

The production and inactivation of pyocines

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Most strains of *Pseudomonas aeruginosa* produce substances known as pyocines which inhibit the growth of other strains of the same species. Pyocines, first described by Jacob (1954) and studied in detail by Hamon (1956), belong to the class of substances known as bacteriocines (Jacob, Lwoff, Siminovitch & Wollman, 1953) and the fact that they are active only on closely related organisms differentiates them from the many other antibiotic substances produced by *Ps. aeruginosa* (Emmerich & Löw, 1899; Schoental, 1941; Hays *et al.* 1945; Young, 1947). There is considerable variation in pyocine activity of different strains within the species and it has been suggested that this could form the basis of a typing scheme (Holloway, 1960; Papavassiliou, 1961). It was clear, however, from preliminary experiments in this laboratory that such a scheme could not be employed until a more reliable method of testing for pyocine production had been devised. The degree of inhibition of one strain by another varied with the medium used and other cultural conditions. Several strains which had been described by other workers as non-producers of pyocines were found to be active. It was also noted, in cross-streaking experiments, that an area of growth of the indicator strain often occurred in the centre of a zone of inhibition, suggesting that *Ps. aeruginosa* strains produced, as well as pyocines, other substances which antagonized their action. Brubaker & Surgalla (1961) made similar observations on an inhibitory substance while investigating the pesticines produced by *Pasteurella pestis*.

This work describes experiments to determine the optimum conditions for the production and demonstration of pyocines and the nature of the pyocine-inactivating substances.

MATERIALS AND METHODS

Strains

Pseudomonas aeruginosa

(a) Producing pyocines: N.C.T.C. 1999, B 55, M 163, S 178 and H 194. Although all these strains had different ranges of activity when tested on a large series of indicator strains, the first four of them inhibited all four indicator strains listed under (c); strain H 194, however, inhibited only the indicator strain M 8.

(b) not producing pyocines: W 96 and P 501. These two strains did not inhibit any of a large series of indicator strains including those listed under (c).

(c) Indicator strains: M 8, B 10, S 17 and B 26. The first of these was inhibited by all the strains listed under (a) and the other three were inhibited by all except strain H 194.

Proteus vulgaris

A non-motile strain (N.C.T.C. 8313) was used for the production of bacterial proteinase.

Media

(a) Nutrient broth: (Oxoid no. 2) containing 1% 'Lab Lemco' beef extract, 1% peptone (Oxoid L 37), 0.5% sodium chloride pH 7.5.

(b) Nutrient agar (Oxoid no. 2) containing similar ingredients to the nutrient broth, plus 1% 'Ionagar' no. 2 (Oxoid) pH 7.5.

Technique

The procedure used in most of the experiments was similar to that employed by Abbott & Shannon (1958) in studying *Shigella sonnei* colicine production.

Thick nutrient agar plates (8–10 mm. deep) were inoculated with the strain under test as a central streak 1 cm. wide, and incubated at 37° C. for varying periods of time. After incubation, the plates were exposed to chloroform vapour for 1 hr. and the growth was then scraped off with a microscope slide. The plates were re-exposed to chloroform for 1 hr. and left for a further hour with their lids off, to allow the chloroform to evaporate. Four-hour nutrient broth cultures of each of the four indicator strains were then streaked across each plate at right angles to the line of the original inoculum. After re-incubation for 16 hr. the plates were examined for evidence of inhibition of growth of the indicator strains. Any deviation from this technique is reported in the text.

EXPERIMENTAL

(1) *Formation of pyocines on solid media*

Five nutrient agar plates were inoculated with strain N.C.T.C. 1999, and incubated for 16, 24, 36, 48 and 72 hr., respectively. After killing and removing the growth, each plate was inoculated with the four indicator strains and re-incubated. The width of the zone of inhibition of each indicator strain on each plate is shown in Text-fig. 1. Large zones appeared on the plate on which the pyocinogenic strain had been growing for 24 hr. On the plate incubated for 36 hr., however, the zones were distinctly smaller, though they were again larger on the plates incubated for 48 and 72 hr. This suggested that a period of production of pyocines was followed by a period in which inactivating substances were produced.

