The Growth *in vitro* of Killer Particles from *Paramecium aurelia* and the Axenic Culture of this Protozoon

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SUMMARY

Paramecia belonging to certain strains of *Paramecium aurelia* (syngens 1, 2 and 8) were transferred from dual culture with bacteria to axenic media, where the growth of some stocks continued, with weekly subculturing. The only axenically grown stock found to be capable of supporting growth of mu particles indefinitely was stock 138 (syngen 8).

Lambda and mu particles from axenically grown *Paramecium aurelia* were cultivated *in vitro* in a highly complex medium at 27° under aerobic conditions. The particles retained their characteristic killing action on certain *P. aurelia* stocks but could not infect sensitive *paramecia*. The particles divided at approximately one fission per day and achieved a maximum density of only $16\text{--}20 \times 10^3/\text{ml}$. The implications of these studies for the interaction of the nuclear genes and the killer particles are discussed.

INTRODUCTION

Symbiotic particles in the cytoplasm of *Paramecium aurelia* (Sonneborn, 1959; Beale, Jurand & Preer, 1969) may enable the ciliate to kill other *paramecia* which lack particles. Electron microscope studies of the particles suggest that they are bacteria (Beale & Jurand, 1960; Beale *et al.* 1969; Jurand & Preer, 1969). They possess DNA, RNA, proteins, carbohydrates and lipids (van Wagten-donk & Tanguay, 1963; Smith-Sonneborn & van Wagten-donk, 1964; Stevenson, 1969). Mu particles contain diaminopimelic acid and possibly muramic acid which are characteristic of bacterial cell walls (Stevenson, 1967). The DNA content is similar in amount to that of free-living bacteria (Soldo, van Wagten-donk & Godoy, 1970; M. Chance, unpublished), ribosomal RNA is present (Baker, 1970) and many key enzymes of metabolic pathways have been found (Kung, 1968; J. Williams, unpublished). The kappa particles of stock 51 have been shown to possess typical bacterial cytochromes (Kung, 1970). It might be anticipated, then, that these particles would grow outside the ciliate. Lambda particles (stock 299, syngen 8) have been maintained in a highly complex medium but no characteristics of their growth cycle have been provided (van Wagten-donk, Clark & Godoy, 1963). *In vivo* they depend on host nuclear genes for their maintenance and replication (Preer, 1968), each kind of particle requiring the presence of a specific nuclear gene or genes. Organisms with one copy of the nuclear gene possess approximately half the number of particles compared with those with two copies of the gene (Chao, 1953; Balsley, 1966). The rate of replication of the particles is geared to that of the *paramecium* (Soldo, 1963).

The work reported here was concerned with the cultivation of the particles in various media outside the host organism and also with their maintenance within the host when the *paramecia* themselves were cultivated in axenic medium.

METHODS

Strictly aseptic conditions were observed throughout.

Culture of paramecia in axenic media. Cultures of *Paramecium aurelia* are normally kept in grass medium containing *Aerobacter aerogenes*. Before attempting to cultivate the killer particles *in vitro* it was necessary to grow the paramecia without these bacteria. Transfer to axenic cultivation was carried out in one of two ways.

(a) Washing in sterile yeast extract: 100 to 200 paramecia from a dual culture with bacteria were washed through 0.25 % yeast extract contained in five three-welled depression slides using a micropipette to transfer single organisms from well to well. The paramecia that remained alive at the end of this procedure were inoculated into 5 ml. axenic medium contained in test tubes, 10 organisms per tube, and incubated at 27°, unless otherwise stated.

(b) Antibiotic treatment: this method was not used for stocks bearing killer particles since the particles are sensitive to antibiotics. A small inoculum of paramecia was placed in 0.5 ml. axenic medium containing 0.07 to 0.05 ml. of a stock solution of 1×10^6 units of penicillin and 0.2 g. streptomycin per 20 ml. in well slides. When the swimming movements of the ciliates indicated that they had begun to be affected by the antibiotics, the remaining organisms were inoculated into 5 ml. axenic medium contained in test tubes, 10 organisms per tube, and incubated at 27° unless otherwise stated.

