Nutritional Requirements of *Pasteurella tularensis* for Growth from Small Inocula

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SUMMARY

The ability of *Pasteurella tularensis* to grow from small inocula (less than 10^5 organisms) was found to be critically dependent upon the supply of materials produced by this organism (growth-initiating substance, GIS). In contrast, large inocula showed no requirement for added GIS. The growth-promoting activity of GIS was enhanced in the presence of blood. The evidence favours the conclusion that the inoculum-dependent growth characteristics of *P. tularensis* are attributable to a nutritional heterogeneity of the bacterial population. According to this interpretation, GIS is produced by a relatively small number of bacteria in the population and is excreted by them into the surrounding culture fluid. The excreted material enables growth of the GIS-requiring organisms which constitute a major proportion of the total population. This commensal relationship appears to account for the characteristic all-or-none type of growth response elicited by GIS.

INTRODUCTION

Previous studies elucidated the essential nutritional requirements of *Pasteurella tularensis* and led to the development of a chemically defined medium which supported luxuriant growth of various strains of this organism (Mager, Traub & Grossowicz, 1954; Traub, Mager & Grossowicz, 1955). These strains, however, required large inocula (in excess of 10^5 organisms) for growth initiation, both in the defined medium and in complex and specially enriched media, and did not produce colonies in the conventional viable (colony) count procedure. It was thought that the inoculum-dependent growth might reflect a demand of this organism for some endogenously produced metabolite. This assumption was borne out by finding that growth of *P. tularensis* from small inocula could be ensured by supplementing the culture medium with aqueous extracts of the same organism (Mager, 1964). Subsequent work showed that growing cultures of *P. tularensis* produced and accumulated in the medium material capable of initiating growth from single organisms. The present paper describes the conditions which govern the elaboration of the growth-initiating substance (GIS) by *P. tularensis* cultures and the characteristics of its growth-promoting action.

METHODS

Organisms. The various strains of *Pasteurella tularensis* used in this work (see Table 1) originated from the culture collections of Dr C. R. Owen (Rocky Mountain Laboratory, Hamilton, Montana, U.S.A.) and Dr L. Foshay (University of Cincinnati,

Ohio, U.S.A.). The cultures were maintained by weekly transfers on cysteine blood agar (see below).

Media and cultural procedures. The cysteine blood agar (CB) medium used for colony counts was composed of (% w/v): 0.8, dehydrated nutrient broth (Difco); 1, glucose; 0.5, NaCl; 0.05, cysteine HCl; 2, agar; and 2 % (v/v) sterile defibrinated rabbit blood. The liquid medium (cysteine broth) was of the following composition (% w/v): 2, Proteose peptone (Difco); 0.5, NaCl; 0.5, glucose; 0.05, cysteine HCl. The minimal medium used for cultivation of a *Rhizopus* strain was constituted as follows (% w/v): 0.7, Na_2HPO_4 ; 0.3, KH_2PO_4 ; 0.1, NH_4Cl ; 0.025, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.0015, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; 1, glucose; with thiamine 5 $\mu\text{g./ml.}$, ZnCl_2 15 $\mu\text{g./ml.}$ The media were prepared in distilled water, adjusted to pH 7 and autoclaved at 120° for 20 min. Heat-labile materials were sterilized by filtration through ultrafine sintered-glass filters or Millipore membranes (0.45 μ pore size; from Millipore Filter Corporation, Bedford, Mass., U.S.A.). The sterile solutions and defibrinated blood were added aseptically to the autoclaved basal medium.

