THE RELATIONSHIP BETWEEN pH TOLERANCE, VIRULENCE, AND PROTEOLYTIC ENZYMES IN BACTERIA. II. SHIGELLA*

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A previous paper showed that virulent strains of *Bacillus anthracis* were capable of growing in broth of higher pH values than would comparable numbers of an avirulent variant. The pH tolerance of the virulent bacteria was shown to be correlated with a production of alkaline-effective proteolytic enzymes greater than that of the avirulent strain. The addition of trypsin to broth increased the pH tolerance of the avirulent strain. It was also found that heating serum to destroy the serum protease-inhibitor made it a better medium for bacterial growth. It was stated that pH tolerance is related to the production of a trypsin-like enzyme (or enzymes) and that this enzyme is probably a determining factor in the virulence of the organism.

Roughness of colonial growth is associated with virulence among strains of *B. anthracis,* and some of the results obtained might be ascribed to the character of "roughness" rather than to virulence. Also, this bacterium is gram-positive. For these reasons, strains of Shigella, which are opposite in these two characteristics, were chosen for study.

Materials and methods

Procedures were similar to those described in the previous paper.[2]

Cultures. The bacteria used in this study were Shigella species, *S. dysenteriae,* and *S. paradysenteriae* Flexner. Cultures D-1, D-2, D-3, D-4, and D-5 were obtained from Dr. H. P. Treffers of Yale University School of Medicine.

Shigella species is probably a member of the para-Shiga group and is designated by the code "D-1." It is virulent.

S. dysenteriae is an avirulent organism typical in its reactions, and is referred to as "D-2."

S. paradysenteriae Flexner. Three original strains typical in their biochemical reactions were used. They were "D-3", and "D-4" (both virulent), and "D-5" (avirulent).

Dissociation of cultures. Strains VD-3, VD-4, and VD-5R were dissociated from the original cultures D-3, D-4, and D-5, respectively ("V" in the above designations means "variant"), by inoculating the original cultures into tubes of nutrient-tryptose broth at about pH 7.0 and incubating at 37°C. for 25 days; the

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cultures being transferred once every 7 days. At the end of this time the broth cultures were streaked on nutrient-tryptose agar plates, rough colonies were transferred to broth, the broth incubated, and then streaked on to plates from which rough colonies were transferred to slants. Strain VD-5S, a smooth variant of strain D-5, was obtained by streaking a broth culture of D-5 without any extended period of incubation. Strain 4AD-3 was obtained from VD-3 by passing this culture through alkaline broth of increasing pH up to 8.65 (seven transfers) and then through nine transfers in broth of the same pH. Strain 1D-3 was obtained by passing D-3 through one mouse.

Enzyme titration. Enzyme solution was added to equal volumes of 1 per cent Bacto-peptone solution, and a modification of the formol titration method used by Brown[1] was applied to 1 and 2 ml. of the enzyme-peptone solution. Distilled water was added to the aliquot to bring the total to 10 ml. Five drops of phenol red indicator were added (except when the Beckmann glass electrode was used in place of the comparator block), the reaction adjusted to pH 8, 4 drops of phenol red added and followed by the addition of 8 ml. of formalin. The contents of the tube were then mixed gently and titrated with *N*/20 NaOH to pH 8. The titration of a formalin blank was subtracted from the above titration. In a few cases the enzyme solution was inactivated by boiling and the inactivated enzyme was added to peptone to serve as a control.

Experimental results

Virulence: The cultures to be tested were grown in Difco-brain-heart-infusion broth for from 19 to 24 hours and 0.5 ml. of the diluted broth culture were added to 4.5 ml. of 5 per cent mucin[4] to give the desired dilution of bacteria. Of the resulting suspension, 0.5 ml. were injected intraperitoneally into each of three 18 to 22 gm. white mice and the animals were observed for three weeks. As variations in the effectiveness of the mucin influence the virulence of the bacteria tested, the virulent and avirulent bacteria were tested at the same time and with the same batch of mucin.

