STUDIES ON STRUCTURE AND FUNCTION OF BACTERIAL FIMBRIAE. REPORT III. FIMBRIATION AND ACID AGGLUTINATION

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ABSTRACT

The present studies provide an evidence suggesting that the occurrence of acid agglutination is one of the remarkable characteristics of the fimbriate strains of E. coli, Sh. flexneri or S. marcescens, and that the fimbriae may significantly influence the net charge of the bacterial surface. Three types of pattern of acid agglutination are recognizable and may be interpreted as presenting the qualitative and quantitative differences in the constituents of the fimbriae or in the antigenic structure.

INTRODUCTION

Electron microscopy reveals in certain groups of bacteria fine filamentous appendages 5–10 mμ in thickness known as "fimbriae" (Duguid, Smith, Dempster and Edmunds 1955) or "pili" (Brinton and Baron 1960), usually invisible by any other method. They occur in many strains of most genera of the family Enterobacteriaceae. They are arranged around the periphery in the number of 100 to 300 per cell, and are apparently distinguished from the flagella in being shorter and thinner, more numerous and straight, and in radiating in all directions from the cells (Brinton, Buzzell and Lauffer 1954; Smith 1954; Duguid and Gillies 1957, 1958; Duguid 1959; Okamoto 1958; Muto 1959; Shedden 1962; Hashimoto et al. 1963). Although their function in bacterial cells is not yet clarified, they seem to confer to the cells certain properties to adhere to the surface of the cells of various animal and plant origin and to play a significant role in the transfer of the gene (s) from the fimbriate strain to the non-fimbriate strain (Brinton and Baron 1958; Brinton, Gemski, Falkow and Baron 1961; Hashimoto et al. 1965).

Many workers quoted above, in working with the fimbriate cells, observed that the fimbriate cells have a faster electrophoretical mobility

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than that of the non-fimbriate cells, and that they form smaller, more translucent colonies on agar plates. Thus, the colony variations observed on the isolation plates of any strain may show striking differential characteristics between the fimbriate cells and the non-fimbriate cells in connection with their differences in electrophoretical mobility.

These observations led to the examination of the acid agglutination optima of the two types of strains in fimbriation in order to obtain further understanding as to the structure and function of the bacterial fimbriae from the physico-chemical point.

Materials and Methods

Method for acid agglutination: The method for the determination of the range and the optima of the acid agglutination was the slightly modified one of Michaelis (1911), and Beniasch (1911). The buffer series of McIlvaine were prepared according to Table 1.

Table 1. Preparation of Buffer Series of McIlvaine
0.1M $C_6H_8O_7$ (20—X) ml+0.2M Na_2HPO_4 Xml

X ml	pH	X ml	pH
0.40	2.2	7.71	4.0
1.24	2.4	8.28	4.2
2.18	2.6	8.82	4.4
3.17	2.8	9.35	4.6
4.11	3.0	9.86	4.8
4.94	3.2	10.30	5.0
5.70	3.4	10.72	5.2
6.44	3.6	11.15	5.4
7.10	3.8		

Acid agglutination was carried out by adding 0.2 ml of bacterial suspension of the cells in question to 1.8 ml of each of the buffer series just prepared. The tubes were carefully shaken, incubated in water bath at 45°C and read for the occurrence of the aggregation of the cells after 15 minutes and one hour. The range of acid agglutination was considered to be that zone of pH where the aggregation of the cells was noticed, and the degree of the agglutination was recorded as −, +, ╫ and trace according to the completeness of the reaction. The agglutination optimum was determined to be that zone of pH where complete aggregation occurred leaving a water-clear supernatant fluid.

Preparation of bacterial suspension: Preparation of bacterial suspension of the fimbriate strain was as follows: all of the organisms used were cul-

tured in nutrient broth at 37°C for 24 hours. Growth of each of the strains in nutrient broth was of diffuse and uniformly turbid appearance with no signs of detectable granular growth. After washing twice with normal saline the sediments were thoroughly shaken in a volume of normal saline equal to that of the original culture, and the final suspensions were carefully brought to a uniform turbidity. The bacterial suspension of the non-fimbriate strains was prepared in the same manner.

The preparation of bacterial suspension of the cells in the non-fimbriate phase of the fimbriate strain was obtained as follows: the stock culture on the agar slant of the strain to be tested was successively transferred on to the agar slants at intervals of 24 hours for at least 10 days, and a non-fimbriate variant without any hemagglutinating activity was obtained. The lawns were harvested from the surface culture, and the yields were weighed and suspended in a concentration of 1 mg/ml in normal saline. After washing once, the suspension of the same concentration of the cells was prepared.