Media used for the axenic cultivation of paramecia. The axenic medium was that of Soldo, Godoy & van Wagtendonk (1966). This medium had a ratio of TEM-4T: (diacetyl tartaric acid esters obtained from tallow monoglycerides) stigmasterol of 100:5 µg./ml. ('100:5' axenic medium). In a series of experiments folic acid, one of the constituents of this medium, was left out and attempts were made to grow killer and sensitive stock 299 animals in it. We also used axenic medium with a ratio of TEM-4T: stigmasterol of 40:4 µg./ml. ('40:4' axenic medium). Other axenic media occasionally used were as follows. Medium A: this was the axenic medium of Soldo (1960) with many constituents, including Edamine S. However, the non-dialysable yeast fraction was replaced by TEM-4T, trypticase and yeast nucleic acid in the same concentrations as used in the '100:5' axenic medium (Soldo *et al.* 1966). Medium B: this was the same as medium A except that lactalbumin was used at a final concentration of 10.0 mg./ml. and replaced the Edamine S.

Test for sterility of axenic cultures of paramecia, and of sterility of particle preparations. Axenic stocks and preparations of particles were checked for bacterial contamination in nutrient agar (Oxoid), nutrient broth (Oxoid), glucose-nutrient agar, glucose-nutrient broth, thioglycollate broth (Oxoid), Sabouraud broth (Oxoid), tryptone soya broth (Oxoid), axenic medium and axenic agar, i.e. axenic medium containing 1.5 to 2.0 % agar. They were considered sterile if no growth appeared on any of the media at 27° or 37° after 14 days.

Maintenance of axenic stocks of paramecia. The axenic stocks were subcultured weekly by means of a 1 ml. inoculum of the 7-day culture in 5 ml. of fresh medium, incubated at 20° and 27°.

Isolation of particles from axenic paramecia. Stocks 299 and 138, containing lambda and mu particles respectively, and growing in axenic medium were the sources of the particles. 500 ml. paramecium culture were centrifuged at 600g for 10 min. in an MSE oil-testing centrifuge, the pellet of paramecia homogenized by syringing with a hypodermic needle (21-gauge $\times$ 1½ in.) until no intact paramecia remained, and the homogenate then checked for sterility in the same way as the axenic stocks. The preparations of particles so obtained were inoculated into a wide variety of media and incubated at different temperatures. One ml. cultures of particles *in vitro* were subcultured every 4 to 5 days into 5 ml. medium.

Media used for the cultivation of the particles. Medium C: this was the medium of van Wagtenonk *et al.* (1963) except that the non-dialysable yeast fraction was replaced by yeast RNA (B.D.H.) 1 mg./ml., TEM-4T 100 µg./ml. and trypticase (BBL) 5 µg./ml. (Soldo *et al.* 1966). Also the stigmasterol concentration was altered to 5 µg./ml. Medium D: this was similar to medium C except that lactalbumin 0.5 mg./ml. replaced Edamine S. Medium E: paramecia (either stock 562 with alpha particles, or stock 299 containing lambda particles, or stock 540 with mu particles) were cultured in bacterized media for 7 days at 27° and this culture was used instead of distilled water to prepare nutrient agar, pH 7.0. Medium F: stock 540 paramecia (without mu particles) were cultured at 27° in '100:5' axenic medium for 7 days and then harvested aseptically. The pellet of organisms was broken by freezing and thawing once and then 8 ml. of this homogenate was added to 32 ml. of sterilized nutrient agar cooled to 40°, and plates were poured. The agar medium contained therefore sterile paramecium homogenate which had not been autoclaved. Sterility was tested by placing a sample plate at 27°. Medium G: this was PPLO broth (Difco) containing Bacto PPLO serum fraction (Difco) at a final concentration of 1 %.

