

GROWTH-INHIBITING METABOLITES OF *PROTEUS MIRABILIS*

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PLATES XIV AND XV

SWARMING by proteus bacilli, first described by Hauser (1885), has continued to puzzle microbiologists. It is thought to be caused by negative chemotaxic metabolites (Lominski and Lendrum, 1947; Hughes, 1957; Hoeniger, 1964; Brogan, Nettleton and Reid, 1971) that accumulate during growth. When a threshold concentration is reached, division is inhibited and the bacteria develop into highly motile long forms which swarm down the concentration gradient. On escape from the inhibitory concentration, the long forms fragment into short rods which multiply until the metabolites again accumulate to the critical swarming concentration. The process is then repeated and results in the well-known pattern of concentric circles seen on certain media. These chemotaxic metabolites have not been isolated and their chemical composition is unknown (Hughes); they appear to be volatile (Lominski and Lendrum; Hughes) and to be inactivated by ether (Lominski and Lendrum). Hughes showed that proteus bacilli also form stable non-volatile substances which may be responsible for the line of demarcation (Dienes, 1946, 1947) between swarms of different strains of *Proteus* when they are matched on agar.

During an investigation of methionine synthesis in a strain of *Proteus mirabilis*, syntrophism could not be detected among auxotrophs with blocks at different steps in the pathway (Grabow and Smit, 1967). It seemed possible that this might be due to the excretion of growth-inhibiting substances similar to those of some *Escherichia coli* mutants (Böck and Neidhardt, 1966; Zwaig and Diéguez, 1970) or to the protoplast-bursting factor of *Bacillus amyloliquefaciens* (May and Elliott, 1970). This communication describes experiments which confirm that proteus bacilli form growth-inhibiting metabolites. These metabolites could not be distinguished from those held responsible for swarming, but they differ significantly from the growth-inhibiting factors of *E. coli* and *B. amyloliquefaciens*.

MATERIALS AND METHODS

The minimal agar medium (MM) was that of Grabow and Smit. Syntrophism tests by parallel streaking were performed on this medium enriched with 2 µg per ml DL-methionine or DL-arginine hydrochloride for methionineless and arginineless auxotrophs respectively.

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The medium also contains 0.01 per cent. (w/v) 2,3,5-triphenyltetrazolium chloride. This indicator causes inocula to become intensely red when about half the maximal growth is attained. Swarming does not occur on this medium. Thirty ml of medium in 9-cm glass petri dishes was used throughout. Closed plates were dried by overnight incubation. Cultures were incubated at 37°C for 24 hr.

Organisms used are listed in the table. Streak inoculations were made with a stirrup-shaped wire loop (Grabow and Smit). Chloroform and ether treatment of bacterial growth on solid media was done by inverting the petri dishes over these liquids contained in the lids. Exposure was for 3 hr at room temperature.

TABLE
Organisms used in tests on growth inhibition and syntrophism

Strain	Reference
<i>Proteus mirabilis</i> no. 13	Coetzee and Sacks (1960)
Mutants of <i>Proteus mirabilis</i> no. 13: <i>argF</i> 602; <i>argE</i> 208	Prozesky (1967)
<i>metA/B</i> 291; <i>metE</i> 709	Grabow and Smit (1967)
FM3 <i>mot</i> ; FM4 <i>fla</i> ; FM5 <i>fla</i>	Appelbaum, Hugo and Coetzee (1971)
<i>Proteus mirabilis</i> no. 197 and no. 256	Locally isolated by Professor J. N. Coetzee
<i>Proteus vulgaris</i> no. 56 and no. 69	Locally isolated by Professor J. N. Coetzee
<i>Proteus morganii</i> no. M50	Prozesky, De Klerk and Coetzee (1965)
<i>Proteus rettgeri</i> no. R325	Coetzee (1963a)
Providence no. P29	NCTC no. 9211 (Coetzee, 1963b)
<i>Escherichia coli</i> no. CA-7	Grabow and Smit (1967)