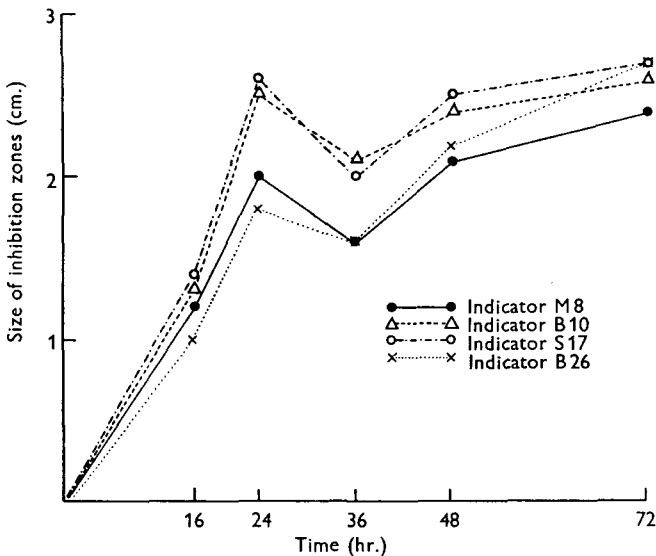
When incubation was done for periods longer than 72 hr. (up to 7 days) zones of inhibition of a more or less constant width were obtained.

The same experiment was repeated with strains B 55 and S 178 and similar results were obtained. The reduction in zone size after the 24 hr. period differed slightly with the different pyocinogenic strains, but was always demonstrated.

(2) *Demonstration of the pyocine-inactivating substances*

(a) A plate was inoculated with strain N.C.T.C. 1999 in the usual manner and incubated for 24 hr. After the growth had been killed and scraped off, the same area was re-inoculated with the non-pyocine-producing strain W 96. After 24 hr.

re-incubation, the resulting growth was killed and removed. The indicator strains were then inoculated at right angles to the original streaks and the plate was examined after a further 16 hr. incubation. No inhibition of the indicator strains was seen. A control plate, prepared in the same way but not inoculated with strain W 96, showed the usual zones of inhibition, indicating that strain W 96 produced substances which inactivated the pyocines elaborated by strain N.C.T.C. 1999.



Text-fig. 1. Zones of inhibition of four indicator strains produced after different periods of growth of the pyocinogenic strain N.C.T.C. 1999.

(b) A streak of strain M 163, 2 cm. wide, was made to one side of the middle line of a 14 cm. nutrient agar plate (streak A, Pl. 1, fig. 1). After 48 hr. incubation, the growth was sterilized and removed. Strain P 501 was then inoculated in a similar manner on the other side of the middle line, in the upper half of the plate only (streak B). After a further 48 hr. incubation this growth was killed and removed, and the four indicator strains were each streaked across the plate, once on the upper half of the plate and again on the lower half. Pl. 1, fig. 1 shows the result of this experiment. On the upper half of the plate there was no inhibition of growth of the indicator strains on the side on which strain P 501 had grown, suggesting that this strain produced pyocine-inactivating substances but no pyocines. On the side on which M 163 had been grown, however, the usual inhibition of the indicators occurred. In the lower control half of the plate the zone of inhibition extended symmetrically on both sides of the primary streak of strain M 163.

(c) Strains N.C.T.C. 1999, B 55 and S 178 were stabbed with a straight wire into a nutrient agar plate at 3 points on a horizontal line 2 cm. apart. After 48 hr. incubation, the resulting macrocolonies were killed by chloroform and scraped off. Strain W 96 was then heavily streaked on the plate, parallel to the previous line and at a distance of 2 cm. from it. After another 48 hr. incubation, the resulting growth was killed and removed, and the plate flooded with a 4 hr. broth culture

of indicator strain M 8. Pl. 1, fig. 2 shows this plate after 16 hr. re-incubation. The effect of the pyocines produced by strains B 55 and S 178 appears as larger inhibition zones of the indicator strain on the side of the macrocolonies away from the line where strain W 96 had been grown, than on the other side. With strain N.C.T.C. 1999, there is no zone of inhibition. It was concluded that the pyocines produced by strains B 55 and S 178 were partially inactivated by substances derived from strain W 96 and that produced by N.C.T.C. 1999 was completely inactivated.