Media used for growth of paramecia stocks and also particles. Medium H: this was a 1-day *Aerobacter aerogenes* culture in grass medium mixed with axenic medium ('100:5') in ratios of 25:27; 50:50; 75:25. The pH was adjusted to 7.0 and the mixture autoclaved.

Counting methods used to estimate the number of particles/ml. in vitro. A sample of growth medium containing particles (5 ml. from test tubes, 15 to 50 ml. from flasks) was filtered through a 0.45 µm. white plain millipore filter (24 mm. diameter). The cells were resuspended in 0.5 ml. of filtered sterile distilled water plus 0.5 ml. latex suspension containing 10⁶ latex particles/ml. (DVB latex 12.0 µm. diameter, obtained from Coulter Electronics, Dunstable, Bedfordshire) mixed well on a whirlimix and placed in a haemocytometer (Hawksley, depth 0.2 mm., area $\frac{1}{400}$ mm.²) and counted by the following methods under the microscope at a magnification of ×640. The ratio of latex particles: killer particles was determined by counting 50 fields, and the number of particles/ml. in the original culture calculated. The number of organisms on the whole grid, i.e. 20×20 squares, were counted and the number/ml. calculated from this.

Test for in vitro growth of particles by their ability to kill sensitive paramecia. Paramecia containing killer particles are known to kill certain paramecia which do not possess the particles, e.g. stock 299 containing lambda releases a toxic substance into the medium which can kill stock 92; mu particles kill by a different method and require prolonged cell to cell contact such as occurs in mating.

Mu particles (0.15 ml.) grown *in vitro* from stock 138 were added to ten ciliates of various paramecia stocks (stock 299 without lambda particles, stock 540 without mu particles, or stock 92) all contained in 0.5 ml. of '100:5' axenic medium. The mixtures were examined over a period of 60 min. for the killing action of the particles.

Lambda particles (0.15 ml.) growing *in vitro* from stock 299 were added to the same stocks in a similar way and observed over the same period of time.

In another experiment, 0.1 ml. lambda particles growing *in vitro* were added to 0.4 ml. sensitive stock 92 in bacterized culture in well slides and left at room temperature. The mixtures were again examined over a period of 60 min. for the typical behaviour of organisms that were being killed by toxic agents from lambda particles.

RESULTS

Growth of particle-bearing and non-particle-bearing stocks in axenic medium. The results of growing paramecia with and without particles in axenic medium are shown in Table 1.

Stock 138 without mu particles was obtained by growing stock 138 containing mu particles at 32° and subculturing every 3 to 4 days for approximately 28 days. Spontaneous loss of particles from stocks 299 and 562 occurred after growth of the paramecia in axenic medium. Stock 138, however, maintained its mu particles when grown in both '100:5' and '40:4' axenic medium at 20° and 27°.

Table 1. *The growth of killer and sensitive paramecia in different axenic media*

Cells were removed from growth in bacterized culture by various techniques, and placed in different axenic media at different temperatures. They were examined to see if they grew and if they continued to contain killer particles.