Unless otherwise indicated, the following procedure was used to study the growth-initiation requirements of *Pasteurella tularensis*. Inocula of the various strains were prepared from 24 hr cysteine broth cultures. The organisms were washed once with fresh cysteine broth and suspended in the same medium to a final turbidity of 100 units on the scale of the Klett-Summerson photoelectric colorimeter fitted with a no. 42 filter. These suspensions, containing about 5×10^9 organisms/ml. (as estimated by colony counting on CB plates), served for preparing the final inocula by serial dilution in cysteine broth. Two-tenths ml. of bacterial suspension were added to 5 ml. cysteine broth dispensed in Erlenmeyer flasks (25 ml.) and the cultures incubated at 36–37° with continuous shaking in the New Brunswick gyrotory incubator-shaker. Growth was measured turbidimetrically in the Klett photometer (no. 42 filter) or in the Bausch and Lomb Spectronic-20 Colorimeter at 500 m μ . All the growth experiments were run in duplicate and the purity of the cultures routinely examined microscopically.

RESULTS

Colony counts made by a procedure described below established that the minimal size of inoculum required to secure growth in cysteine broth varied with different strains of *Pasteurella tularensis* from 10^5 to 10^8 organisms (Table 1). Confluent growth on the solid CB medium was consistently produced with massive inocula, whereas attempts to obtain colonial growth with appropriately diluted suspensions were unsuccessful. These observations suggested that the growth-determining role of the inoculum size might reflect a need of individual organisms for mutual 'feeding'. To test this, the surface of a CB agar plate was evenly inoculated by spreading about 1000 organisms of the *Pasteurella* s & D strain and a loopful of a 24 hr culture of the same strain in broth was streaked across the plate to produce a narrow zone of confluent growth. During incubation discrete colonies sprang up in successive crops at increasing distances from the streak of confluent growth, the sequential growth onset of the colonies being manifested by their distally diminishing size. It appeared reasonable to conclude that material produced by the organisms in the zone of confluent growth and excreted by them into the surrounding medium had enabled the more distant isolated organisms to produce colonies.

The presence of a growth-initiating factor was shown more directly in 24–48 hr broth cultures of *Pasteurella tularensis* sterilized by Millipore membrane filtration. Such sterile culture filtrates when added in small amounts to fresh cysteine broth gave growth of the various *P. tularensis* strains from as few as 10–100 organisms. The minimal quantity of culture-filtrate required for growth initiation was inversely related to the size of inoculum (Table 2). With a constant inoculum size the time lag

Table 1. *Inoculum-dependent growth of various strains of Pasteurella tularensis; effect of growth-initiating substance (GIS)*

Each strain was grown in 5 ml. cysteine broth. Where indicated, (GIS) was added in the form of 0.2 ml. sterile filtrate of a 70 hr culture of *P. tularensis* strain S & D. Growth was followed during 6 days of incubation at 37° with continuous shaking. The signs + and – denote maximal growth and no growth, respectively.

Strain	Inoculum size (no. of organisms)									
	10 ²		10 ⁴		10 ⁵		10 ⁷		10 ⁸	
	+ GIS	– GIS	+ GIS	– GIS	+ GIS	– GIS	+ GIS	– GIS	+ GIS	– GIS
Growth response										
SCHU highly virulent	–	+	–	+	+	+	+	+	+	+
SCHU-M16 highly virulent	–	+	–	+	+	+	+	+	+	+
VAVENTLY moderately virulent	–	+	–	+	–	+	+	+	+	+
425-F4G moderately virulent	–	+	–	+	–	+	+	+	+	+
30110 slightly virulent	–	+	–	+	–	+	–	+	+	+
JAP avirulent	–	+	–	+	–	+	–	+	+	+
VACCINE avirulent	–	+	–	+	+	+	+	+	+	+
S & D avirulent	–	+	–	+	–	+	–	+	+	+

Table 2. *Growth-promoting effect of Pasteurella tularensis strain S & D culture filtrate in relation to inoculum size*

The standard inoculum of *Pasteurella tularensis* strain S & D contained 2×10^9 organisms. The culture filtrate was derived from a 48 hr cysteine broth culture of *P. tularensis* strain S & D.

