**, 265.
- DUGUID, J. P. (1951). The demonstration of bacterial capsules and slime. *J. Path. Bact.* **63**, 673.
- DUGUID, J. P. & WILKINSON, J. F. (1953). The influence of cultural conditions on polysaccharide production by *Aerobacter aerogenes*. *J. gen. Microbiol.* **9**, 174.
- EDWARDS, P. R. & FIFE, M. A. (1952). Capsule types of *Klebsiella*. *J. infect. Dis.* **91**, 92.
- EDMUNDS, P. N. (1954). Further *Klebsiella* capsule types. *J. infect. Dis.* **94**, 65.
- HOTCHKISS, R. D. (1948). A microchemical reaction resulting in the staining of polysaccharide structures in fixed tissue preparations. *Arch. Biochem.* **16**, 131.
- KAUFFMANN, F. (1947). The serology of the coli group. *J. Immunol.* **57**, 71.
- KAUFFMANN, F. (1949). On the serology of the *Klebsiella* group. *Acta path. microbiol. scand.* **26**, 381.
- LANKFORD, C. E., HOYO, H. & LUTTERINGER, J. F. (1951). Intracellular polysaccharide of Enterobacteriaceae. *J. Bact.* **62**, 621.
- MACPHERSON, I. A., WILKINSON, J. F. & SWAIN, R. H. A. (1953). The effect of *Klebsiella aerogenes* and *Klebsiella cloacae* polysaccharides on haemagglutination by and multiplication of the influenza group of viruses. *Brit. J. exp. Path.* **34**, 603.
- MORGAN, H. R. & BECKWITH, T. D. (1939). Mucoid dissociation in the colony-typhoid-salmonella group. *J. infect. Dis.* **65**, 113.
- PICKETT, M. J. & CABELLI, V. J. (1953). The precipitating antigens of Friedländer's bacillus. *J. gen. Microbiol.* **9**, 249.
- WILSON, G. S. (1935). The bacteriological grading of milk. *Spec. Rep. Ser. med. Res. Coun., Lond.* no. 206, p. 156.

EXPLANATION OF PLATE

- Figs. 1-3. Colonies of *Aerobacter aerogenes* on MacConkey's agar after 24 hr. at 35°. ($\times 7$)
 Fig. 1. Mucoid colony of strain A3. Fig. 2. Smooth colony of strain A3 (O). Fig. 3. Rough colony of strain D.301 (R).
- Fig. 4. Cells of *A. aerogenes* strain A1 suspended in antiserum for strain A3. Unstained wet film. Shows capsule 'swelling' and agglutination. ($\times 3750$.)
- Fig. 5. *A. cloacae* strain NCTC 5920 grown on lactose peptone agar for 14 days at 15-20°. In wet film with indian ink. Bacilli with indefinite capsules and much slime (light areas). ($\times 2000$.)
- Figs. 6-8. *Escherichia coli* strain A55. Intracellular polysaccharide stained by periodate-Schiff method. ($\times 3000$.)
- Fig. 6. After 4 days at 35° on 250 mg. lactose and 7.5 mg. ammonium sulphate/50 ml. medium. Minimal polysaccharide staining ($\perp$).
- Fig. 7. After 4 days at 35° on 250 mg. lactose and 7.5 mg. ammonium sulphate/50 ml. medium. Moderate polysaccharide staining ($++$).
- Fig. 8. After 14 days at 15-20° on 250 mg. lactose and 7.5 mg. ammonium sulphate/50 ml. medium. Maximal polysaccharide staining ($++++$).

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