(3) *Effect of proteolytic enzymes on pyocines*

(a) Strains N.C.T.C. 1999, B 55, M 163 and S 178 were stabbed with a straight wire into a nutrient agar plate in two rows about 4 cm. apart. After 48 hr. incubation, followed by exposure to chloroform and then air, a strip of thick sterile blotting paper (1 × 7 cm.) soaked in a 1:1000 solution of crystalline trypsin was placed in the central area between the macrocolonies. The plate was then incubated for 2 hr., the paper was removed, and the plate flooded with a 4 hr. nutrient broth culture of the indicator strain M 8. After 16 hr. re-incubation, it was found that there was no inhibition of the indicator strain by strains N.C.T.C. 1999 and B 55, since their pyocines had been completely inactivated. With strain M 163, however, slight inhibition of the indicator strain occurred as a result of partial inactivation of pyocine, while with strain S 178 normal inhibition occurred as the pyocine had not been affected. The results suggested that the pyocines produced by different strains varied in their susceptibility to the inactivating action of trypsin.

(b) A thick sterile blotting paper strip was soaked in trypsin solution and placed to one side of the middle line of a nutrient agar plate. A thin layer of agar was then poured over the whole surface of the plate. When this had solidified, strain B 55 was inoculated over the blotting paper area and to a distance of 1 cm. to one side of it. After 24 hr. incubation, the growth was killed and removed, and the plate then flooded with a 4 hr. nutrient broth culture of the indicator strain M 8. After a further 16 hr. incubation, the pyocinogenic activity of strain B 55 could only be demonstrated by an ill-defined zone of inhibition, away from the blotting paper area, indicating the ability of trypsin to inactivate pyocines.

(c) The previous two experiments were repeated, replacing the trypsin with a 20% solution of papain in 0.2 M phosphate buffer containing 0.001 M versene and 0.005 M cysteine. Inactivation of pyocines by papain could not be demonstrated.

(4) *Effect of a bacterial proteinase*

A set of experiments similar to 2b and 3a was performed, replacing the strain W 96 and the blotting paper strip respectively by a streak of the *Proteus vulgaris* strain N.C.T.C. 8313, and incubating the plates for 48 hr. The rest of the procedures were then followed as before. The results showed that this strain of *Proteus* completely inactivated the pyocines produced by strains N.C.T.C. 1999 and B 55. Those pyocines produced by strain M 163 were partially inactivated and those produced by strain S 178 remained unaffected. These results were similar to those obtained using trypsin and suggest that the effect of the *Proteus* on pyocines may be due to proteinase action.

(5) Passage of pyocines through membrane filters

Membrane filters (Oxoid size 4 cm., porosity 0.5–1.0 μ) were placed in the centre of three nutrient agar plates. Strain N.C.T.C. 1999 was inoculated in a $\frac{1}{2}$ cm. wide band across the filter. After 24, 48 and 72 hr. incubation, respectively, the filters were removed and 4 hr. nutrient broth cultures of the four indicator strains streaked on the area previously occupied by the membrane filter and at right angles to the position of the original streak. After another 16 hr. incubation, growth and inhibition zones were recorded. Strain N.C.T.C. 1999 was selected for this experiment since excessively motile strains tended to grow over the edge of the membrane filter within 48 hr.

Inhibition of indicator strain B 10 appeared after 48 hr. but was not detected at 24 or 72 hr. The other indicator strains were not inhibited at any time. From these results it was concluded that only some pyocines were able to pass through the membrane filter. The fact that pyocines could be detected after 48 hr., but not later, suggested that the inactivating substances were formed later than the pyocines and also passed through the membrane. The inability to demonstrate pyocines affecting indicator strains other than B 10 suggested the simultaneous passage of certain pyocines and their inactivating substances through the membrane.

(6) Effect of the composition of the medium on the production of pyocines and the suppression of the inactivating substances

A common finding in cross-streaking experiments was the appearance of discrete colonies or central growth in the inhibition zones of the indicator strains. This interfered with the proper reading of these inhibition zones. The colonies were repeatedly tested and found sensitive to the pyocine-producing strains with very rare exceptions, suggesting the involvement of a mechanism different from resistance. When the presence of pyocine-inactivating substances was demonstrated in the previous experiments, a trial was made for the suppression of the central growth.