Dilution of the standard inoculum	Amounts of culture filtrate added to fresh cysteine broth (% v/v)				
	0	1	2.5	5	10
	Growth after incubation for 100 hr (Klett units)				
None	330	310	320	340	300
10 ⁻²	0	320	320	310	320
10 ⁻³	0	340	330	320	310
10 ⁻⁵	0	0	0	310	310
10 ⁻⁷	0	0	0	0	340
10 ⁻⁸	0	0	0	0	0

for the appearance of visible growth decreased with increasing concentrations of filtrate (Fig. 1). Once initiated, however, growth proceeded in all instances at a nearly equal rate, attaining the same final yield irrespective of the duration of the lag (Table 2; Fig. 1). Essentially similar results were obtained on examining the ability of added culture filtrate to promote colonial growth on CB plates. Here again, the requisite

threshold dose varied within certain limits in roughly inverse proportion to the inoculum size (Fig. 2). The number of colonies, however, yielded by a given inoculum remained practically unaltered on increasing the amount of the filtrate added above the minimal effective dose. Thus, the all-or-none type of growth-promoting effect exhibited by the culture filtrates differed essentially from the graded dose response observed in conventional bioassays of vitamins and other essential metabolites.

Fig. 1

Fig. 2

Fig. 1. Growth curves of *Pasteurella tularensis* in cysteine broth supplemented with different amounts of culture filtrate as a source of growth-initiating substance (GIS). Sterile filtrate from a 48 hr culture of *P. tularensis* strain s & D was added to fresh double-strength cysteine broth and the final volume was made up with water to 15 ml. After inoculation with about 2×10^8 organisms of *P. tularensis* strain s & D, growth was determined turbidimetrically at intervals. Amounts of culture filtrate added: ○—○, 2 ml.; ●—●, 1 ml.; △—△, 0.5 ml.; ▲—▲, 0.1 ml.

Fig. 2. Dose response curve of *Pasteurella tularensis* strain s & D to growth-initiating substance (GIS) as a function of inoculum size. One unit of GIS is the amount required for growth of 200 organisms of *P. tularensis* strain s & D on blood-cysteine agar medium after incubation for 48 hr at 37°.

Requirement of different Pasteurella tularensis strains for growth-initiating substance

Apart from the avirulent *Pasteurella* s & D strain which was used throughout this study, a random assortment of 7 other strains *P. tularensis* of diverse origin and of widely different degrees of virulence was examined for their GIS requirements. All the strains tested showed essentially the same all-or-none pattern of response to GIS, although they differed considerably with regard to the minimal inoculum capable of growth without added GIS (Table 1). This quantity, which will be referred to as 'critical inoculum size', was fairly constant for each strain, provided that the conditions of subcultivation were rigidly standardized. It may be concluded, therefore, that the requirement for GIS constitutes a characteristic attribute of the *P. tularensis* species.

Occurrence of the Pasteurella growth-initiating substance in other micro-organisms

The production of GIS by organisms other than *Pasteurella tularensis* was not systematically studied, but its excretion by widely different species of bacteria and

moulds was frequently noted when contaminants appeared on the agar plates used for GIS assays. Of particular interest in this respect was an unidentified *Rhizopus* species which produced GIS in amounts largely exceeding those yielded by *Pasteurella* s & D. The formation of GIS by the *Rhizopus* in a minimal medium (see Methods) was markedly stimulated by trace amounts of Zn^{2+} . Further, as with *P. tularensis*, only a small portion of GIS elaborated by the *Rhizopus* was retained in the mycelium; the bulk of it was excreted into the culture medium.

Fig. 3

Fig. 4

Fig. 3. Sparing effect of blood on requirement of *Pasteurella tularensis* for growth-initiating substance (GIS). The numbers on the ordinate refer to threshold amounts of GIS required for growth of an inoculum of 200 organisms of *P. tularensis* strain s & D on CB agar in the presence of various amounts of blood.

Fig. 4. Time-course of growth and GIS production by cultures of *Pasteurella tularensis* in cysteine broth. Inoculum: 2.5×10^8 organisms of *P. tularensis* strain s & D. Dashed line: growth; solid line: GIS produced.

