EFFECT OF TEMPERATURE ON GROWTH AND VIRULENCE OF *PASTEURELLA PESTIS*

Physical and Nutritional Requirements for Restoration of Virulence

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Fukui et al. (1959; 1960) reported previously that *Pasteurella pestis* from cultures grown at 5 C consistently manifested lowered virulence. It was further demonstrated that subsequent incubation of such cultures at 37 C for 6 to 8 hr restored maximal virulence in the absence of significant cellular multiplication. Since previous studies were performed with a complex medium under one set of physical and chemical conditions, studies were undertaken to define the physical and nutritional requirements for the restoration of virulence.

MATERIAL AND METHODS

The Alexander strain of *P. pestis* was employed in these studies. At the outset, this strain was passed through guinea pigs, and several blood agar base (Difco) slants were inoculated from a virulent single colony isolate. After incubation at 26 C for 24 hr, the slants were layered with sterile mineral oil and stored at −20 C. One slant was removed from storage each week to serve as an inoculum for a series of blood agar base working slants which were also incubated at 26 C for 24 hr before being used as inocula for broth cultures. The growth from a single working slant served as the inoculum for 50 ml of heart infusion broth (Difco) contained in a 500-ml Erlenmeyer flask and supplemented with 0.25% xylose and 0.06% $MgSO_4 \cdot 7H_2O$. The broth cultures were incubated on a reciprocating shaker at 5 C for 4 days. They were then transferred to fresh medium at the rate of 10 ml of culture per 50 ml of medium and again incubated at 5 C for 4 days before being used in an experiment. The resulting culture, yielding approximately 1×10^9 viable cells per ml, was harvested by centrifugation at 5 C. After washing once by resuspending the cells in glass-distilled water and recentrifuging, the cells were suspended in a volume of distilled water about one-tenth that of the original volume of the culture. Portions of this suspension were then added to measured volumes of the test media and incubated on a reciprocating shaker at 37 C for 6 hr.

Virulence titrations and viable cell assays of cultures were determined by methods described in our previous paper (Fukui et al., 1960).

RESULTS

Effects of gaseous environment on restoration of virulence. Cultures grown at 5 C under various gaseous environments were incubated at 37 C on a reciprocating shaker for 6 hr. Desiccators were used to contain the culture flasks, and the air was removed by alternately evacuating the jars to approximately 0.25 atmospheric pressure by means of a vacuum pump and filling with nitrogen gas. The desired environment was attained following the final evacuation either by sealing under vacuum or by allowing gas of known composition to flow into the desiccator to approximately 0.98 atm.

Preliminary experiments indicated that air was required for maximal restoration of virulence by *P. pestis* incubated at 37 C after growth at 5 C in a liquid medium. To confirm this point and at the same time determine the efficiency of the techniques used in removing air from the desiccators, an experiment was performed in which the only variable was the number of times the containers were evacuated and flushed before they were filled with nitrogen and sealed. The results of this experiment (Table 1) show that some component of air other than nitrogen (probably oxygen) was required for the reactions concerned with the restoration of virulence under these conditions. It was also evident that to block completely the reactions responsible for restoration of virulence, it was necessary to evacuate and flush the desiccators six times, a procedure which was followed in all subsequent experiments concerned with gaseous environment. Since the medium was not buffered in this

TABLE 1
Requirement of air for the restoration of virulence at 37 C

Evacuation Cycles*	Culture Values after Incubation for 6 hr†		
	Viable cells/ml	pH	LD_{50}‡
None	1.0×10^9	6.7	40 (26–65)
1	1.0×10^9	6.6	70 (42–102)
3	9.9×10^8	5.6	150 (90–260)
6	8.1×10^8	5.8	430 (280–700)

* Culture evacuated and flushed with nitrogen at each cycle.

† Values for culture before incubation at 37 C were: 1.1×10^9 viable cells/ml; pH 7.5; and LD_{50} 350 (210–600).

‡ Intraperitoneal mouse LD_{50} with 95% confidence limits.

experiment, the pH of the cultures dropped quite drastically in those environments of greatly reduced oxygen tension. However, separate studies on the effect of pH on the restoration of virulence under aerobic conditions, indicated that the restoration of virulence occurs at a pH as low as 5.5.