The following substances were added either alone or in various combinations to the basal nutrient agar medium:

Calcium chloride	0.1 M	Sodium citrate	0.1 %
Manganese chloride	0.01 M	K ₂ HPO ₄	0.1 %
Cupric sulphate	10 ⁻⁶ M	Protamine sulphate	1 %
Iodoacetic acid	10 ⁻⁵ M	Ethylenediamine tetra-acetic acid	1 %

Strain B 55 was inoculated by the streak technique on to plates containing each medium. Primary incubation was for 48 hr. and after inoculation of the four indicator strains, further incubation was for 16 hr. The inhibition zones were measured and any appearance of discrete colonies or confluent growth in the centre of the zones was recorded. A control nutrient agar plate was included in the series.

From Table 1 it can be seen that the addition of cupric sulphate increased the size of the inhibition zones of indicator strains M 8 and S 17, suggesting that this

substance stimulated the production of particular pyocines. Calcium chloride and manganese chloride had a small enhancing effect on the production of pyocines but there was apparently a simultaneous increase in the activity of the inactivating substances, as discrete colonies and even confluent central growth appeared in the inhibition zones. Sodium citrate and dipotassium hydrogen phosphate did not increase the inhibition zones but suppressed the appearance of any colonies in the zone. Another advantage of the medium containing citrate and phosphate was the absence of slime production. It had been observed that when a strain of *Ps. aeruginosa* formed abundant slime it was difficult to remove all the growth from

Table 1. *Effect of eight substances on the inhibition zones produced by pyocinogenic strain B 55 on the indicator strains M 8, B 10, S 17 and B 26*

Substance	Concentration used	Indicator strains			
		M 8	B 10	S 17	B 26
Cupric sulphate	10 ⁻⁶ M	3.4	2.0	3.6	2.0
Calcium chloride	0.1 M	1.8 c	2.0 g	2.0 g	2.0 g
Manganese chloride	0.01 M	1.7 c	1.8 c	2.0 c	1.8 c
Sodium citrate	0.1 %	1.4	1.5	1.6	1.8
Dipotassium hydrogen phosphate	0.1 %	1.5	1.4	1.5	1.8
Iodoacetic acid	10 ⁻⁵ M	1.4	1.4	1.8	2.0
Protamine sulphate	1 %	1.5	1.3	1.6	1.8
EDTA	1 %	1.4 c	1.4 c	1.6	1.9 c
Combination of citrate, phosphate and iodoacetic acid	—	1.5	1.4	1.7	2.0
Control (nutrient agar)	—	1.5 c	1.5 c	1.7	1.8

c = discrete colonies in centre of inhibition zone.

g = confluent growth in centre of inhibition zone.

the plate by scraping, and the remnants of slime sometimes caused an irregular deposition of the indicator strain on the plate. Iodoacetic acid and protamine sulphate had an effect similar to that of citrate and phosphate, and resulted in continuous, well-defined inhibition zones. Ethylenediamine tetra-acetic acid (EDTA) did not affect the inhibition zones and did not diminish the appearance of growth in the zones.

The medium finally adopted for examining pyocine production contained 0.1 % sodium citrate, 0.1 % dipotassium hydrogen phosphate and 10⁻⁵M iodoacetic acid.

(7) *Effect of temperature on the stability of pyocines*

Twelve nutrient agar plates were inoculated by the streak technique with strain N.C.T.C. 1999 and incubated for 48 hr., and then divided into three sets of four plates each. The first set was kept in the incubator at 37° C., the second at room temperature (approx. 22° C.) and the third at 5° C. One plate from each set was tested with the four indicator strains after 1, 2, 3 and 6 days. Inhibition zones and appearance of isolated colonies or confluent central growth were recorded (Table 2). The size of the zones and the appearance of isolated colonies was not influenced by storage at 5° C., or at room temperature for periods up to 6 days.