RESULTS

Growth inhibition by parallel inocula

Parallel streaks of strains *metE* 709 and *metA/B* 291 (mutants of *Proteus mirabilis* no. 13) were made 1 mm apart on methionine-enriched MM. Syntrophism was not detected, but a small area of growth inhibition was observed at adjacent sides of the inocula (fig. 1A). With inocula 1 cm apart inhibition was not observed. A third inoculum of either strain *metE* 709 or *metA/B* 291 streaked between growths separated by 1 cm grew poorly (fig. 1B) but grew almost normally if the original inocula were separated by more than 1 cm (fig. 1D). When streaks of *metE* 709 and *metA/B* 291 were made 1 cm apart and after incubation were treated with chloroform, before another was streaked between, the middle inoculum grew normally (fig. 1C). Similar results were obtained with *Escherichia coli*, although there is reason to believe (Zwaig and Diéguez) that wild-type strains of this species do not excrete self-inhibitory metabolites.

Growth inhibition by circular inocula

Inhibition of growth was also observed with a circular inoculum (diameter 1 cm) of the wild-type *P. mirabilis* no. 13 on MM. After incubation the inoculum showed more growth on the outer side (fig. 2A and B). When a small inoculum of strain no. 13 was at this stage made in the centre of the ring it did not grow (fig. 2B). An *E. coli* inoculum made in a grown circle of *P. mirabilis*

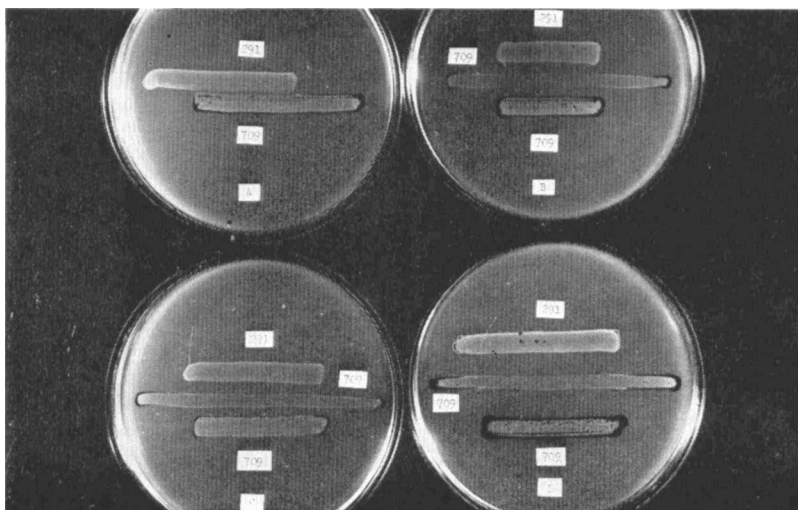
GROWTH INHIBITION OF *PROTEUS*

FIG. 1.—(A) Parallel streaks of methionineless *Proteus mirabilis* auxotrophs on enriched minimal medium. Adjacent sides show slight growth inhibition. (B) Inhibition of growth of an inoculum placed between two streaks of growth. (C) Parallel streaks exposed to chloroform after incubation do not inhibit growth of an inoculum placed between them. (D) Parallel growths further apart do not affect a third inoculum.

(Dark areas in this and the subsequent figures indicate reduction of 2,3,5-triphenyltetrazolium chloride.)