Stock	Particles present in original culture	Growth in axenic	Ratio of TEM-4T/stig-masterol in media supporting axenic growth	Method of freeing paramecium from bacteria	Temp. of growth	Maintenance of particles in axenic paramecia	Duration of maintenance of stocks
513 (Syngen 1)	—	+	100:5	Antibiotics	27°	No particles	<div style="display: inline-block; vertical-align: middle;"> <div style="display: inline-block; vertical-align: middle;"> Indefinitely in 100:5 Indefinitely in 100:5 Indefinitely in 100:5 Maintained indefinitely </div> </div>
60 (Syngen 1)	—	+	100:5				
90 (Syngen 1)	—	+	100:5				
540 (Syngen 1)	—	+	100:5				
540 (Syngen 1)	Mu	—	<div style="display: inline-block; vertical-align: middle;"> <div style="display: inline-block; vertical-align: middle;">100:5</div> <div style="display: inline-block; vertical-align: middle;">40:4</div> </div>	<div style="display: inline-block; vertical-align: middle;"> <div style="display: inline-block; vertical-align: middle;">Yeast extract</div> <div style="display: inline-block; vertical-align: middle;">Antibiotics</div> </div>	<div style="display: inline-block; vertical-align: middle;"> <div style="display: inline-block; vertical-align: middle;">27°</div> <div style="display: inline-block; vertical-align: middle;">20°</div> </div>	Cells never obtained in axenic medium	
562 (Syngen 2)	Alpha	+	100:5	Derived from 562 with alpha	<div style="display: inline-block; vertical-align: middle;"> <div style="display: inline-block; vertical-align: middle;">27°</div> <div style="display: inline-block; vertical-align: middle;">20°</div> </div>	No particles	Maintained indefinitely in axenic medium
114 (Syngen 2)	Sigma	—	100:5	Yeast extract	<div style="display: inline-block; vertical-align: middle;"> <div style="display: inline-block; vertical-align: middle;">27°</div> <div style="display: inline-block; vertical-align: middle;">20°</div> </div>	Cells never obtained in axenic medium	
138 (Syngen 8)	Mu	+	<div style="display: inline-block; vertical-align: middle;"> <div style="display: inline-block; vertical-align: middle;">100:5</div> <div style="display: inline-block; vertical-align: middle;">40:4</div> <div style="display: inline-block; vertical-align: middle;">100:10</div> </div>	Yeast extract	<div style="display: inline-block; vertical-align: middle;"> <div style="display: inline-block; vertical-align: middle;">27°</div> <div style="display: inline-block; vertical-align: middle;">20°</div> </div>	Maintained indefinitely in two media at both temperatures (i.e. 100:5, 40:4)	Maintained indefinitely
138 (Syngen 8)	—	+	<div style="display: inline-block; vertical-align: middle;"> <div style="display: inline-block; vertical-align: middle;">100:5</div> <div style="display: inline-block; vertical-align: middle;">40:4</div> </div>	Derived from 138 killers	<div style="display: inline-block; vertical-align: middle;"> <div style="display: inline-block; vertical-align: middle;">27°</div> <div style="display: inline-block; vertical-align: middle;">20°</div> </div>	No particles	Maintained indefinitely
299 (Syngen 2)	Lambda	+	100:5	Washing in yeast extract	<div style="display: inline-block; vertical-align: middle;"> <div style="display: inline-block; vertical-align: middle;">27°</div> <div style="display: inline-block; vertical-align: middle;">20°</div> </div>	Lambda maintained for 5 weeks, i.e. approx. 100 fissions at 20° and 27°	After loss of lambda particles, sensitive stock was maintained indefinitely
299 (Syngen 8)	—	+	100:5	Derived from 299 killers	<div style="display: inline-block; vertical-align: middle;"> <div style="display: inline-block; vertical-align: middle;">27°</div> <div style="display: inline-block; vertical-align: middle;">20°</div> </div>	No particles	Maintained indefinitely

Stock 114 containing sigma could not be grown in axenic medium. Many attempts were made to grow stock 540 mate killers (containing mu particles) in a wide variety of axenic media (including media A and B) and under different conditions. The only media used which

gave partial success were 50 % axenic '100:5' medium in which the ciliates lived for 19 days at 27°, and 50:50; 25:75; 72:25 medium H in which the paramecia lived for 6 to 7 days, but on subculturing they did not grow and eventually died. In all other media, e.g. '100:5' axenic medium, they died within 3 to 4 days.

Stock 299 cells without lambda particles and stock 138 without mu particles have also been grown in medium A *without* folic acid for 12 weekly subcultures.