On the plates kept at 37° C., the size of the zones was also unaffected, but confluent central growth started to appear on the second day with indicator strains B 10 and B 26, suggesting an increased action of the inactivating substances at 37° C.

Table 2. *Effect of the storage temperature on the pyocines produced by strain N.C.T.C. 1999 as assayed by the inhibition zones on four indicator strains*

(Zone sizes expressed in cm.)

Storage conditions	Indicator strains			
	M 8	B 10	S 17	B 26
Incubator 1 day	1.5	1.4	1.5	1.6
2 days	1.3	1.1g	1.4	1.5g
3 days	1.3	1.5g	1.6	1.3g
6 days	1.4	1.4g	1.4	1.7g
Room temperature 1 day	1.5	1.4	1.5	1.5
2 days	1.3	1.4c	1.4	1.5c
3 days	1.4	1.4	1.7	1.4c
6 days	1.6	1.4c	1.7	1.5
Cold room 1 day	1.2	1.1	1.4	1.3
2 days	1.3	1.3c	1.4	1.3c
3 days	1.4	1.7	1.5	1.4
6 days	1.3	1.4	1.4	1.3

c = discrete colonies in centre of inhibition zone.
g = confluent growth in centre of inhibition zone.

Table 3. *Effect of fifty subcultures on the production of pyocines of strains N.C.T.C. 1999, B 55 and H 194 as assessed by the inhibition zones on four indicator strains*

(Zone sizes expressed in cm.)

Pyocinogenic strain		Indicator strains			
		M 8	B 10	S 17	B 26
N.C.T.C. 1999	{before subculture	1.6	1.4	1.6	1.5
	{after 50 subcultures	1.5	1.5	1.4	1.6
B 55	{before subculture	1.8	1.9	2.0	1.9
	{after 50 subcultures	1.9	1.7	2.0	1.6
H 194	{before subculture	0.7	—	—	—
	{after 50 subcultures	0.8	—	—	—

(8) *Effect of frequent subculture and prolonged storage on the production of pyocines*

It has been claimed that *Ps. aeruginosa* strains lose their ability to produce pyocines on storage (Hamon, Véron & Péron, 1961). Three pyocinogenic strains, two with a wide range of activity (N.C.T.C. 1999 and B 55) and one with a narrow range (H 194) were used in the following experiment. Each was subcultured 50 times over a period of 3 months both on nutrient agar slopes and in broth tubes and tested for the production of pyocines every 10 subcultures by the usual

cross-streaking technique with the four indicator strains. Inhibition zones remained constant throughout the experiment (Table 3).

Other strains which were known to have been kept for long periods under unfavourable cultural conditions were also examined; they included 10 strains which had been stored on nutrient agar slopes at room temperature for 5–8 years, with 6- to 12-monthly subcultures. All were good producers of pyocines and their patterns were similar to those of freshly isolated strains.

DISCUSSION

In studying the production of bacteriocines by *Shigella sonnei* by the cross-streaking method, Abbott & Shannon (1958) noted that discrete colonies, or even confluent growth, often appeared in the centre of the zone of inhibition of an indicator strain. They named these appearances 'D zones', and considered them to be due to the growth of a minority of organisms that were resistant to the bacteriocine. On testing the sensitivity of these colonies in comparison with the original culture it was found, however, that they were rarely truly resistant (Abbott, pers. com.). Similar findings have now been obtained with *Pseudomonas aeruginosa* cultures. The results suggest that the pyocine, which is first formed at the centre of the primary streak and diffuses outwards, is inactivated by substances which are produced at a later stage of the growth cycle. At the periphery of the growing pyocinogenic strain, where the inactivating substances have not yet been produced, the indicator strains are inhibited by the pyocines formed early in the growth phase. The motility of a particular pyocinogenic strain may also play a part in the formation of the D zones, as an actively motile strain will tend to move the bilateral area of inhibition further towards the periphery. It has been demonstrated that this phenomenon was not simply due to the diffusion of the pyocines into the periphery; diffusion alone would have resulted in a gradual increase in zone size with or without central growth of the indicator strains, but in fact the zone size decreased between the 24 and 48 hr. periods. This could only be explained by the appearance of some other substances interfering with the activity of the pyocines. The appearance of 'resistant' colonies, or confluent control growth, is therefore due to the inactivation of the pyocines. When there is little pyocine inactivation, a few discrete colonies will appear, but when most of the pyocines have been inactivated, confluent growth (D zones) is obtained. Further, it has been shown that different pyocines vary in their susceptibility to the inactivating substances. It is probable that much of the difficulty experienced by some workers in demonstrating the pyocinogenic activity of some strains of *Ps. aeruginosa* may have been due to simultaneous production of pyocine-inactivating substances.

