THE PIGMENTATION OF *PASTEURELLA PESTIS* ON A DEFINED MEDIUM CONTAINING HAEMIN

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ISOLATED cells of *Pasteurella pestis* will not grow under aerobic conditions unless haemin or some other substance capable of destroying hydrogen peroxide is added to the medium (Herbert, 1949).

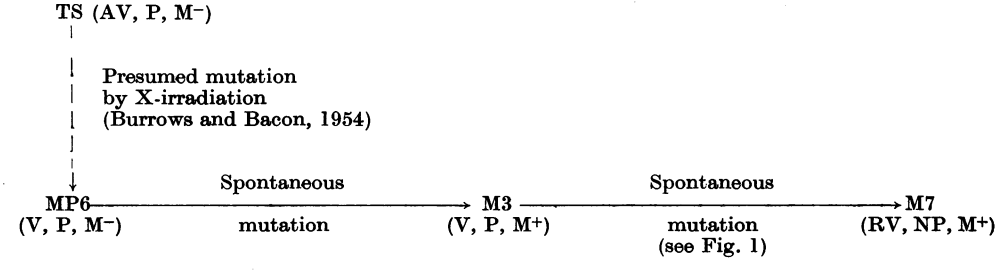
Using a concentration of haemin greatly in excess of the minimum requirement for growth, we have observed an additional effect. On a suitable medium, under well-defined conditions, many strains of *P. pestis* produce dark brown colonies, while others produce colonies lacking the brown pigment. After prolonged incubation, non-pigmented secondary colonies usually develop from pigmented colonies (Fig. 1). Our interest in this phenomenon was greatly increased when we observed that a pure non-pigmented strain, isolated from the secondary growth of a highly virulent pigmented strain, displayed reduced virulence for mice. It seemed that this phenomenon merited further investigation.

This paper is concerned with the development of a medium giving maximum differentiation between pigmented and non-pigmented strains, the chemical nature of the pigment and a preliminary survey of the pigmentation characteristics of a number of strains. The reduced virulence of non-pigmented mutants of virulent strains will be dealt with in a subsequent paper.

MATERIALS AND METHODS

Strains

The strains mostly studied were labelled TS, M3 and M7. TS is a convenient abbreviation for "Tjiwidej" smooth, a classical protective avirulent (AV) strain (Otten, 1936). It is pigmented (P) and requires methionine for growth (M^-). M3 is highly virulent (V), pigmented, and independent of methionine (M^+). M7 is also methionine-independent, but is non-pigmented (NP) and has reduced virulence (RV). The relationship between these strains is shown in the following scheme:



Non-pigmented mutants were also readily selected directly from MP6. Dependence or independence on methionine did not appear to affect the characters controlling pigmentation or virulence.

Other strains examined comprised a collection made in this Establishment from numerous sources.

Cultures and suspensions

Strains were stored as dried cultures, prepared by the method of Stamp (1947). For routine use, cultures were prepared by incubating in casein acid hydrolysate-galactose liquid medium (Ross, Hakes and Herbert, unpublished) for 48 hr. at 28°, with shaking. These cultures were stored at 2° for up to one month; full viability was maintained throughout this period.

Inocula for pigmentation tests were taken from these liquid cultures. Dilutions were made in phosphate buffer, pH 7·4.

Basal chemically-defined medium

The basal medium, on which the difference in colonial pigmentation between strains was first observed, had the following composition (for approximately 1 litre).

KH_2PO_4, 4·12 g. + 300 ml. water, adjusted to pH 6·8 or 8·0 with N-NaOH; glucose, 2·24 g. (3·7 ml. of a 60 per cent solution); NH_4Cl, 0·53 g. and $(NH_4)_2SO_4$, 0·53 g. dissolved in 100 ml. water: DL-phenylalanine, 0·12 g. (30 ml. of a 0·4 per cent solution); DL-methionine, 0·06 g. (5 ml. of a 1·2 per cent solution); L-cystine, 0·048 g. (10 ml. of a 0·48 per cent solution); DL-glutamic acid, 0·132 g. (10 ml. of a 1·32 per cent solution); glycine, 0·05 g. (5 ml. of a 1·0 per cent solution); DL-valine, 0·048 g. (5 ml. of a 0·96 per cent solution); L-leucine, 0·027 g. (12·2 ml. of a 0·224 per cent solution); DL-isoleucine, 0·055 g. (15 ml. of a 0·363 per cent solution); DL-serine, 0·020 g. (5 ml. of a 0·4 per cent solution); DL-threonine, 0·048 g. (5 ml. of a 0·96 per cent solution); pantothenate, 0·25 ml. of a 0·1 per cent solution of the calcium salt; biotin, 1 ml. of a solution containing 1·25 mg./l.; haemin, 0·025 g. (10 ml. of a 0·25 per cent solution in 0·01 N-NaOH); $MgSO_4.7H_2O$, 0·1 g. (10 ml. of a 1 per cent solution); $ZnSO_4.7H_2O$, 1 ml. of a 0·01 per cent solution; $MnSO_4.7H_2O$, 1 ml. of a 0·01 per cent solution; lactic acid, 1 ml. of a 4 per cent v/v solution; agar, 20 g. + 500 ml. water, adjusted to pH 7 with N-NaOH.