Strains tested: Fourteen strains of *Escherichia coli,* eight strains of *Shigella flexneri* (reference numbers 1–9 except 2) and one of *Sh. dysenteriae,* and eight strains of *Serratia marcescens* (reference numbers 14, 24, 31, 33, 34, 44, 45 and 46) were used.

Experimental

Variability of the range and optimum of the acid agglutination of E. coli, Sh. flexneri (including Sh. dysenteriae) and S. marcescens.

1. Acid agglutination of *E. coli.*

Fourteen strains of *E. coli,* of which eleven strains were the fimbriate and the remainder were the non-fimbriate, were subjected to test for acid agglutination. The results are shown in Table 2. From the table it is noticed that the fimbriate strains proved positive for acid agglutination because of the occurrence of the aggregation of the cells in a wider range of pH to be tested, while the non-fimbriate strains did not aggregate in the same range of pH. Therefore, there was a remarkable difference in the acid agglutination between the fimbriate and the non-fimbriate strains.

Among the fimbriate strains, there were three types of acid agglutination recognized. The first type was differentiated from the other by the occurrence of the aggregation of the cells in the more acid region ranging from pH 2.2 to 3.4 having an optimum of 2.8 to 3.0. *E. coli* 4465 and 30-2A-B belong to this type. In the second type, consisting of *E. coli* 5556, 6196, Nr, CS 101, No. 7 and No. 11, aggregation occurred in the pH series ranging from 3.0 to 4.2 presenting an optimum pH of about 3.6 to 3.8. The third type containing the remainder of the fimbriate strains showed an alkaline

Table 2. Acid Agglutination of E. coli

	Strains	Hemagglutination	Agglutination in pH of															
			2.2	2.4	2.6	2.8	3.0	3.2	3.4	3.6	3.8	4.0	4.2	4.4	4.6	4.8	5.0	5.4	5.8
Fimbriate Strains	5556	++	—	—	—	—	+	+	++	++	++	++	++	+	+	—	—	—	—
	5557	++	—	—	—	—	—	—	—	—	+	++	++	++	++	+	+	+	—
	4465	+	+	+	++	++	+	+	+	—	—	—	—	—	—	—	—	—	—
	6196	+	—	—	—	—	+	++	++	++	++	++	—	—	—	—	—	—	—
	D 130/58	+	—	—	—	—	—	—	—	—	+	++	++	++	++	+	—	—	—
	Kauffmann	+	—	—	—	—	—	—	—	—	tr	+	+	++	+	+	—	—	—
	30–2A–B	+	+	+	+	+	++	++	++	+	+	tr	—	—	—	—	—	—	—
	Nr	+	—	—	—	—	+	+	++	++	++	++	+	—	—	—	—	—	—
	CS 101	++	—	—	—	+	+	+	+	++	+	+	+	—	—	—	—	—	—
	no. 7	+	—	—	—	—	—	—	+	+	++	++	+	+	+	—	—	—	—
	no. 11	+	—	—	—	—	—	—	+	+	++	++	++	++	tr	—	—	—	—
Non-fimbriate	1064	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	0 124	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	communis	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Table 3. Acid Agglutination of Shigella flexneri

	Strains			Hemagglutination	Agglutination in pH of														
					2.2	2.4	2.6	2.8	3.0	3.2	3.4	3.6	3.8	4.0	4.2	4.4	4.6	4.8	5.0	5.2
Sh. flexneri	Fimbriate	Sh-1	1a	++	—	—	—	—	—	—	+	+	++	++	++	++	++	+	—	—
		Sh-3	1a	+	—	—	—	—	—	—	+	+	++	++	+	+	tr	—	—	—
		Sh-5	3a	++	—	—	—	—	—	—	+	+	++	++	++	++	++	+	—	—
	Non-fimbriate	Sh-4	2a	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		Sh-6	2a	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		Sh-7	3a	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		Sh-8	1a	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		Sh-9	1a	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Sh. dysenteriae			1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

shift in producing the aggregation of the cells in the pH range from 3.8 to 5.0 with the optimum at 4.2 or 4.4.

It will be noted, however, that the occurrence of acid agglutination in the buffer series to be tested is characteristic of the fimbriate strains of *E. coli*. Although the range and optima of agglutination are not always the same for all of the fimbriate cells of *E. coli* in that some strains show a slight acid shift and some strains show a more alkaline shift, these shifts are not so great as to reject the difference between the fimbriate and the non-fimbriate strains of *E. coli*.