Sparing effect of blood on requirement for growth-initiating substance

The quantitative requirement for GIS was substantially decreased by the addition of rabbit blood to the medium. The adjuvant effect of blood on GIS activity was nearly maximal at a 2% (v/v), whereas without added GIS no growth resulted even when the amount of blood was increased to 20% (v/v). An experiment on the sparing effect of blood on GIS requirement is shown in Fig. 3. The precise quantitative aspects of this relationship varied considerably in different experiments, for reasons not yet apparent. When plasma and washed red blood cells were tested separately, the adjuvant activity proved to be present mainly in the intact red cells. The activity of blood declined rapidly upon haemolysis induced by osmotic shock or by freezing and thawing.

Assay procedure

Based on the information derived from the above experiments, the following procedure for assaying GIS activity was adopted. Serial dilutions of culture fluid or other material to be tested for GIS activity were added to 30 ml. portions of the basal

CB medium (without blood) dispensed in suitable flasks. After autoclaving at 120° for 20 min., the medium was cooled in a water bath to about 50° and sterile defibrinated rabbit blood added aseptically to a final concentration of 2 % (v/v). After thorough mixing, the contents of each flask were poured into two sterile Petri dishes and allowed to solidify. The surface of each pair of duplicate plates was inoculated with about 20 and 200 organisms of *Pasteurella* s & D, respectively, by spreading 0.1 ml. of a culture suitably diluted in saline solution. The plates were examined after incubation for 48 hr at 37°. The value of 1 unit of GIS was assigned to the amount which enabled growth of the 200-organism inoculum but not of the 20-organism inoculum.

Kinetics of production of growth-initiating substance by Pasteurella tularensis

In an attempt to study the kinetics of GIS production in relation to the rate of growth proliferation, *Pasteurella* s & D was grown in cysteine broth and samples withdrawn at various intervals for colony counts and GIS titrations, which were run concurrently. As evident from Fig. 4, contrary to the typical lag-phase or even initial decline of the colony count displayed by the growth curve, there was no detectable delay in the start of GIS production. On the other hand, during the logarithmic and stationary phases of the growth cycle the time-course of GIS production closely paralleled the rate of division of the organisms.

Partition of growth-initiating substance between organisms and culture fluid

The distribution of GIS was determined in 100 ml. samples of 24 hr cultures of *Pasteurella* s & D. The organisms deposited by centrifugation for 20 min. at 20,000g at 5° were washed three times with 0.85 % NaCl solution to remove traces of GIS. The washed organisms were suspended in 20 ml. distilled water and after incubation for 30 min. at 37° were deposited by centrifugation as above. This treatment liberated into the suspending medium GIS activity which amounted to about 0.5–1 % of its overall titre in the culture (100 units out of about 10,000 units GIS/100 ml. culture fluid). A similar pattern of GIS distribution was found with other *P. tularensis* strains tested (SCHU, VAVENTLY, 3001). The release of the cell-bound GIS activity appeared to be attributable to the injurious effect of the hypotonic environment (distilled water) on the permeability barrier of the cell (Mager, 1959), since similar treatment with 0.85 % NaCl solution did not release detectable amounts of GIS. The leakage of GIS coincided with the accumulation in the suspending fluid of considerable amounts of material with a maximum extinction at about 260 m μ . The yields of cell-bound GIS extracted by heating the organisms for 5 min. in a boiling water bath or by ultrasonic treatment for 5 min. in a 10 KC Raytheon oscillator were nearly identical to the amount of GIS liberated by osmotic shock.

Inoculum-dependent requirement for growth-initiating substance as a reflection of population heterogeneity

The available data did not permit a decision as to whether the requirement for added GIS was governed by the initial population density of the culture, or by the number of organisms in the inoculum. By varying the size of the inoculum and the volume of cysteine broth it was found that the ability of *Pasteurella tularensis* to multiply without added GIS was determined solely by the number of organisms pro-

vided in the inoculum (Table 3). Thus, an inoculum of *P. tularensis* strain s & D, which was within the minimal size range required for growth initiation in the absence of an exogenous source of GIS, exhibited an unimpaired capacity for growth, even when the initial population density was reduced up to 400-fold by increasing the volume of the culture medium. Furthermore, when 100 ml. broth were inoculated with the same 'minimal' number of organisms as above and dispensed in 5 ml. portions to 20 flasks, only 4 flasks showed growth without supplementation by GIS.