Solutions were sterilised by autoclaving except those of glucose and L-cystine which were sterilised by filtration. The solutions of ammonium salts, buffer and agar were pooled immediately after autoclaving and the mixture was stored in screw-capped glass bottles at room temperature. When plates were required, bottles of the mixture were melted in a steam bath and the other constituents were added immediately before pouring. The pH values quoted for media are those to which the buffer was adjusted before mixing with the other constituents.

Spectroscopic measurements

These were made with the Hartridge reversion spectroscope.

RESULTS

Development of a medium giving high pigmentation

Modifications of the basal medium were made to increase the differentiation of pigmented and non-pigmented strains. The variables considered were nutrients, pH of medium, temperature of incubation and haemin concentration. The pigmented strain TS and the non-pigmented strain M7 were used throughout these tests.

It was found that the intensity of pigmentation of TS increased as the haemin concentration was raised from the original level of 25 μg./ml. to 100 μg./ml. The higher level was used thereafter.

Growth of isolated cells was entirely reliable at 28° on media of pH either 6·8 or 8·0, but was uncertain at 37°, especially at the higher pH. At 28°, pigmentation of TS was inferior at the lower pH, while at 37° M7 showed some tendency to pigment. It was therefore decided to use media of pH 8·0 at 28°. High glucose

concentration greatly improved the reliability of growth at 37° (3 per cent glucose seemed to be optimum, compared with the standard concentration of 0·2 per cent). This advantage was offset, for our purposes, by the tendency of M7 to become somewhat pigmented at high glucose concentrations.

Further increase in the pigmentation of TS was achieved by eliminating some of the amino acids from the medium. Those retained were phenylalanine, methionine and cysteine, which were generally essential for growth of TS, threonine, which greatly stimulated growth and was essential for satisfactory pigmentation, and valine and isoleucine, which further augmented growth and pigmentation (they were essential for growth at 37°). Ammonium salts did not appear to affect pigmentation, but their omission led to very slow growth and small colony size.

In the presence of the six above amino acids, the effect of substituting other sugars for glucose was investigated, using sugar concentrations of 2 g./l. This had no effect on pigmentation: the difference between TS and M7 was equally obvious with glucose, galactose, xylose, fructose, maltose or mannose. The colonies were inclined to spread with glucose, fructose, maltose or mannose, but much less so with galactose or xylose. Although the connection is not clear, this difference in colonial morphology parallels observations on the sugar metabolism of *P. pestis* in liquid medium (Ross, Hakes and Herbert, unpublished). Much more acid is produced with glucose, fructose, maltose or mannose than with galactose or xylose. The more compact type of colony was preferable for our work, and galactose was therefore chosen for use as routine carbon source.