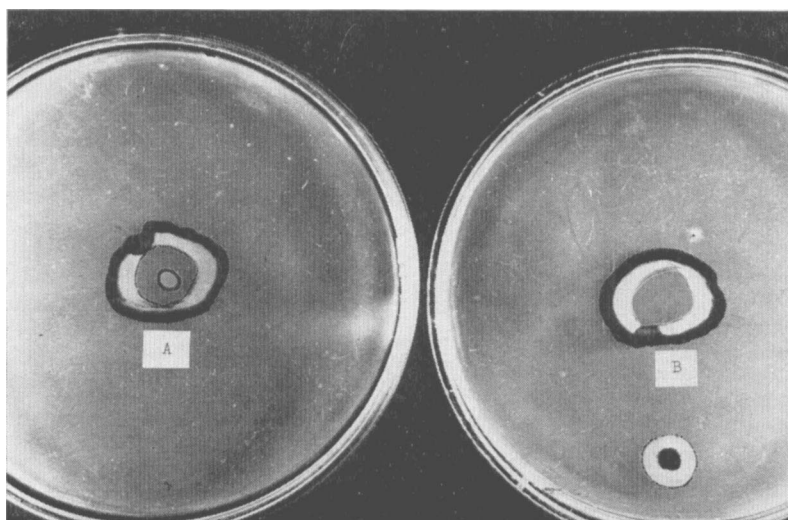


FIG. 2.—(A) Circular streak of wild-type *P. mirabilis* attains more growth at the periphery. Inoculum of *P. mirabilis* at the centre of the circle grows almost normally after the circular growth was treated with chloroform. (B) An inoculum of *P. mirabilis* at the centre hardly grows if the circular growth is not treated with chloroform.

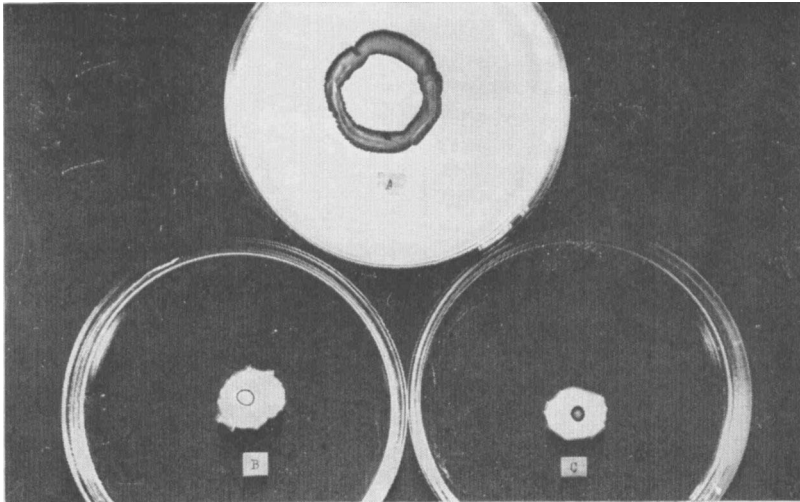
GROWTH INHIBITION OF *PROTEUS*

FIG. 3.—(A) Circular streak of wild-type *P. mirabilis*. (B) Inoculum of *P. mirabilis* grows poorly on remaining island after circular growth and surrounding medium has been removed. (C) Inoculum of *P. mirabilis* grows almost normally on chloroform-treated island.

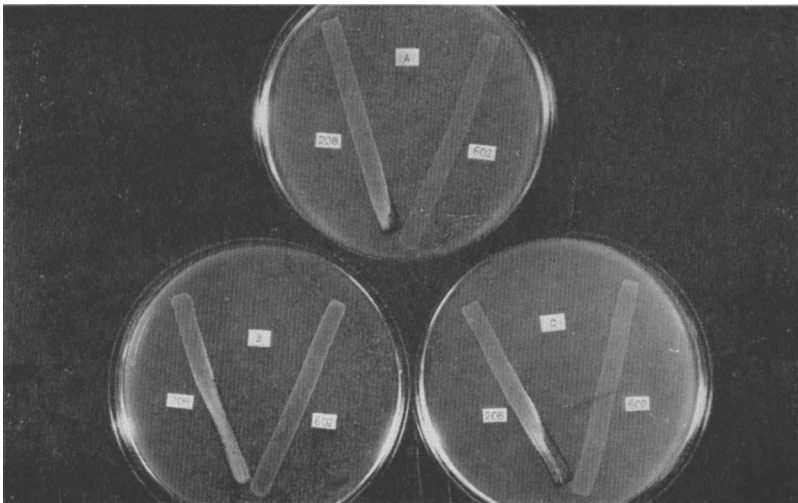


FIG. 4.—(A) Syntrophism is present at apex of V where simultaneously inoculated streaks of arginine-less *P. mirabilis* auxotrophs grow on enriched minimal medium. (B) No syntrophism at apex when the feeder-strain (*argF* 602) is incubated before inoculating the recipient strain (*argE* 208). Feeding is present where the streaks are more distant since feeder-strain has grown for 48 hr. (C) Syntrophism is present at apex if the feeder-strain (*argF* 602) is incubated and exposed to chloroform before the recipient strain (*argE* 208) is inoculated.

showed much more growth. A circular inoculum of *E. coli* also showed more growth at the periphery. Inocula of *P. mirabilis* and *E. coli* within the ring grew to about the same extent. After treatment of a circular growth of *P. mirabilis* with chloroform, both *P. mirabilis* (fig. 2A) and *E. coli* attained almost normal growth at the centre.