Table 3. *Effect of inoculum size and initial population density on growth of Pasteurella tularensis without added growth-initiating substance*

The various inocula, as defined by the number of colony-forming units of *P. tularensis* strain s & D, were added each to different amounts of cysteine broth, as indicated in the Table. Final growth was recorded after incubation for 5 days at 37° with continuous shaking.

Size of inoculum (colony count)	Volumes of culture medium (ml.)				
	2.5	5	25	100	1000
	Growth*				
5×10^7	+	+	+	+	+
2.5×10^7	+	+	+	+	+
10^7	—	—	—	—	—

* +, Maximal growth; —, no growth.

These results suggested that the growth-controlling effect of inoculum size reflected a heterogeneity of the bacterial population, the latter comprising a minor proportion of 'autotrophic' organisms and a majority of organisms which were 'auxotrophic' with respect to GIS. This conclusion agreed with the additional observation that the critical inoculum size was not appreciably affected by repeated washing with fresh broth or 0.85 % NaCl solution, thus arguing a possible role of carry-over of traces of GIS adherent to the organisms.

Corroborative evidence in favour of the above interpretation was afforded by an experiment to test the ability of *Pasteurella* s & D, prevented from multiplying by streptomycin, to serve as a source of GIS for a subminimal inoculum of a streptomycin-resistant *Pasteurella* mutant (Table 4). This experiment was similar in principle to that of Puck & Marcus (1955), in which non-proliferating animal cells obtained by X-ray radiation were shown to function as growth-supporting 'feeders' for the untreated cells. As evident from Table 4, however, an inoculum of the streptomycin-resistant *Pasteurella* mutant, which was about 5-fold lower than the critical size required for GIS-independent growth, was not induced to multiply in the presence of streptomycin, by adding streptomycin-sensitive pasteurellas in a 20-fold excess over the critical inoculum size. In a separate control experiment streptomycin did not interfere with the excretion of GIS by the streptomycin-resistant mutant strain.

Numerous unsuccessful attempts were made to arrive at segregation of a uniform population by rapid successive passages of large inocula, repeated subcultivation of growth produced with highly dilute inocula in the presence of GIS, and serial streaking of single colonies. The only positive result of these efforts was a transient shift in the ratio of the two 'nutritional' varieties as reflected in the difference of critical inoculum size.

Further insight into the nature of the population-dependent growth phenomenon was gained by depleting the organisms of their endogenous GIS content by osmotic shock treatment. The organisms so treated lost the ability to multiply without added GIS, even when the inoculum used was 1000-fold greater than that required for GIS-independent growth of the untreated organisms. When supplemented with GIS, however, the osmotically shocked culture proved to contain nearly the same number of colony-forming units as did the untreated control. The progeny of the osmotically shocked culture reverted after one to two passages to the original population-dependent growth pattern.

Table 4. *Inability of streptomycin-inhibited organisms of Pasteurella tularensis strain S & D to supply growth-initiating substance (GIS) to streptomycin-resistant mutants of the same strain*

The GIS-feeding capacity of non-growing organisms of *P. tularensis* strain S & D was tested in 5 ml. cysteine broth supplemented with streptomycin sulphate (200 µg./ml.), using an inoculum composed of a subminimal amount (10^7) streptomycin-resistant organisms of *Pasteurella* strain S & D (i.e. an amount incapable of growth without added GIS) and an amount (10^9) of streptomycin-sensitive mutant organisms representing about a 200-fold excess of the critical inoculum size of this strain. The culture was incubated for 70 hr at 37° with continuous shaking. Parallel controls were run with separate inocula of each mutant strain grown in the presence and in the absence of streptomycin. Growth of the parent strain was completely inhibited by streptomycin sulphate at 10 µg./ml. medium; the resistant mutant grew normally with amounts of streptomycin sulphate as high as 2 mg./ml. medium. In the absence of streptomycin both mutant strains showed equal growth response to GIS supplied in the form of a sterile filtrate from a 48 hr culture of *P. tularensis* strain S & D.