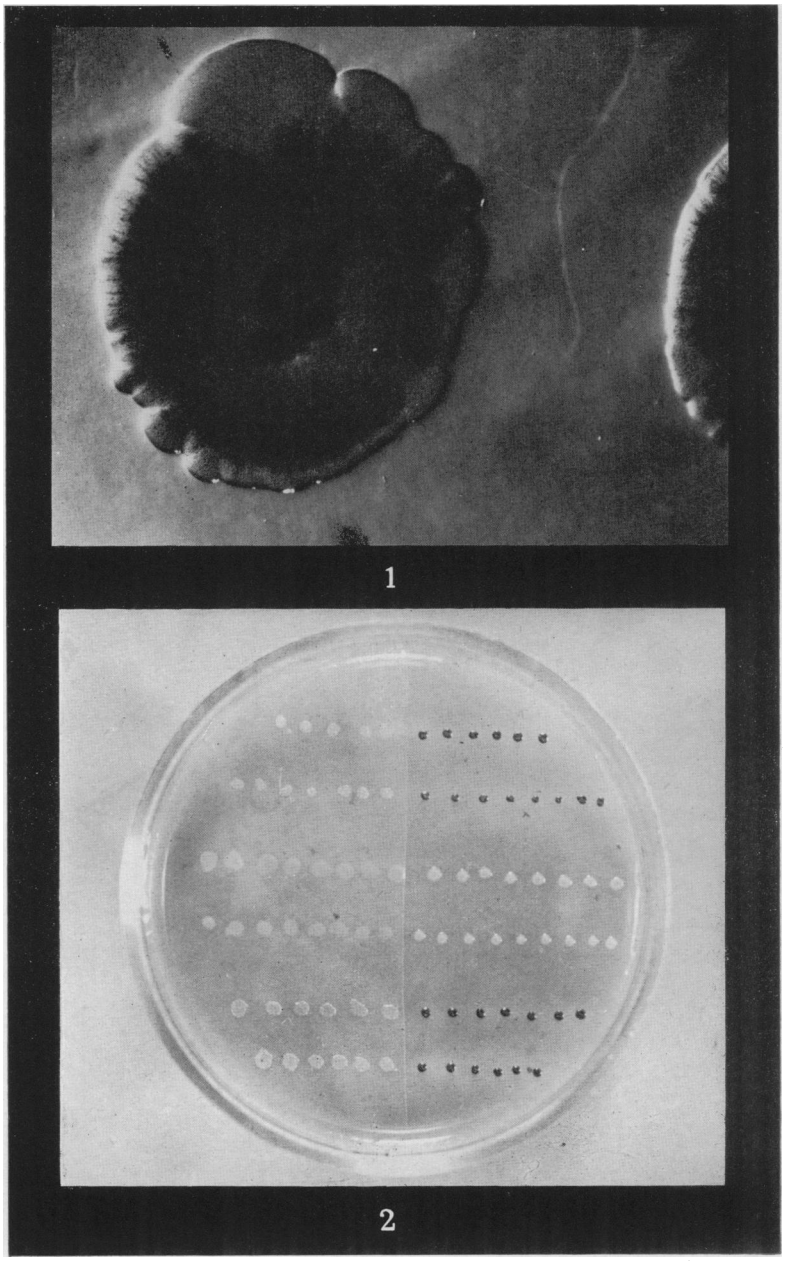
Subsequent to this change of carbon source, it was observed that glycine was a completely satisfactory alternative to threonine for stimulation of growth and promotion of pigmentation. The substitution of glycine for threonine was the final modification made in devising a medium suitable for reliable growth of TS and M7 and for clearly demonstrating the pigmentation of TS. On this medium, of which the composition is given in full below, colonies could be counted after 2 or 3 days' incubation at 28°, and the pigmentation of TS was clearly established in 3 or 4 days. M7 remained non-pigmented for about 8 days: on more prolonged incubation some pigment did appear, but the intensity of pigmentation never approached that of TS.

The composition of *ca.* 1 litre of the final pigmentation medium was; KH_2PO_4, 4·12 g. + 300 ml. water adjusted to pH 8·0 with N-NaOH: galactose, 2 g. (10 ml. of a 20 per cent solution): NH_4Cl, 0·53 g. and $(NH_4)_2SO_4$, 0·53 g. dissolved in 100 ml. water: DL-phenylalanine, 0·12 g. (30 ml. of a 0·4 per cent solution): DL-methionine, 0·06 g. (5 ml. of a 1·2 per cent solution): L-cysteine hydrochloride, 0·048 g. (5 ml. of a 0·96 per cent solution): DL-valine, 0·048 g. (5 ml. of a 0·96 per cent solution): DL-isoleucine, 0·055 g. (15 ml. of a 0·363 per cent solution): glycine, 0·05 g. (5 ml. of a 1·0 per cent solution): haemin, 0·10 g. (40 ml. of a 0·25

EXPLANATION OF PLATE

FIG. 1.—Pigmented colony with non-pigmented outgrowths. M3 colony after 12 days' incubation at 28° (M7 was cultured from the secondary growth). × 12·5.

FIG. 2.—Dependence of pigmentation on haemin. Petri dish containing pigmentation medium with left half overlayed with "Cellophane," inoculated with droplets of suspensions of 10^8 bacteria per ml. and incubated at 28° for 4 days. The impermeability of "Cellophane" to haemin, while not affecting growth, completely prevents pigmentation of M3 (top two rows) and TS (bottom two rows); M7 (middle two rows) is not pigmented even when haemin is accessible.



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per cent solution in 0·01 N-NaOH) : $MgSO_4.7H_2O$, 0·1 g. (2·5 ml. of a 4 per cent solution) : $ZnSO_4.7H_2O$, 1 ml. of a 0·01 per cent solution : $MnSO_4.4H_2O$, 1 ml. of a 0·01 per cent solution : lactic acid, 1 ml. of a 4 per cent v/v solution : agar, 20 g. + 500 ml. water, adjusted to pH 7 with N-NaOH.