2. Acid agglutination of *Sh. flexneri*.

Three fimbriate strains (reference numbers 1, 3 and 5 of *Sh. flexneri*) and six non-fimbriate strains including one strain of *Sh. dysenteriae* (reference numbers 4, 6, 7, 8 and 9) were tested for acid agglutination. As seen from Table 3, there is a clear-cut difference between the fimbriate and the non-fimbriate strains in that the fimbriate strains produced an aggregation of the cells over the pH range of 3.4 and 4.8 in contrast to the complete lack of aggregation of the non-fimbriate cells. In addition, the pattern of the agglutination of the fimbriate strains of this genus was similar irrespective of the antigen type, and it is clearly shown that the type of agglutination may be comparable to the third type of *E. coli* in the pH range and optimum of acid agglutination.

3. Acid agglutination of *S. marcescens*.

All strains of *S. marcescens* under examination became fimbriate by culturing in nutrient broth only once. The acid agglutination performed with these strains proved to be positive with a close correspondence to the experiments described above. The pattern of agglutination of this genus, as shown in Table 4, showed a sharp acid shift with a rather wide range of pH from 2.2 to 3.2, except one strain of reference number 44, which produced aggregation over pH 2.4 to 4.6 with the optimum between pH 3.2 and 3.6.

Then, most representative individuals of this genus may belong to the first type of acid agglutination of *E. coli* in their agglutination pattern, although there may exist a few belonging to the second type of acid agglutination of *E. coli*.

Variability of acid agglutination accompanied by fimbrial variation.

In the preceding experiments, it is shown that the fimbriate strains produced aggregation of the cells over a wider range of pH to be tested with the respective optimum of acid agglutination, whereas detectable aggregation has never been noticed in the non-fimbriate over the same range of

Table 4. Acid Agglutination of S.marcescens

Strains			Hemagglutination	Agglutination in pH of														
				2.2	2.4	2.6	2.8	3.0	3.2	3.4	3.6	3.8	4.0	4.2	4.4	4.6	4.8	5.0
S. marcescens		14	+	+	+	+	+	tr	—	—	—	—	—	—	—	—	—	—
〃	〃	24	+	+	+	+	+	—	—	—	—	—	—	—	—	—	—	—
〃	〃	31	++	+	+	+	+	+	—	—	—	—	—	—	—	—	—	—
〃	〃	33	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—	—
〃	〃	34	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—	—
〃	〃	44	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—	—
〃	〃	45	++	—	+	+	+	+	++	+	+	+	+	—	—	—	—	—
〃	〃	46	++	+	+	+	+	+	tr	—	—	—	—	—	—	—	—	—

Table 5. Variability of Acid Agglutination Accompanied by Fimbrial Variation in E.coli

Strains			Hemagglutination	Agglutination in pH of														
				2.2	2.4	2.6	2.8	3.0	3.2	3.4	3.6	3.8	4.0	4.2	4.6	4.8	5.0	5.4	5.8
Fimbriate strains	in fimbriate phase	5556	++	—	—	—	+	+	++	++	++	+	tr	—	—	—	—	—	—
		4465	++	+	+	++	++	+	+	—	—	—	—	—	—	—	—	—	—
		D 130/58	+	—	—	—	—	—	—	—	—	+	++	++	+	+	—	—	—
		6196	++	—	—	—	—	+	++	++	++	++	+	—	—	—	—	—	—
	in non-fimbriate phase	5556	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		4465	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		D 130/58	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		6196	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Table 6. Variability of Acid Agglutination Accompanied by Fimbrial Variation in Sh.flexneri

Strains			Hemagglutination	Agglutination in pH of												
				2.2	2.4	2.8	3.0	3.4	3.8	4.0	4.2	4.6	4.8	5.0	5.4	5.8
Fimbriate strains	in fimbriate phase	Sh-1	++	—	—	—	—	+	++	++	++	+	—	—	—	—
		Sh-3	++	—	—	—	—	+	++	++	+	+	—	—	—	—
		Sh-5	++	—	—	—	—	+	++	++	++	+	—	—	—	—
	in non-fimbriate phase	Sh-1	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		Sh-3	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		Sh-5	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Table 7. Variability of Acid Agglutination Accompanied by Fimbrial Variation in S.marcescens