Fig. 1. (a) Doubling effect with lambda particles in media G and D at inoculum sizes of 9×10^5 and 11×10^5 particles/ml. ●, Growth in medium G; ×, growth in medium D. (b) Doubling effect with lambda particles in media C and G at inoculum sizes of 9.4×10^6 and 11.4×10^6 particles/ml. ■, Growth in medium C; ▲, growth in medium G.

In vitro growth of particles. On inoculation of lambda and mu particles immediately after isolation from paramecia as described in Methods the particles underwent 'the doubling effect', i.e. approximately doubled their numbers. This phenomenon was observed in all the bacteriological media tested regardless of inoculum size. The media used for these experiments included C, D and G, '100:5' axenic medium and nutrient broth. The cultures were incubated aerobically at 27°. The results are plotted in Figs. 1 (a) and (b) for lambda particles in media G, C and D. The initial concentration of lambda particles on inoculation into, for instance, medium G was 9×10^5 in Fig. 1 (a) and 9.4×10^6 in Fig. 1 (b). After the approximate doubling, the particles rapidly fell in number.

In all the media tested, except media C, D and G, the particles on continued subculturing did not undergo further fissions and disappeared (i.e. in nutrient broth, '100:5' axenic

medium and all the other bacteriological media the particles only divided once immediately after isolation from the paramecia). In media C, D and G, however, on further subculturing when the inoculum size was below $20 \times 10^3/\text{ml.}$, both lambda and mu particles divided and were maintained by subculturing for 6 months as described in Methods. I have called this the 'growth effect'.

The inoculum size of lambda particles from stock 299 (that had undergone the 'doubling effect') in medium C was varied so that the initial concentration was $1 \times 10^3/\text{ml.}$, $3 \times 10^3/\text{ml.}$, $4.5 \times 10^3/\text{ml.}$ or $6 \times 10^3/\text{ml.}$, and after 4 days incubation at 27° the lambda particles reached a concentration of 16 to $20 \times 10^3/\text{ml.}$, then decreased rapidly. Similarly, following inoculation

Fig. 2. Growth of lambda in medium C and mu particles in media G and C. ■, Lambda growth in 500 ml. medium C contained in a Thompson bottle at 27° under aerobic conditions; ●, mu growth in 300 ml. medium G contained in a 500 ml. Erlenmeyer flask at 27° under aerobic conditions; ▲, mu growth in 300 ml. medium C contained in a 500 ml. Erlenmeyer flask, in shaking aerobic culture at 32° .

of mu particles from stock 138 at initial concentrations of $6 \times 10^2/\text{ml.}$, $4 \times 10^3/\text{ml.}$ or $6 \times 10^3/\text{ml.}$, the particles grew until they reached 16 to $20 \times 10^3/\text{ml.}$ before the numbers fell rapidly. This decrease was not due to a change in pH. The fission rate has been studied in this growth period and is shown in Fig. 2 for lambda and mu particles. Mu particle growth is shown in media G and C, while lambda growth is shown in medium C only. Both particles divided approximately once per day and the number of fissions depended on the initial inoculum size. The maximum concentration the particles achieved *in vitro* was 16 to $20 \times 10^3/\text{ml.}$

No success in increasing the maximum density or growth rate of lambda or mu particles *in vitro* was obtained by adding homogenates of paramecia to the media, by renewing the growth media, or by adding additional nutrients such as L-cysteine, L-glutamine, 'Marmite',

nicotinamide adenine dinucleotide, ATP and KCP. Lambda and mu particles did not grow in medium G containing crystal violet (Difco) which selects for mycoplasmas and inhibits all bacterial growth.

Killing experiments with in vitro grown particles. Since the particles when present in paramecium confer killer properties on the organism, proof that the particles we have been observing are indeed the killer particles would be to show that they can kill certain sensitive stocks of paramecium.