Experiments to distinguish between growth inhibition by inhibitory metabolites and competition for nourishment

Circular streaks (internal diameter 2 cm) of wild-type *P. mirabilis* no. 13 were made on MM (fig. 3A). After incubation the growth was removed aseptically together with the surrounding medium (fig. 3B and C). The remaining island of medium was expected to contain inhibitory metabolites excreted from the circular growth and to be relatively depleted of nutrients. Diffusion of nutrients to this island was effectively prevented. An inoculum of *P. mirabilis* grew poorly on it (fig. 3B) but markedly better growth was obtained (fig. 3C) if the island was exposed to chloroform before inoculation. Exposure to ether vapour at room temperature also deprived the islands of their growth inhibiting property. These experiments were repeated to ensure that differences in the area of the island did not influence the results. Inocula of *E. coli* attained the same growth on chloroform-treated and untreated "proteus" islands. The experiments were repeated with circular streaks of *E. coli*. In this case *P. mirabilis* and *E. coli* inocula grew equally well on the islands.

After leaving plates with "proteus" islands for 48 hr on the bench or exposing them to 60°C for 30 min., no difference was observed in the growth of *P. mirabilis* inocula on chloroform-treated and untreated islands. In other experiments, the circular inocula were separated from the central area by sections of dialysis tubing inserted into the unsolidified medium after plates were poured. Inhibition was observed on islands obtained by removal of the circular growth and dialysis tubing. This indicated that the growth-inhibiting metabolites are dialysable.

All the strains of *P. mirabilis* and *P. vulgaris* listed in the table excreted growth inhibiting metabolites. The metabolites affected only the growth of *P. mirabilis* and *P. vulgaris* and not that of *P. rettgeri*, *P. morgani*, Providence or *E. coli*.

Effect of growth-inhibiting substances on syntrophism

Strains *argF* 602 and *argE* 208 were simultaneously streaked on an arginine-enriched MM to form the limbs of a V (fig. 4A). After incubation, syntrophism was observed where the ornithine-requiring mutant (*argE* 208) was nearest to the ornithine-excreting (*argF* 602) strain. In a second experiment, strain *argF* 602 was inoculated on the plate and incubated before strain *argE* 208 was streaked to form the second limb. In this case syntrophism was observed only where the mutants were some distance apart (fig. 4B). This showed that strain *argF* 602 had excreted into its surroundings metabolites which prevented syntrophism by growth inhibition. In a third experiment the inoculum of strain *argF* 602

was exposed to chloroform after incubation. In this case the subsequent growth of strain *argE* 208 was stimulated at an area nearest the feeding mutant *argF* 602 (fig. 4C). This indicated that the inhibitory metabolites excreted by strain *argF* 602 were inactivated by the chloroform and, since the growth had also now been killed, no more of these metabolites were being formed. Ornithine survived the chloroform treatment and was present in the immediate vicinity of mutant *argF* 602.

DISCUSSION

The inhibition of growth in closely adjacent streaks and in inocula placed between streaks of existing growth might be caused by inhibiting metabolites, by competition for nutrients, or both. There was no inhibition between more widely separated streaks, and this is consistent with either hypothesis. When the grown culture was treated with chloroform there was no inhibition of an organism subsequently inoculated between closely spaced streaks (fig. 1C), either because the inhibiting metabolites were inactivated by the chloroform or because the medium was no longer being depleted of nutrients as a result of the killing of the growth.

Wild-type *Escherichia coli* is known not to excrete any self-inhibitory metabolites (Zwaig and Diéguez, 1970), and experiments with this organism showed that growth inhibition was due simply to competition for nutrients. Competition for nourishment probably also plays a major role in the effects seen with *Proteus mirabilis*, but this organism in addition evidently excretes substances that affect its own growth.