Composition of inoculum (no. of organisms)		Growth*	
Streptomycin-sensitive	Streptomycin-resistant	Without streptomycin	With streptomycin 200 µg./ml.
10^9	0	+	—
5×10^7	0	+	—
10^7	0	—	—
0	5×10^7	+	+
0	10^7	—	—
10^9	10^7	+	—

* + Maximal growth; — no growth.

DISCUSSION

The requirement of *Pasteurella tularensis* for an endogenously produced metabolite (GIS), as a factor essential for initiating division, accounts for the inoculum-dependent growth characteristics displayed by virulent and avirulent strains of this organism in artificial culture media. Such a requirement is patently inconsistent with the notoriously high virulence of the tularaemia agent, which implies a virtual capacity of a single organism to multiply *in vivo* and to produce a fatal infection in mice. The possibility that a substance similar to GIS or a functionally equivalent compound may be present in the host tissues appears rather unlikely, in view of the negative outcome of an extensive search for such a growth factor in a variety of tissue extracts and other natural materials (Traub *et al.* 1955; Halmann & Mager, 1967). Another possible explanation is that the requirement for GIS exhibited by small inocula *in vitro* stems

from a metabolic derangement due to the inadequacy of the artificial environment in imitating the requisite physico-chemical conditions prevailing *in vivo* (see Traub *et al.* 1955). It is not unlikely that such a mechanism may underlie also the sparing effect exerted by blood on GIS requirement, which appears to be mediated by some labile and as yet elusive factor apparently associated with intact red cells. The currently available evidence, however, does not warrant a definite choice between these alternative conceptions.

The seeming paradox implicit in the demand of *Pasteurella tularensis* for the exogenous supply of a factor which is abundantly synthesized by the same organism in the course of its growth appears to be resolved by the evidence for a genetically determined nutritional heterogeneity of the cell population prevailing in the *P. tularensis* cultures. The coexistence of a minority of autotrophic cells and a vast majority of auxotrophic mutants is based on the ability of the autotrophs to manufacture and excrete a large excess of GIS, largely sufficient to cater to their auxotrophic offsprings. This commensal relationship offers an explanation for the apparent autocatalytic kinetics of GIS implied by its all-or-none action pattern. The considerable stability observed in the population dynamics of the different strains of *P. tularensis* seems to be determined on the one hand by their high mutation rates and on the other hand by the lack of selection pressure inherent in the 'feeding' phenomenon which cancels the advantage of autotrophy for the survival of the species.

The association of an enhanced requirement for added GIS with the release of the cell-bound GIS activity induced by osmotic shocking of the cells suggests that the autotrophic growth capability is critically dependent upon a certain minimum level of endogenous GIS. The as yet unexplored question of the chemical identity of the cell-associated and diffusible forms of GIS may be relevant to the understanding of the factors governing the retention and excretion of GIS by *Pasteurella tularensis* cells.

The inoculum-dependent nature of the GIS requirement and some general characteristics of its growth-promoting action bear a distinct resemblance to those of the 'lag-reducing factors' described by Lankford and his associates in certain *Bacillus* species and designated by them as 'schizokinens' (Sergeant, Lankford & Traxler, 1957; Lankford, Walker, Reeves, Nabbut, Byers & Jones, 1966). On the other hand, the mechanism underlying the population-dependent growth of *Pasteurella tularensis* differs essentially from that observed in mammalian cell cultures, in which the requirement for diffusible endogenous factors appears to be governed by the density of the cell population (see Eagle, 1965).

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