Two different experiments were prepared to test for the killing action of lambda particles grown *in vitro*. A 1-day lambda culture (0.15 ml.) in medium C (i.e. 16 to 20×10^3 lambda/ml.) was mixed with ten paramecia, none of which contained particles, of each of the following stocks: 92 (syngen 3), 540 (syngen 1), 299 (syngen 8), all contained in 0.5 ml. medium. After 10 min. all ten of the stock 92 organisms were dead. The ten stock 540 paramecia were alive for 60 min. but with stock 299, five organisms were killed in 60 min. In the control wells, with paramecia contained in 0.5 ml. axenic medium, the ciliates remained alive for 60 min. *Paramecium aurelia* stock 299 containing lambda killed stock 92 and stock 299 without particles but not stock 540.

In the second experiment a 4-day culture of lambda in medium C was used. In the control, i.e. stock 92 alone in bacterized medium, the paramecia were alive after 60 min., and in the second control, i.e. stock 92 + medium C without lambda particles, the paramecia were alive after 15 min. In the experimental well, i.e. 0.4 ml. stock 92 + 0.1 ml. lambda in medium C after 5 min. the paramecia began to swim in an odd fashion typical of lambda killing, and within 15 min. all the organisms were dead.

To study the killing action of mu *in vitro*, 0.15 ml. of mu from an *in vitro* culture in medium C was used, i.e. 15 to 20×10^3 mu/ml., and mixed with 10 paramecia of each of the three same stocks, in the same way as for the first lambda killing experiment above. With stock 299 all the organisms were dead within 30 min; however, with stocks 92 and 540 all the paramecia were alive after 60 min. *Paramecium aurelia* stock 138 containing mu particles killed certain sensitive stocks in syngen 8 but not stocks in other syngens as it was a mate killer.

Infection experiments with in vitro grown particles. Various attempts to infect stock 299 lacking lambda particles with the *in vitro* grown lambda were unsuccessful. On the other hand, particles prepared from paramecia growing in axenic medium were found to infect stock 299 sensitives. This was done by adding a serial dilution of a homogenate of stock 299 grown axenically and containing lambda to a bacterized culture stock 299 without lambda contained in welled slides at room temperature. The wells were topped up every day with fresh bacterized medium, examined over a period of 10 days, and scored for infection. Infection was first observed after approximately 2 days. Attempts to infect stock 138 without mu particles with the *in vitro* grown mu were also unsuccessful. These results seem to indicate either that the infective ability of the particles is altered during the *in vitro* growth, or that the conditions of the infection experiment are not the same for *in vivo* and *in vitro* particles.

DISCUSSION

The most interesting feature of the symbionts which occur in the nucleus and cytoplasm of *Paramecium aurelia* is that morphologically and biochemically they resemble bacteria. Lambda can be grown *in vitro* (van Wagtendonk *et al.* 1963) and the present work has shown that mu and lambda can grow outside the host ciliate.

The particles would grow *in vitro* to a concentration of 16 to 20×10^3 /ml. and when inside

a paramecium the maximum concentration of the particles was approximately $2.7 \times 10^9/\text{ml.}$, i.e.

Vol. of a paramecium = 3.75×10^{-7} ml. (paramecium considered as a cuboid with a square base of side $50 \mu\text{m.}$ and height $150 \mu\text{m.}$)

Particles/organism = 10^3

$\therefore$ Concentration of particles = $2.7 \times 10^9/\text{ml.}$ in cytoplasm of organism.

This indicates three possibilities. First, the gene was obviously not necessary for limited growth outside the ciliate and possibly the gene protects the particles from destruction by the paramecium; secondly the nuclear gene might have influenced the number and/or the fission rate of the particles, thus affecting the density of the particles present in the cell. Thirdly the optimum medium for particle growth has not yet been found and there is no fundamental reason why the particles cannot grow to numbers approaching 2.7×10^9 *in vitro*.

The low fission rate may be an adaptation to a symbiotic mode of life because the paramecium divides approximately once every 8 h. If the particles multiplied at the rate of *Escherichia coli* (approximately one fission every 24 min.) the host would soon become overpopulated.