This medium was found suitable for the growth of the majority of the strains tested, but a few strains required arginine in addition. To permit growth of these strains, a solution of 104 mg. arginine hydrochloride in 10 ml. water was added. This had no marked effect on the behaviour of other strains.

Chemical nature of the pigment

From an early stage of the investigation, it was clear that pigmentation was dependent on the presence of haemin in the medium. This was readily demonstrated by the following experiment.

One half of a plate of pigmentation medium was covered with a semi-circular sheet of " Cellophane ", the other half being left uncovered. Droplets of TS, M7 and M3 suspensions were seeded across the plate, at right angles to the straight edge of the " Cellophane " sheet, and the plate was incubated at 28°. The " Cellophane ", which is impermeable to haemin, had no effect on growth, but entirely prevented the pigmentation of TS and M3. Pigmentation was normal on the uncovered half of the plate, showing that absorption of haemin from the medium was essential for pigmentation (see Fig. 2).

The texture of pigmented colonies was very much harder and drier than that of non-pigmented colonies. The latter readily gave a homogeneous suspension on shaking with buffer, but this was very difficult to achieve with pigmented colonies. These were easily washed off agar surfaces, but tenaciously retained their structure. Vigorous agitation resulted in free suspension of some of the bacteria, but the majority remained bound together in quite hard flakes. This effect was very striking with confluent growths (from heavy inocula), from which most of the bacteria could be washed off as a continuous sheet, brittle enough to be broken into small flakes by shaking. Under the microscope, wet preparations made from pigmented bacteria shaken with buffer were strikingly heterogeneous. Some free organisms appeared colourless, but the majority were bound together in closely packed masses, in which the cell walls were markedly thickened and yellow in appearance, the colour presumably being due to absorbed haemin.

Whether haemin was bound to bacterial substance after absorption was uncertain. However, it could be demonstrated experimentally that a large amount of haematin was recoverable from pigmented TS. A heavy suspension of TS was prepared after 6 days' growth on pigmentation medium, and the organisms dissolved by heating with N-NaOH. Following reduction with sodium hydrosulphite, an absorption band at 553 mμ wavelength was observed on spectroscopic examination. Addition of pyridine intensified the band, and led to the observation of a second band at 523 mμ. With M7 bacteria treated in the same way only a faint absorption at 553 mμ occurred. The bands observed with TS closely corresponded to those of pyridine haemochromogen, which, in our controls, prepared from haemin, appeared at 555 and 525 mμ. The pink colour of the complex was visible to the naked eye in the TS preparation and in the haemin control, but not in the M7 preparation.

These results were further confirmed by spectroscopic observations after the addition of potassium cyanide to dissolved bacteria. With TS, a faint cyanhaema-

tin absorption band was observed at 547 mμ, corresponding to that in the haemin control. In both cases, reduction with sodium hydrosulphite gave a visible pink colouration, and the absorption bands of reduced cyanhaematin, a very strong one at 535·5 mμ and a somewhat weaker one at 566 mμ, were observed. With M7, there was no detectable absorption before reduction, and only very faint absorption in the region of 534–537 mμ after addition of hydrosulphite.

It was clear that the pigmentation of TS was due to absorption and retention of haemin. The failure of M7 to show comparable pigmentation might have been due either to failure to absorb haemin or to destruction of haemin after absorption. A test of these alternative hypotheses was made by measuring the change in haemin concentration of liquid pigmentation medium when exposed to suspensions of M3 and M7 bacteria. It is clear from the results (Table I) that M7 absorbed haemin poorly compared with M3, which would satisfactorily account for their difference in pigmentation on our medium.

TABLE I.—*Absorption of Haemin from Liquid Pigmentation Medium by Suspended Bacteria*

Bacteria in suspension.	Haemin concentration (μg./ml.) in supernatant liquid after rotation at 28° for— 4·5 hr.	23 hr.
None	98	98
	99	100
M3	25	17
	28	17
M7	73	75
	73	73

Organisms harvested from growth on pigmentation agar medium for 5 days at 28° were suspended to a density of *ca.* 10^{10} organisms per ml. in liquid pigmentation medium contained in 4¼ in. × ⅛ in. (10·8 × 1·3 cm.) glass tubes. The suspensions were rotated at 28° for the times shown and haemin in the supernatants, after centrifugation, was estimated colorimetrically, using the EEL portable colorimeter with No. 621 blue filter.