Several experiments were performed to compare the effect of nitrogen, a mixture of 95% nitrogen and 5% carbon dioxide, air, and vacuum on the virulence restoration phenomenon. Cultures grown at 5 C under nitrogen and under a mixture of 95% nitrogen and 5% carbon dioxide were compared with the cultures grown in air. To minimize pH changes during growth at 5 C and during the 6 hr incubation at 37 C, the xylose concentration in the medium was reduced from 1.0% to 0.1% and potassium phosphate buffer was added to give a final concentration of 0.02 M phosphate at pH 7.5. The data from a typical experiment with cultures of similar physiological age are summarized in Table 2.

The results indicated that the synthesis of virulence factors at 37 C was definitely dependent on the presence of air. Since virulence was not restored during incubation under N_2, $N_2 + CO_2$, or in vacuum, an absolute requirement for oxygen was indicated. In contrast, cells grown under N_2 or $N_2 + 5\%$ CO_2 at 5 C consistently elicited greater initial virulence than those cells grown in air. Also the addition of 5% CO_2 to nitrogen during growth at 5 C usually yielded cells of greater virulence. In each case, however, an increase in virulence was noted when the cells were subsequently incubated at 37 C under aerobic conditions.

Effects of pH on restoration of virulence. To define the pH limits for the development of virulence, cultures grown at pH 7.5 in the usual manner were pooled, samples were transferred to sterile Erlenmeyer flasks, and sterile 1.0 M

TABLE 2
Effect of various gaseous environments on the growth at 5 C and the restoration of virulence at 37 C

Sample*	Culture Count/ml		pH		LD_{50}†	
	5 C‡	37 C‡	5 C‡	37 C‡	5 C‡	37 C‡
Air/air	9.0×10^8	1.2×10^9	7.5	7.5	3,000 (2,000–4,600)	20 (13–31)
Air/vacuum	9.0×10^8	1.0×10^9	7.5	7.2	3,000 (2,000–4,600)	500 (300–850)
Air/N_2	9.0×10^8	8.0×10^8	7.5	7.1	3,000 (2,000–4,600)	500 (300–850)
Air/$N_2 + CO_2$	9.0×10^8	7.4×10^8	7.5	6.9	3,000 (2,000–4,600)	200 (1,200–3,400)
N_2/air	1.8×10^9	2.3×10^9	7.5	7.9	150 (97–250)	33 (22–54)
$N_2 + CO_2$/air	9.5×10^8	1.2×10^9	7.2	7.3	30 (18–50)	9 (5–15)

* Gaseous environment for growth at 5 C/gaseous environment during incubation at 37 C.

† Intraperitoneal mouse LD_{50} with 95% confidence limits.

‡ Denotes values obtained with cultures grown at 5 C and after incubation at 37 C for 6 hr.

TABLE 3

Effect of pH on the restoration of virulence at 37 C

Sample* (Initial pH)	pH (after 6 hr)	Mouse Intraperitoneal LD_{50}† with 95% Confidence Limits
5.5	5.5	30 (16–55)
6.5	6.6	7 (4–12)
7.5	7.5	6 (4–10)
8.4	8.0	10 (6–18)
9.3	8.7	1,000 (600–1,700)

* The initial viable cell count in each case was 1.2×10^9/ml. No significant changes in viable count were noted during incubation at 37 C for 6 hr.

† The intraperitoneal mouse LD_{50} value before incubation at 37 C was 800 with 95% confidence limits of 490 and 1,400.

NaOH or 1.0 M H_2SO_4 was added to adjust each sample to the desired pH. The cultures were then incubated at 37 C for 6 hr. Virulence for mice was determined before and after exposure to 37 C. The results of a typical experiment are presented in Table 3. It was evident that pH had no effect on the restoration of virulence over the range of 5.5 to 8.4, but the mechanism for the synthesis of virulence factors was completely blocked at pH 9.3. In other experiments it was found that a drastic reduction in viable count occurred at pH 5.0 and 5.2.