The doubling effect was observed only when the particles were first isolated from the host paramecium and was probably caused by the particles completing a division that had begun in the host cell. This is analogous to the division of chloroplasts on first isolation from plants (Ridley & Leech, 1970). If the particles were allowed to divide and complete the doubling effect and were then inoculated at high concentrations, i.e. above $20 \times 10^3/\text{ml.}$, into fresh medium, this doubling effect was not observed and they disappeared rapidly until the numbers reached $16\text{--}20 \times 10^3/\text{ml.}$ This effect may also be observed for *Escherichia coli* with inocula above maximum concentration.

Another approach to the problem of particle maintenance was to grow the paramecia themselves in axenic medium, since it is known from enzyme studies that the host physiology is different in axenic medium from bacterized medium (Rowe, Gibson & Cavill, 1971). Only with stock 138 killers were the mu particles found to be maintained indefinitely and this raises the question of why the other particles, for example alpha and lambda are less easily maintained in axenic medium than bacterized medium. The particles can also be maintained in other protozoa (Sonneborn, 1965; Gibson, 1965).

Previously it has been reported that '100:5' axenic medium supported the growth of particle bearers in the even but not the odd numbered syngens of *Paramecium aurelia* (Soldo *et al.* 1966) and that the TEM-4T:stigmaterol ratio was important in the maintenance of stocks. Stock 138 (syngen 8) containing mu particles, however, has been maintained in the present work in axenic medium with weekly subculturing at both 27° and 20° for 12 weeks in TEM-4T: stigmaterol ratios of 100:5, 40:4 and 100:10 without loss of particles in any of the media. After this period the paramecia growing in 100:10 medium began to die although particles were present up to death. The paramecia in the 100:5 and 40:4 medium remained healthy and so it would seem that not only the ratio of TEM-4T: stigmaterol but also the final concentration is important in maintenance of the stock as the cells lived in 40:4 but died in 100:10 axenic medium.

Soldo *et al.* (1966) observed that axenic medium (100:5) supports the growth of the even but not the odd number syngens of *Paramecium aurelia* with the exception of stock 92 (syngen 3). In the same paper Soldo reported that stock 299 containing lambda was supported indefinitely.

It has been shown above that syngen 1 stocks 513, 60, 90 and 540 (without particles) are maintained indefinitely in this medium. In 100:5 axenic medium stock 299 containing lambda were maintained for only 5 weeks at 20° and 27°, after which period the paramecia lost their particles. It is reported that stock 540 with mu particles will grow and maintain the particles in 40:4 medium (van Wagtenonk, Goldman & Smith, 1970). However, I have not been able to repeat this. In the same paper van Wagtenonk *et al.* claim to have maintained mu particles in stocks 548 and 551 of syngen 1 in 40:4 medium. They also disclose that strains within a syngen respond differently to '40:4' and '100:5' media. This is at variance with my observations.

Soldo (1963) claimed that while both stock 299 containing lambda particles and stock 299 without lambda particles grew in the complete axenic medium, only the particle-bearing stock grew in axenic medium without folic acid. The sensitive stock died. He concluded from these experiments that the lambda particles provided the host paramecium with folic acid. I have not been able to demonstrate this. In my experiments stock 299 without lambda and stock 138 without mu survived at least 12 weekly subcultures into 100:5 axenic medium without folic acid.

From the growth of the particles *in vitro* it seems reasonable to envisage the particles as bacteria or of bacterial origin. They may represent a stage in evolution between a free-living bacterium and an obligate parasite as they can live in the paramecium and grow *in vitro* (although with a low fission rate and to a low maximum density) with a growth curve typical of most bacteria. It would be interesting to study the growth of other particles outside the host paramecium since there may be different degrees of integration in terms of replication rate and mechanism of maintenance involved with different particles.

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