Survey of a collection of strains of Pasteurella pestis *for ability to produce pigmented colonies*

Tests of ability to produce pigmented colonies were made for a number of strains of varied origin which had been stored as dried cultures (Stamp, 1947) for 2 years or more, and subsequently freeze-dried in " *Mist. desiccans* " a mixture of one part of broth and three parts of serum, with 7·5 per cent glucose added (Fry and Greaves, 1951). The freeze-dried cultures were suspended in broth, and streaked on pigmentation agar supplemented with arginine (essential for four of the strains). After incubation for 4 days at 28°, it was obvious that most of the cultures were heterogeneous, giving rise to a mixture of pigmented (P) and non-pigmented (NP) colonies. A suitable region of each plate, containing 50 to 100 discrete colonies, was marked off, and the numbers of colonies of each type in this region were counted. The results are compiled in Table II. On sub-culture, P colonies again gave mixtures, usually with P colonies in the majority, and with NP colonies mostly appearing as secondaries. In contrast, sub-cultures from NP colonies were, invariably, homogeneously NP.

TABLE II.—*Pigmentation of Different Strains of* Pasteurella pestis

		Percentage of pigmented colonies.
Avirulent strains	A1122	98
	EV76	0
	53H1	41
	Java	0
	14	23
	Soemedang	100*
	Tjiwidej	100*
Virulent strains	F9581	20
	I-72	25
	L27	100
	L36	98
	L37	100*
	139L	81
	New Mexico 499559	100*
	„ „ 499668†	93
	Shasta	100
	WCDCPp5A2258†	36
	„ 6†	98
	„ 54†	90
	Yokohama	15

* Few NP colonies among dense growth.
† Arginine-dependent strains.

DISCUSSION

Of twenty strains tested (after prolonged storage) only four gave populations homogeneous in pigmentation character, two strains being homogeneously P and two homogeneously NP. The other sixteen strains gave mixtures of P and NP colonies.

Mutation from P to NP occurred in all the strains which we have examined, but the reverse mutation, from NP to P, was never observed. It is therefore probable that all strains showing heterogeneity were originally of P rather than of NP type. In many cases, NP type predominated in heterogeneous populations, suggesting that this type was preferentially selected during culture or storage *in vitro*.

The classification of strains of *P. pestis* into the two types, P and NP, on the basis of high or low haemin absorption, was quite unequivocal under our conditions. In addition to haemin, P strains absorbed basic dyes from liquid medium more efficiently than did NP, suggesting that the mechanism responsible for ability to absorb haemin was not specific for this substance. While P and NP strains harvested from pigmentation medium differed widely in ability to absorb haemin from liquid medium (Table I) this difference was considerably less evident with organisms harvested from other media (*e.g.*, tryptic meat agar). P and NP strains thus appear to differ quantitatively in their ability to absorb haemin, and growth on pigmentation medium serves to enhance this quantitative difference.

Preliminary tests have shown that NP mutants of virulent strains generally have reduced virulence for mice. Mutation to NP type followed by preferential selection may therefore contribute to loss of virulence in laboratory cultures.

While loss of ability to pigment leads to reduction in virulence, possession of the ability obviously does not, by itself, confer high virulence. Ability to pigment

appears to be one of a number of properties collectively determining high virulence in *P. pestis*. Further, it is evident that ability to pigment is not connected with ability to resist phagocytosis, which sharply differentiates virulent from the majority of protective avirulent strains (Burrows, 1955).

Investigations of the changed virulence of NP strains, which are reported in a subsequent paper, indicate that such strains are population-limited *in vivo* by iron deficiency. It is tempting to speculate that the low ability to absorb haemin characterising such strains *in vitro* is, in some way, instrumental in producing the limitation observed *in vivo*.

SUMMARY

Many strains of *P. pestis*, when cultivated on a chemically defined medium containing haemin, form dark brown colonies. The dark brown pigmentation results from absorption of haemin from the medium. After prolonged incubation, pigmented colonies produce non-pigmented secondary colonies, which on sub-culture give only non-pigmented colonies. Pigmented and non-pigmented strains can most easily be differentiated on a medium containing galactose, amino acids and 100 μg./ml. of haemin, buffered at pH 8·0 with phosphate and incubated at 28°.

Of 20 laboratory strains tested after prolonged storage, 16 consisted of mixtures of pigmented (P) and non-pigmented (NP) types. On sub-culture, P strains gave predominantly P type with some NP mutants, but NP strains remained pure NP. In nature *P. pestis* is probably P type, and NP organisms accumulate during culture or storage *in vitro*.

NP mutants derived from P virulent strains are of reduced virulence for mice.

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