The presence of pyocine-inactivating substances was also demonstrated by the membrane filter experiment, where pyocines were detected on the second day but not on the third, suggesting that the pyocines passed the membrane first and the inactivating substances later. Their presence can also explain the curves in Text-fig. 1, where production of pyocines occurs first, followed at a later stage by the inactivating substances. This resembles the formation and subsequent

destruction by bacterial proteases of staphylokinase (Elek, 1953) and of staphylococcal coagulase (Lominski, Smith & Morrison, 1953).

Gratia & Betz-Bareau (1946) and Frédéricq (1948) reported that colicines produced by coliform bacilli were inactivated by trypsin and bacterial proteinases. According to Hamon (1956), pyocines are also protein in nature. The experiments described here, in which pyocines were inactivated similarly by strains of *Ps. aeruginosa*, a strain of *Proteus vulgaris*, and the proteolytic enzyme trypsin, provide some evidence that the inactivating substances were proteinases. It was also shown that calcium, which enhances the proteinases of pseudomonas and proteus cultures (Haines, 1932), has a similar effect on the pyocine inhibitors. Hamon & Péron (1962) found that some of the bacteriocines of *Ps. aeruginosa* were resistant and others sensitive to the action of trypsin.

Maschmann (1937) showed that strains of *Ps. aeruginosa* produced a wide variety of different proteinases and used phosphates to inhibit them. Elliott (1945) found that iodoacetic acid inhibited the proteolytic enzymes produced by *Streptococcus pyogenes*, and Gorini (1950) reported that bacterial proteinases were inhibited by citrates. In view of these observations, experiments were made in which iodoacetic acid, phosphates and other substances were tested for their effect on pyocine inhibitors. Finally, a nutrient agar medium was devised containing 10^{-5} molar iodoacetic acid, 0.1 % sodium citrate and 0.1 % dipotassium hydrogen phosphate.

The pyocine-inactivating substances appear to be different from some other pseudomonas proteinases, particularly the one described by Fisher (1960) which is inhibited by thiol groups and stimulated by sodium iodoacetate.

The fact that strains of *Ps. aeruginosa* growing on this medium produce little slime is an additional advantage. Holland (1962) found that the presence of bacterial slime protected otherwise sensitive organisms against the lethal activities of megacine, the bacteriocine produced by *Bacillus megaterium*. Indicator strains which may in fact be sensitive to the bacteriocine may, with abundant slime production, appear to be resistant.

It is considered that the proposed medium will give more consistent and reliable results than simple nutrient media in examining pseudomonas cultures for pyocinogenic activity and is thus of value in investigating the use of pyocine production as the basis for a typing scheme of *Ps. aeruginosa*.

SUMMARY

Certain strains of *Ps. aeruginosa* produce, in addition to pyocines, substances which inhibit pyocine activity. These pyocine inhibitors are probably proteolytic enzymes.

In order to investigate the production of pyocines by various strains of *Ps. aeruginosa*, a nutrient agar medium was devised in which the action of the pyocine-inhibiting substances is suppressed by incorporating 10^{-5} M iodoacetic acid, 0.1 % sodium citrate and 0.1 % dipotassium hydrogen phosphate. This medium also diminished slime production.

Pyocine production is a stable characteristic which is not lost on repeated

sub-culture or prolonged storage, and might form the basis of a typing system for *Ps. aeruginosa*.