The virulence titrations showed that strain D-1 killed with 116,000 bacteria, while strain D-2 required 34,000,000 cells. The titrations of the *S. paradysenteriae* Flexner strains showed that D-3 was virulent in that 7,500 organisms killed mice, whereas the rough variant VD-3 dissociated from it was avirulent, for only 56,000,000 bacteria produced death. The results of D-4 showed that it killed with 3,100 cells, while the VD-4 variant derived from it required 9,000,000 organisms to kill.

pH tolerance: This section presents the results of determinations of the pH tolerance of the bacteria under study and will show that the virulent bacteria are more tolerant to alkaline broth than are the avirulent organisms.

Cultures were grown on nutrient-tryptose agar slants and some of the

growth was suspended in 7 ml. of distilled water in Klett-Summerson tubes and standardized to a density of "12" in the colorimeter using a blue filter. Of each standard suspension 0.1 ml. was inoculated into a duplicate set of tubes containing 10 ml. of sterile broth of the desired pH value. The strains tested all gave about equal growth at pH 5.15 and at pH 7.75, but at pH 8.65 all the virulent strains (D-1, D-3, D-4) grew in 19 hours whereas the avirulent strains (D-2, VD-3R, D-5, VD-5R) failed to grow. When this experiment was repeated and counts made upon the suspensions so the numbers of cells inoculated into the test-tubes were known, similar results were obtained, but it was also observed that the distilled water killed the avirulent cells. Further tests elicited the information that distilled water, tap water, and physiological saline all produced a decrease in the numbers of avirulent bacteria. The virulent strains were adversely affected also, but to a lesser extent. This led to the use of broth as the suspending medium, the dilution tubes being kept in water at 4°C. to prevent growth of the bacteria.

The results of inoculating strains D-3, VD-3, 4AD-3, and 1D-3 into broth of various pH values are given in Table 1. It is seen that there is a difference between the virulent D-3 strain and the avirulent VD-3 strain, this difference being quite apparent at each of the pH values tested. While at pH 8.50 all tubes of D-3 and VD-3 of comparable inocula showed growth, the growth was greater in the D-3 inoculated tubes. At pH 8.65, even 6 organisms of the virulent D-3 strain produced a heavy growth in 18 hours, while not even 3,400,000 VD-3 bacteria were able to yield visible growth in this time and an inoculum of 680,000 after incubation for 24 hours failed to show growth. After this time all VD-3 inoculated tubes showed growth, but the amount was less than in the tubes inoculated with culture D-3. The differentiation is complete at pH 8.80 where none of the inocula of the VD-3 strain showed growth after 72 hours of incubation. The 1D-3 strain which had been passed through a mouse without a resultant increase in virulence also showed no increase in pH tolerance, the results being very similar to those obtained with the original D-3 strain. Strain 4AD-3 was somewhat more resistant to alkaline pH values than was VD-3, for at pH 8.65, 410,000 bacteria produced visible growth in 18 hours, while with VD-3 no growth was apparent at this time with inocula of either 680,000 or 3,400,000 bacteria. The difference remained at 24 hours, but after 48 hours of incubation the growth of both strains was equal. The 4AD-3 strain also grew better than did the VD-3 strain at the higher pH value of 8.80. A comparison of the results with the virulent D-3 and 1D-3