The loss of inhibition on storage or heating of the plates demonstrated that the inhibiting metabolites formed by *P. mirabilis* are either inactivated by heat, or are volatile and lost when the producer organisms are killed. The possibility that restoration of growth might have resulted from diffusion of nutrients to the centre—since the organisms in the circular inoculum were no longer metabolising—was excluded by isolating the central area as an island from the surrounding medium. The fact that these growth-inhibiting substances can exert such a marked inhibitory effect on growth over a period of 24 hr must mean that they reach a high initial concentration in the circle of growth.

Strains of *P. mirabilis* and *P. vulgaris*, as well as auxotrophic mutants and mutants that do not swarm because the flagella are absent or paralysed, were all shown to excrete metabolites that inhibit the growth of each other, but the growth of *P. morganii*, *P. rettgeri*, Providence and *E. coli* was not affected. The question arises of the relationship between these substances and the negative chemotactic agents thought to be the cause of swarming (Lominski and Lendrum, 1947; Hughes, 1957; Hoeniger, 1964; Brogan *et al.*, 1971). My finding that the growth-inhibitory substances remain active for a considerable time in agar, that they are dialysable, volatile and inactivated by ether, is consistent with observations on the metabolites associated with swarming (Lominski and Lendrum; Hughes; Hoeniger). The susceptibility of the former to chloroform has not been reported for the metabolites involved in swarming. These similarities, and the finding that growth-inhibiting metabolites are formed only

by strains of *P. mirabilis* and *P. vulgaris*, suggest that both swarming and growth inhibition may be caused by the same substances. This conclusion is in agreement with the observation (Moltke, 1929; Hughes) that the mortality among the swimmers is high, and with the suggestion (Hughes, 1956, 1957; but see Hoeniger) that the metabolites responsible for swarming may exert toxic effects on proteus organisms similar to those caused by penicillin and ultraviolet light. It has however not previously been shown clearly that the metabolites associated with swarming indeed inhibit the growth of the organism because earlier experiments were made on media on which proteus bacilli can swarm. The minimal medium used here does not allow swarming and the proteus organisms were therefore not able to "escape" from the growth inhibiting metabolites. My findings may explain the observation (Lominski and Lendrum) that the clear zones between the concentric rings of growth in a swarming culture are at first practically sterile and only gradually become populated. The intermediate areas may again become habitable when the volatile growth-inhibiting substances evaporate.

A bactericidal product is excreted by mutants of *E. coli* with a glycerol kinase that is insensitive to feedback inhibition by fructose-1,6-diphosphate when grown in the presence of glycerol. This differs from the *P. mirabilis* metabolites in that it is not produced by wild-type organisms, affects a wide range of bacteria and is not volatile (Böck and Neidhardt, 1966; Zwaig and Diéguez). Although the protoplast-bursting factor of *Bacillus amyloliquefaciens* is excreted by wild-type organisms, it also differs from the present metabolites in that it affects a relatively wide range of bacteria and is not volatile (May and Elliott, 1970).

The present findings indicate that since the growth-inhibiting substances—unlike growth-promoting intermediates—are volatile and exert an effect only in high concentrations, syntrophism studies are unlikely to be influenced when the streaks are made simultaneously. On the other hand, prior growth of the feeder strain may result in accumulation of sufficient inhibitory substances to affect syntrophism adversely. That growth-inhibiting substances are not responsible for the negative results in syntrophism experiments with methionineless *P. mirabilis* auxotrophs is in agreement with findings (Grabow and Smit, 1967) that these auxotrophs do not accumulate precursors of metabolic blocks.

SUMMARY

Strains of *Proteus mirabilis* and *P. vulgaris* produce metabolites which inhibit their own growth and that of other members of the group, but not that of *P. rettgeri*, *P.morganii*, Providence or *Escherichia coli*. These metabolites are dialysable, volatile and inactivated by ether and by chloroform. They are active in solid media for a long time after excretion. These properties are like those of the negative chemotactic metabolites that have been held responsible for the swarming of proteus organisms. These metabolites do not influence parallel-streak tests for syntrophism between auxotrophs of *P. mirabilis*.

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