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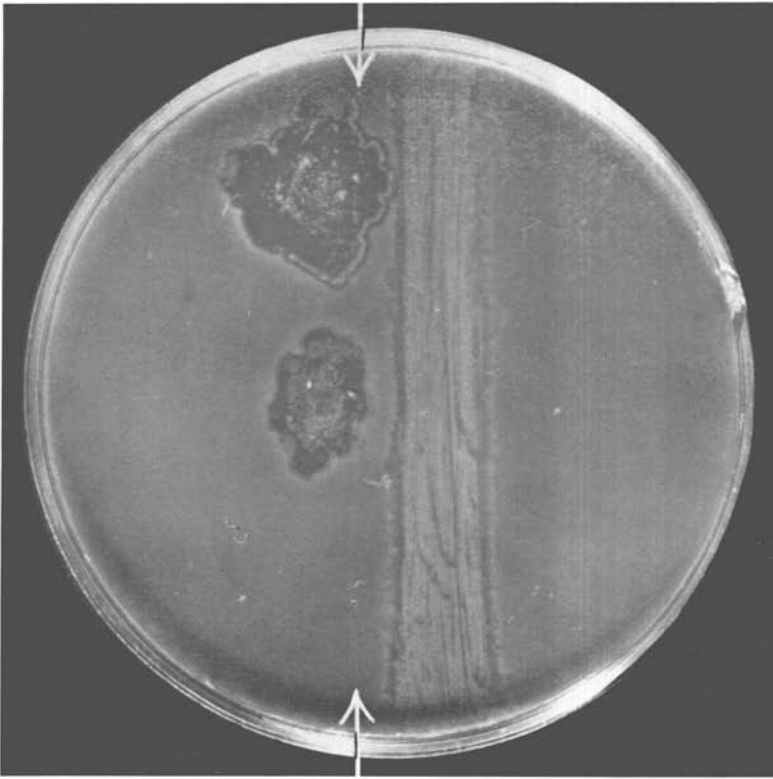


Fig. 2

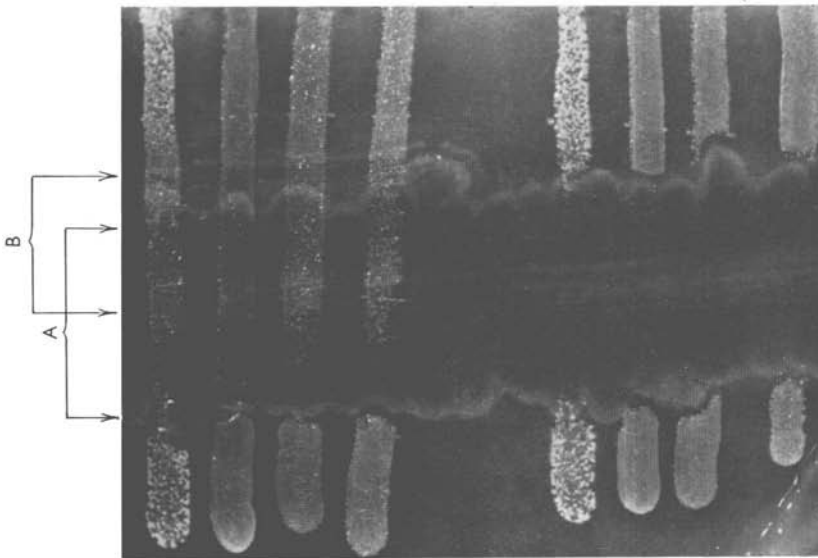


Fig. 1

EXPLANATION OF PLATE 1

Fig. 1. Inactivation of the pyocines of strain M 163 (Streak A) by the non-pyocinogenic strain P 501 (Streak B). In the upper half of the plate, the inactivation of pyocines is shown by growth of the indicator strains on the side of the primary streak where strain P 501 had been grown. Inhibition of indicators occurred on the other side. In the lower half of the plate, where only strain M 163 had been grown, there was inhibition of the indicator strains on both sides.

Fig. 2. Inactivation by the non-pyocinogenic strain W 96 of pyocines produced by strains N.C.T.C. 1999, B 55 and S 178. From left to right: the complete inactivation of pyocines produced by strain N.C.T.C. 1999 is shown by an absent inhibition zone of the indicator M 8. Partial inactivation of pyocines produced by strains B 55 and S 178 is shown by smaller inhibition zones to the side of strain W 96 than to the other side. The arrows point to the line of inoculation of the three pyocinogenic strains.