TABLE 1

pH TOLERANCE OF STRAINS OF *SHIGELLA PARADYSENTERIAE* FLEXNER

		pH 8.50 Incubation (hours)				*pH 8.65 Incubation (hours)*				*pH 8.80 Incubation (hours)*			
Strain	*Inoculum*	*18*	*24*	*48*	*72*	*18*	*24*	*48*	*72*	*18*	*24*	*48*	*72*
D-3	5,500,000	x*	x	x	x	x	x	x	x	1	1	1	2
	550,000	x	x	x	x	x	x	x	x	0	1	1	2
	55,000	x	x	x	x	3	3	3	3	0	0	1	2
	5,500	3	3	3	3	4	4	4	4	0	0	1	2
	550	3	3	3	3	4	4	4	4	0	0	1	2
	55	3	3	3	3	4	4	4	4	x	x	x	x
	6	3	3	3	3	4	4	4	4	x	x	x	x
	1	0	3	3	3	x	x	x	x	x	x	x	x
1D-3	3,070,000	x	x	x	x	x	x	x	x	0	1	1	2
	307,000	x	x	x	x	x	x	x	x	0	0	1	2
	30,700	x	x	x	x	x	x	x	x	0	0	1	2
	3,070	3	3	3	3	3	3	3	3	0	0	1	1
	307	3	3	3	3	3	3	3	3	0	0	0	0
	31	3	3	3	3	2	3	3	3	x	x	x	x
	4	3	3	3	3	1	3	3	3	x	x	x	x
	1	0	0	0	0	0	0	0	0	x	x	x	x
VD-3	3,400,000	2	2	2	2	0	2	2	2	0	0	0	0
	680,000	x	x	x	x	0	0	2	2	0	0	0	0
	340,000	2	2	2	2	0	0	2	2	0	0	0	0
	68,000	x	x	x	x	0	0	2	2	0	0	0	0
	34,000	2	2	2	2	0	0	2	2	0	0	0	0
	3,400	2	2	2	2	0	0	2	2	0	0	0	0
	340	1	2	2	2	x	x	x	x	x	x	x	x
4AD-3	4,100,000	x	x	x	x	2	2	2	2	0	0	1	1
	820,000	x	x	x	x	x	x	x	x	0	0	1	1
	410,000	2	3	3	3	2	2	2	2	0	0	0	1
	82,000	x	x	x	x	x	x	x	x	0	0	0	1
	41,000	2	3	3	3	0	2	2	2	0	0	0	0
	4,100	1	3	3	3	0	0	1	1	0	0	0	0
	410	0	2	2	2	0	0	1	1	x	x	x	x
	41	0	1	2	2	x	x	x	x	x	x	x	x

*Numbers indicate amount of growth.
0=no growth, 1=slight growth, 2=good growth, 3=very good growth, 4=heavy growth, x=not done.

strains and those of the avirulent strains shows that the virulent bacteria are more tolerant to high pH values than are the avirulent organisms.

The change in pH of uninoculated broth tubes was watched by taking the pH of such tubes after 72 hours of incubation. There was no

change in the pH 8.50 tubes and only a slight change in the broth adjusted to pH 8.65 and pH 8.80.

An important factor which might be responsible for the results obtained would be the change in pH caused by the addition of the inoculum, for if the inoculum of the strain showing growth at the higher pH values had the ability of causing an immediate drop in pH it would explain the results obtained. The pH of the broth was determined with the Beckmann meter immediately before and after inoculation and then again after incubation. The results (Table 2) show that no change in pH resulted from the addition of the inoculum, but was due to the growth of the organisms inoculated.

TABLE 2

pH VALUES OF BROTH BEFORE AND AFTER INOCULATION

Strain	*Before inoculation*	*After inoculation (hours)* 0		21.5
	pH	pH	pH	Growth
D-3	8.60	8.60	8.30	2
D-5	8.60	8.60	8.55	0
Uninoculated	8.65	x	8.65	0
D-3	8.75	8.75	8.60	1
D-5	8.75	8.75	8.65	0
Uninoculated	8.75	x	8.70	0

Proteolytic enzymes: It has been inferred that the virulence and the pH tolerance of bacteria are associated with the presence of enzymes which differ quantitatively or qualitatively in the bacteria. Previous studies with *B. anthracis*[2] have suggested that the virulent bacteria possess more proteolytic enzyme activity in the alkaline range than do the avirulent bacteria.

The amount of enzyme activity produced by the virulent Flexner D-4 and the avirulent Flexner VD-5R strains was studied. Bacteria were inoculated into brain-heart-infusion broth (Difco) and after incubation at 37°C. for 11 days the cultures were filtered through Chamberland L-5 candles and the bacterial filtrates were used as the enzyme solutions. Four milliliters of the filtrate were added to 4 ml. of a 1 per cent Bacto-peptone solution. In the first experiment, a portion of the enzyme solution was inactivated by autoclaving, and 4 ml. of this inactivated filtrate were added to 4 ml. of peptone solution. These mixtures were adjusted to pH 8.70 and incubated at 37°C. for 4 days, after which the formol titration was carried out with $N/20$ NaOH. The results of such an ex-

periment show that the D-4 strain produced enzymes which were active at pH 8.70, while no such activity was exhibited by the VD-5R strain.