The effect of pH of the growth medium on virulence of cultures grown at 5 C was also determined. Heart infusion broth adjusted to the desired pH was dispensed into 500-ml Erlenmeyer flasks (50 ml per flask). After sterilization by autoclaving (121 C for 15 min), suitable additions of sterile 25% xylose solution and sterile 1.0 M PO_4 buffer of the desired pH were made to give final concentrations of 0.1% and 0.02 M, respectively. Cultures were transferred twice at each pH before they were finally adjusted to pH 7.0 and tested for virulence before and after incubation at 37 C for 6 hr. As shown in Table 4, cultures grown at pH values below 7.0 exhibited markedly greater virulence than those grown at higher pH values. Nevertheless, all cultures increased in virulence during the 6 hr incubation at 37 C.

Effect of growth temperature on virulence. P. pestis incubated with aeration in heart infusion broth at 37 C usually yielded mouse intraperitoneal LD_{50} values between 1 and 10; whereas the same cultures incubated at 5 C yielded LD_{50} values between 300 and several thousand. Therefore it was of interest to determine the virulence of cultures of comparable physiological age grown at intermediate temperatures. The results of several experiments indicated that cultures grown at 11 C were consistently more virulent than the 5 C culture and slightly more virulent than the 8 C culture, thus indicating a

TABLE 4

Effect of pH on the virulence of cultures grown at 5 C and on the restoration of virulence at 37 C

Initial pH of Culture Grown at 5 C	Culture Count/ml		pH During Incubation at 37 C		LD_{50}*	
	5 C†	37 C†	0 Hr	6 Hr	5 C†	37 C†
5.7	2.5×10^8	3.1×10^8	7.15	7.5	180 (100–300)	9 (5–16)
6.1	6.3×10^8	9.5×10^8	7.0	7.3	73 (50–102)	7 (4–12)
6.6	8.8×10^8	9.0×10^8	7.0	7.2	70 (42–103)	7 (4–12)
7.0	9.0×10^8	9.1×10^8	7.0	7.2	625 (400–1,000)	130 (70–230)
7.8	5.9×10^8	5.9×10^8	7.0	7.2	600 (330–1,100)	30 (18–50)
8.2	4.7×10^8	3.8×10^8	7.0	7.5	1,500 (1,000–2,500)	22 (14–38)

* Intraperitoneal mouse LD_{50} with 95% confidence limits.

† Denotes values obtained with the culture grown at 5 C and after incubation at 37 C for 6 hr.

significant effect of 3 to 6 C temperature rise on the virulence of the culture. The cultures grown at 37 C, as expected, manifested the greatest virulence (Table 5).

TABLE 5

Effect of growth temperature on the virulence of Pasteurella pestis

Growth Temperature	Mouse Intraperitoneal LD_{50} with 95% Confidence Limits
C	
5	1,500 (1,000–2,500)
8	350 (220–560)
11	150 (57–180)
26	13 (8–22)
37	7(4.5–11)

TABLE 6

Effect of temperature of incubation for 6 hr on the restoration of virulence to cultures grown at 5 C

Temperature	LD_{50} with 95% Confidence Limits
C	
5	1,950 (557–6,825)
11	460 (192–1,104)
26	210 (81–546)
36	20 (10–42)
37	8.8 (3.1–28)
40	7.2 (1.9–27)
41	6.2 (3.8–13.6)
42	33 (11–99)
43	2,700 (1,227–5,940)
44	3,000 (1,363–6,600)
45	10,000 (3.1×10^3–3.2×10^4)

The results of experiments in which the cultures grown at 5 C were subsequently incubated at various temperatures for 6 hr are presented in Table 6. It would appear from these results that the maximal restoration of virulence occurs between 37 C and 41 C with no sharp optimum. Although no reduction of viable counts was noticed by incubation of cells at higher temperatures generally, the cultures incubated at 45 C usually underwent a reduction of about one log in terms of viable cell count during the 6 hr incubation.