Strains				Hemagglutination	Agglutination in pH of														
					2.2	2.4	2.6	2.8	3.0	3.2	3.4	3.6	3.8	4.0	4.2	4.4	4.6	4.8	5.0
Fimbriate strains	in fimbriate straphase	S.marcescens	33	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—	—
		〃	〃 34	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—	—
		〃	〃 44	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—	—
		〃	〃 45	++	—	tr.	+	+	+	++	++	++	+	+	—	—	—	—	—
	in non-fimbriate phaseriate	〃	〃 13	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		〃	〃 24	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		〃	〃 31	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		〃	〃 46	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

pH. It seems probable from these results that the fimbriae on the surface of the bacterial cells are responsible for the appearance of the acid agglutination demonstrating a striking difference between the fimbriate and the non-fimbriate strains. In order to obtain further evidence for this suggestion, therefore, the following experiments were carried out with the non-fimbriate variants derived from the fimbriate strains by successive transfers on the agar slant.

The non-fimbriate variants were selected from the fimbriate strains of *E. coli* through successive transfers on the agar slant. And, the two different types of the same strains as to the fimbriation were compared for the occurrence of acid agglutination. The results are presented in Table 5. From the table it is clearly realized that the non-fimbriate variants of *E. coli* have lost their aggregating activity in the pH range under examination with the loss of the fimbriation, in spite that the same organisms can produce acid agglutination in their fimbriate phase with the characteristic pattern of the agglutinating types.

Similar results were obtained from the experiments carried out with the non-fimbriate variants derived from the fimbriate strains of *Sh. flexneri* or *S. marcescens,* as shown in Tables 6 and 7.

Effect of heat on acid agglutinating activity of Sh. flexneri.

As it has been pointed out by the preceding authors that the fimbriae were thermolabile by virtue of their protein nature, the effect of heat on acid agglutinating activity of the fimbriate strains of *Sh. flexneri* was examined because they aggregated in the sharp range of pH and showed a consistent optimum pH. Each of the suspensions of the fimbriate strains in normal saline were subjected to heating at 56°C or 100°C for 30 minutes, and after centrifugation the sediments were resuspended in the original volume.

As shown in Table 8 in which are presented the results, the non-fimbriate strains were not influenced by heating because they were not at all able to aggregate before or after the heating. On the contrary, the fimbriate strains changed the capacity of aggregating in the pH range under examination after boiling. Namely, the fimbriate strains remain unchanged their capacity of aggregating after heating at 56°C, but they could not produce any more the acid agglutination with the concomitant detachment of the fimbriae from their surface after boiling at 100°C. These results may be interpreted as evidence suggesting the responsibility of the fimbriae for the occurrence of acid agglutination of the fimbriate strains.

Effect of electrolytes on acid agglutination.

The effect of the presence of any electrolyte in the agglutination system

Table 8. Effect of Heating on Acid Agglutination of Sh.flexneri

	Strains	without heating		with heating at 56°C		with heating at 100°C	
		Fimbriation	Acid Agglutination	Fimbriation	Acid Agglutination	Fimbriation	Acid Agglutination
Fimbriate strains	Sh-1	+	+ (in pH 3.2–4.4)	+	+ (in pH 3.2–4.4)	−	−
	Sh-3	+	+ (in pH 3.6–4.4)	+	+ (in pH 3.6–4.4)	−	−
	Sh-5	+	+ (in pH 3.0–4.4)	+	+ (in pH 3.0–4.2)	−	−
Non-fimbriate strains	Sh-8	−	−	−	−	−	−
	Sh-9	−	−	−	−	−	−

Table 9. Effect of Electrolytes on Acid Agglutination

1. NaCl

pH	Concentration of NaCl in mols						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	0
2.2	−	−	−	−	−	−	−
2.6	−	−	−	−	−	−	−
3.0	−	−	−	−	−	−	−
3.4	−	−	−	+	−	−	+
3.8	++	+	+	+	+	+	++
4.0	+	+	+	+	+	+	++
4.4	+	+	+	+	+	+	++
5.0	−	−	−	−	−	−	−
5.4	−	−	−	−	−	−	−

2. $CoCl_2$

pH	Concentration of $CoCl_2$ in mols						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	0
2.2	−	−	−	−	−	−	−
2.4	−	−	−	−	−	−	−
3.0	−	−	−	−	−	−	−
3.4	−	−	+	+	+	+	+
3.8	−	++	++	++	++	++	++
4.0	−	++	++	++	+	+	++
4.4	·	+	+	++	+	+	++
5.0	·	·	·	·	·	·	−
5.4	·	·	·	·	·	·	−