In the next and in succeeding experiments using the formol titration method, 1 ml. of enzyme solution was added to 9 ml. of peptone solution and this mixture was titrated immediately before and again after incu-

TABLE 3

ENZYMIC EFFECT OF FILTRATES OF STRAINS OF *SHIGELLA PARADYSENTERIAE* FLEXNER ON PEPTONE

	Titration of 1 ml. of peptone + filtrate				
	pH values		*Ml. N/20 NaOH*		*D-4 minus*
Experiment	*D-4*	*VD-5R*	*D-4*	*VD-5R*	*VD-5R*
57	6.70	6.85	.16	.06	.10
56	6.80	6.80	.24	.11	.13
47	7.15	7.00	.16	.07	.09
49	7.40	7.35	0	0	0
51	7.50	7.50	.22	.16	.06
		mean=	.16	.08	.08
43	8.70	8.70	.15	.02	.13
50	8.70	8.60	.12	.02	.10
57	8.70	8.65	.04	.02	.02
58	8.70	8.70	.10	.03	.07
		mean=	.10	.02	.08
51	8.80	8.80	.04	.04	0
55	8.80	8.70	.04	0	.04
56	8.75	8.70	.06	.03	.03
		mean=	.05	.02	.03

bation for 4 or more days. A summary of the results is recorded in Table 3 and shows that of 5 experiments performed at or near pH 7, one showed no activity with either strain, while the other four showed enzymic activity in the filtrates of both strains with the D-4 filtrate being more active than that of VD-5R in each instance. The mean value for the titrations involving the D-4 strain was 0.16 ml. of NaOH, while that for the avirulent VD-5R was only 0.08 ml. The statistical significance of the differences between the virulent and avirulent strains was tested by using the standard error of the differences and gave a probability value of 0.05 to 0.02, indicating significance. Four experiments at pH 8.70 gave a mean value for the avirulent VD-5R of only 0.02 ml. of

NaOH, an insignificant amount. The virulent D-4 strain gave a value of 0.10 ml. These results are statistically significant. The differences at about pH 8.80 are not significant. The series of 12 experiments in Table 3 was tested by the analysis of variance and showed a high significance for the differences between the two strains, the probability being less than 0.001.

These results show that pH tolerance is associated with the greater production (and release into the medium) of alkaline-effective proteolytic enzymes by the virulent bacteria. As pH tolerance has been shown to be related to virulence, this production of trypsin-like enzyme is also related to virulence.

Discussion

The reader is referred to the paper dealing with *Bacillus anthracis* for a complete discussion of the implications of the results of this study. The results obtained with the Shigella group of bacteria were similar to those obtained with *B. anthracis.* That the virulent strains were able to grow at pH values higher than could the avirulent bacteria dissociated from them indicates a close correlation between pH tolerance and virulence. The ability of the bacteria to grow in alkaline broth is apparently due to the production of a proteolytic enzyme which is active at alkaline pH values. Thus, with the bacteria of the Shigella group as well as with *B. anthracis* the probable importance of an alkaline-effective enzyme in the composition of virulence[3] is indicated. The results obtained with the dysentery strains showed that differentiation was most effective at about pH 8.65, whereas with *B. anthracis* it was about pH 9.20. This could mean that either different enzymes were involved or that the *B. anthracis* strains produced more of the same enzyme. The data obtained from enzyme preparations of the two genera of bacteria indicate similarity. It must be emphasized that "enzyme" refers to an extract of the bacterial cells, and more than one enzyme or system of enzymes could be involved. The quantitative aspect of the alkaline-effective enzyme is shown by the initiation of growth in alkaline broth by larger inocula when smaller inocula fail to do so.

The same relationship between pH tolerance, virulence, and the production of alkaline-effective proteolytic enzymes very probably exists among other, if not all, genera of virulent bacteria. The neutralization of the proteolytic enzymes, which is believed to be a cause of the invasive property of the organism, offers a point of attack in interfering with the in vivo growth of virulent bacteria.

References

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