Nutritional requirements. To define the chemical basis for the synthesis of virulence factors at 37 C by nonproliferating cells, a study of the nutritional requirements was undertaken. The results of preliminary studies with heart infusion broth and casein hydrolyzate indicated that, in addition to the nitrogenous compounds, phosphate and an oxidizable sugar were required for the complete restoration of virulence within 6 hr at 37 C. These results were further substantiated with Wessman's chemically defined medium (Wessman, Miller, and Surgalla, 1958). The results shown in Table 7, indicate that the complete defined medium used at double strength with 0.02% xylose was capable of permitting the restoration of virulence. Only partial restoration of virulence was observed when the complete defined medium was used at single strength and no enhancement of virulence was noted in the absence of xylose. When phosphate was omitted from this medium and the medium was buffered with 0.002 M Veronal buffer at pH 7.4, only partial restoration of virulence occurred. Although tris buffer (tris(hydroxymethyl)amino-

TABLE 7

Restoration of virulence in chemically defined medium at 37 C

Modification in Medium*	Viable Cell Count per ml		LD_{50}† with 95% Confidence Limits	
	0 Hr‡	6 Hr‡	0 Hr	6 Hr
+ 0.02% Xylose	3.7×10^8	3.8×10^8	700 (420–1,200)	40 (25–70)
− Xylose	3.7×10^8	4.0×10^8	700 (420–1,200)	2,700 (1,600–4,100)
+ Xylose, − PO_4	1.0×10^9	2.1×10^8	1,200 (600–1,700)	190 (100–310)

* Double strength chemically defined medium of Wessman.

† Intraperitoneal mouse LD_{50}.

‡ Time post 37 C incubation.

methane) at 0.02 M concentrations inhibited the restoration of virulence, Veronal buffer in the concentration used above did not interfere with the restoration of virulence in the complete medium.

In subsequent studies, it was learned that complete restoration of virulence could be obtained with the 13 amino acids contained in Wessman's chemically defined medium when used in double strength, if 0.02% xylose, 0.02 M phosphate (pH 7.2), 0.02 M sodium gluconate, and 0.05% agar were added. The addition of agar was required to maintain the viability of the cells. The omission of any of the amino acids singly or in combination usually resulted in partial restoration of virulence. Of the several amino acid analogues tested (allylglycine, DL-ethionine, DL-leucine, DL-*p*-fluorophenylalanine, DL-β-phenyllactic acid), the analogues for phenylalanine at 16 mM concentrations were the only analogues that consistently blocked the restoration of virulence. The addition of 0.004 M $CaCl_2$ or 0.012 M $NaHCO_3$ which had been reported (Higuchi, Kupferberg, and Smith, 1959; Delwiche et al., 1959) to maintain the virulence of cultures grown at 37 C did not affect the restoration of virulence in the test system described.

DISCUSSION

In contrast to numerous papers published on the nutrition and physical requirements for the growth of *P. pestis* (Hills and Spurr, 1952; Higuchi and Carlin, 1958; Wessman et al., 1958; Sokhey and Habbu, 1943*a*, *b*; Brownlow and Wessman, 1960; and others), relatively few reports have appeared on the requirements specifically directed toward the synthesis of factors required for the expression of virulence (Burrows and Bacon, 1956*a*, *b*; Jackson and Burrows, 1956; Crumpton and Davies, 1956; Fukui et al., 1957*b*, 1959, and 1960; Fox and Higuchi, 1958). The reports by Meyer (1950) and Burrows and Bacon (1956*a*, *b*) have adequately reviewed the correlation between the temperature dependent synthesis of fraction I and V antigens to resistance to phagocytosis. Recently Fukui et al. (1957*a*) have reported that virulent strains of *P. pestis*, grown in vivo or under certain conditions in vitro (37 C), acquired the ability to resist clearance from the lungs of guinea pigs and they related this observation to resistance to phagocytosis. These observations coupled with the demonstration by Fukui et al. (1960, 1961) that virulence could be restored to cultures that had undergone a phenotypic loss of virulence during growth at 5 C by further incubation at 37 C, made it seem apparent to us that the critical requirements for the synthesis of virulence factors might be demonstrated by determining the physical and nutritional requirements for the temperature dependent restoration of virulence.

Although oxygen was required for the rapid restoration of virulence to cultures which had undergone a loss of virulence during growth at 5 C, it was interesting to find that the cultures grown at 5 C in the absence of oxygen were generally more virulent than those cultures grown in the presence of air. Observations concerning the greater virulence of cultures grown at 37 C in the absence of oxygen and under reduced oxygen tension were reported by Delwiche et al. (1959) and Ogg et al. (1958). However, the basis for these apparently similar observations at 5 C and 37 C must not be interpreted to be the same, since Delwiche et al. demonstrated that the growth in the absence of air minimized a genotypic shift in population from virulent to avirulent which occurred in the presence of air, whereas the reduction of virulence during growth at 5 C was attributed to the lack of synthesis of temperature dependent virulence factors (Fukui et al., 1960, 1961).