3. $BaCl_2$

pH	Concentration of $BaCl_2$ in mols						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	0
2.2	−	−	−	−	−	−	−
2.6	−	−	−	−	−	−	−
3.0	−	−	−	−	−	−	−
3.4	−	−	++	++	++	++	+
3.8	·	·	·	·	·	·	++
4.0	·	·	·	·	·	·	++
4.4	·	·	·	·	·	·	+
5.0	·	·	·	·	·	·	−
5.4	·	·	·	·	·	·	−

4. $AlCl_3$

pH	Concntration of $AlCl_3$ in mols						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	0
2.2	−	−	−	−	−	−	−
2.6	−	−	−	−	−	−	−
3.0	−	−	−	−	−	−	−
3.4	−	−	−	++	+	+	+
3.8	−	−	++	++	++	+	++
4.0	−	+	++	++	++	++	++
4.4	+	+	+	+	+	+	++
5.0	−	−	−	+	−	−	−
5.4	−	−	−	−	−	−	−

was examined on the pattern of the acid agglutination of the fimbriate strains of *Sh. flexneri*.

As shown in Table 9, the patterns of acid agglutination are modified by the presence of a higher salt concentration in the system, the effect of which proved to be inhibitory at the concentration of 10^{-1} to 10^{-2} mol.

Discussion

Sobernheim and Seligman (1910) found a strain of *Bacillus enteritidis* to separate into two races, and Beniasch (1911–12) tested the acid agglutination point of this organism and found it to have altered its acid agglutination optimum when tested on two different occasions, a year having elapsed between the two tests. de Kruif (1922) observed a distinct difference in acid agglutination optimum for type D (bacillus of rabbit septicemia) and its mutant form, type G, and suggested that these acid agglutination optima were in the nature of physical constants for the two types and would imply the fundamental difference in the chemical constitution of the organisms.

On the other hand, the polar groups of the bacterial surface may be expected to contribute to the electrophoretical mobility of the organism as a function of their acid and base-binding capacities (Abramson, Moyer and Garin 1942). A study of the electrophoretical mobility of native and altered organisms under the proper conditions of pH, ionic strentgh, etc. should yield approximately information on the titration curve of the surface complex (Cohen 1945; McQuillen 1950). Among them, Cohen pointed out that the chemical modification of *B. proteus* OX-19 resulted in intermediate products and end products whose qualitative behavior in agglutination phenomena was grossly different from that of native organisms with the remarkable, concomitant change in the electrophoretical mobility.

It is well known that the isoelectric point of many bacteria is in the neighbourhood of pH 4.0 and 5.0; but in a neutral solution of low content the charge is negative and the bacteria move towards the anode in the electrophoretical field (Putter 1921; Northrop 1922, 1928; Winslow et al. 1923; and many subsequent workers). It was also pointed out that at pH values where all the bacteria carry an excess of one charge, electrostatic repulsion between particles will tend to impede agglutination of the cells, and with the change in the charge the isoelectric point was reached where the flocculation of the cells occurred, and pH value would present the isoelectric point of the organisms with the cessation of the electrophoretical mobility.

It is also well known that many types of bacteria have on occasion two variants, "rough" and "smooth", which differ in colony morphology, and two variants show a difference in tendency to agglutinate and precipitate

from concentrated suspensions. Moyer (1936) noted that his strain of *E. coli* had a bimodal distribution, and succeeded in showing the connection between the mobility classes and colony morphology variants, of which the fast bacteria were rough and the slow bacteria were smooth. During the morphological investigations of *E. coli* by electron microscope, Brinton, Buzzell and Lauffer confirmed the development of the filaments (at present termed pili by them) on the cell surface easily distinguishable from flagella, and presented a proof of the correlation between the filamentation and the electrophoretical mobility or the colony morphology, in demonstrating that the faster the mobility is the organism is that much more non-fimbriate and a producer of a larger colony, and the slower the mobility is the organism is that much more fimbriate and a producer of a smaller colony.

The present studies adduce an evidence suggesting that the occurrence of acid agglutination is also one of the remarkable characteristics of the fimbriate strains and that the fimbriae may significantly influence the net charge of the bacterial surface. The different patterns of acid agglutination observed in the members of the various genera or of the same species seem to be not so difficult to be interpreted because of the qualitative and quantitative differences in the constituents of the fimbriae or in the antigenic structure as previously demonstrated by some workers (Duguid and Gillies 1956). And the difference, in turn, would influence the net charges of the cell surface and may be responsible for the species or genus specificity of the fimbriae.

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