The pH values for the restoration of virulence at 37 C appeared not to be critical; however, the pH values maintained during the growth of the culture at 5 C were apparently highly critical since the cultures grown at pH 7 or higher consistently manifested reduced virulence in contrast to cultures grown at pH values below pH 7.0. It should be mentioned that Ogg et al. (1958) have reported that the virulence of *P. pestis* cultures could be maintained during growth at 37 C in air if the initial pH of the medium was adjusted to pH 7.8 or 8.0. These findings as well as the limiting pH values of 5.0 and 9.6 and the optimal pH range of 7.2 and 7.6 for the growth of *P. pestis* at the optimal temperature of growth (28 C) reported by Sokhey and Habbu (1943*b*) should be taken into consideration when determining the pH requirements for the growth of virulent strains of *P. pestis*. Likewise the results of our experiments indicated that the optimal temperature range for the restoration of virulence

was 37 to 41 C, whereas Sokhey and Habbu (1943*a*) reported that the optimum for growth was 27 to 28 C. Also it might be noted here that Fukui et al. (1957*b*) demonstrated that the virulence of *P. pestis* grown aerobically at 26 C was maintained, whereas the cultures grown at 37 C rapidly lost virulence due to selective outgrowth of avirulent cells.

It was interesting to note that the addition of sodium gluconate which had been reported by Lawton and Surgalla (1960) to stimulate the synthesis of V antigen was found to enhance the rate of restoration of virulence in the minimal medium containing 13 amino acids and an energy source. Although the function of gluconate may be simply related to energy metabolism associated with the synthesis of virulence factors, its mode of action may be very complex, since Lawton reported that glucose or ribose could not replace the requirement for gluconate in another temperature dependent system and in limited studies we could not demonstrate an adaptation for the oxidation of gluconate within 3 hr at 37 C.

In view of the fact that Higuchi et al. (1959) and Delwiche et al. (1959) have reported that the addition of calcium ions or sodium bicarbonate prevented the loss of virulence of *P. pestis* during growth at 37 C with aeration, it was interesting to find that neither calcium ions nor sodium bicarbonate enhanced the restoration of virulence. The reason for these contrasting observations might be that in the former case, growth (cellular multiplication) was a necessary adjunct for their effects, whereas in the latter case growth was not a necessary prerequisite. Therefore it would appear that calcium and bicarbonate functioned primarily by maintaining the population of the growing culture in favor of virulent cells either by stimulating the growth of virulent cells (calcium) or by suppressing the initiation of growth of the avirulent cells ($NaHCO_3$).

Although this speculation does not exclude a role of calcium or bicarbonate in the restoration of virulence, it would seem that if they have any effect, the effect might be subtle and it would not necessarily be reflected in the rapid restoration of virulence as indicated by the results.

The results of the studies to determine the minimal amino acid requirements for the restoration of virulence indicated that the requirements were similar to those defined for the growth of *P. pestis* at 37 C by Higuchi et al. (1958) and Wessman et al. (1958). Thus these results would imply that although growth in terms of cellular multiplication was not a prerequisite for the synthesis of virulence factors required for the maximal expression of virulence, factors required generally for protein synthesis were involved, i.e., energy, amino acids, and the genetic potentiating factors. In many respects, the findings reported in this paper are an extension of the studies of Burrows and Bacon (1956*a*) in which they defined the requirements for rendering *P. pestis* grown at 28 C resistant to phagocytosis by incubation at 37 C. Furthermore, the results of this study points up the need for the awareness of the contrasting requirements with respect to temperature and oxygen and hydrogen ion concentration for growth and for synthesis of factors required for the expression of virulence.

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SUMMARY

Physical and chemical requirements for the synthesis of virulence factors necessary for the restoration of virulence to cultures of *Pasteurella pestis* that had undergone a phenotypic loss of virulence during growth at 5 C are described. The requirements were incubation for 6 hr in air at temperatures between 37 C and 41 C with an adequate supply of amino acids, oxidizable sugar, phosphate, and gluconate. The restoration of virulence occurred between pH 5.5 and 8.4.

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