BACTERIOPHAGE GROWTH ON STATIONARY PHASE *ACHROMOBACTER* STRAINS

BY

SUSAN MARY ROBB

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CONTENTS

SUMMARY

Achromobacter w.t. and strain 14 both support phage α3a growth in stationary phase, but unlike the w.t. strain, exponential phase cultures of strain 14 block phage development.

A standard method was developed for determining phage growth in stationary phase cultures. Lyophilised cells were used to eliminate variations due to the unstable phenotype of Achromobacter strain 14 cells. Phage α3a growth in stationary phase was characterized by a long and variable latent period of 6 to 9 h and an increased burst size of 709 p.f.u. per cell as compared with 153 p.f.u. per cell in exponential wild type cells. During the latent period the infected cells were very sensitive to changes in growth conditions and in particular, dilution. Pre-conditioning of the bacterial cells by allowing them to stand for 24 h after shaking for 3 days was an important aspect of the stationary phase phage growth system. Cells which had been allowed to stand retained the ability to be infected and to support phage growth for at least 16 days. Shaking cultures gradually lost the ability to support phage growth but the phage could persist in the host cell for 10 days until removal from shaking when the lytic cycle could proceed after allowing the cultures to stand. In comparison the latent period and burst size in Achromobacter w.t. stationary phase cells were reduced to less than 2 h and less than 200 respectively.

Stationary phase cultures differed physiologically and morphologically depending on the aeration conditions. In comparison with non-aerated standing cultures, vigorously aerated cultures showed a decrease in viability, RNA synthesis, membrane transport, intracellular ATP levels, UV resistance and heat resistance but had markedly higher protein synthesis

levels. Aerated cells were small non-motile rods which did not support phage growth. They developed into large motile rods under conditions of limited aeration and were able to propagate phage. It was proposed that changes in the host control mechanisms for macromolecular synthesis may be instrumental in either blocking or permitting phage development.

A spontaneous mutant of Achromobacter strain 14 (14^{x}) which liberated phage and was resistant to superinfection was isolated. The phage-host relationship was unstable and similar to the phage carrier state. The liberated phage were able to grow in exponential strain 14 cells. It was proposed that strain 14 was a defective lysogen and that an immunity phase shift model may account for the differential phage growth in exponential and stationary phase cells.

Host transcriptional control appears to be implicated in control of phage development in exponential and stationary phase cells. Achromobacter Lp only supported phage in exponential phase but a rifampicin resistant mutant of this strain was able to propagate phage in stationary phase. In vitro RNA synthesis assays showed that the rifampicin resistance was caused by an alteration in the RNA polymerase.

Preliminary experiments to determine intracellular phage macromolecular synthesis were carried out using exponential Achromobacter w.t. cells which had been irradiated with UV prior to infection. In irradiated cells, infection with phage resulted in stimulation of DNA synthesis but no stimulation of protein synthesis. Phage production was drastically reduced in cells which had been treated with very low UV doses. It was proposed that α3a development may rely heavily on host cell functions which are destroyed by UV.

Achromobacter mutants with defective leucine transport systems were

isolated. Mutants which lost the leucine uptake system completely were totally resistant to phage infection and were unable to adsorb phage α3a. This is the first report to implicate an amino-acid transport system in phage adsorption.

CHAPTER I

1.1 INTRODUCTION

The halotolerant gram-negative bacterium Achromobacter sp. 2 used in this study was originally isolated from hides and was implicated in leather decay since it was collagenolytic (Thomson, Woods and Welton, 1972). With the view to developing a genetic system for the study of the regulation of collagenase production, bacteriophages specific for Achromobacter were isolated and characterized (Thomson and Woods, 1974).

Four closely related phages which formed plaques on Achromobacter sp. 2 were isolated. Phage α1 came from a hide soak solution and phage α2 was isolated after ultraviolet (UV) irradiation of another Achromobacter species (sp. 9). Phage α3a was isolated from a single spontaneous plaque on a total of 250 lawns of Achromobacter sp. 2 and phage α3b was obtained after UV irradiation of the same strain (Thomson and Woods, 1974; J. Thomson, Ph.D. thesis).

The characteristics of the four phages were similar and they were related serologically. Phage α3a (used in the present study) has an hexagonal head with no visible neck, and a long tail (3450 Å), with short tail fibres near the tip. The nucleic acid was shown to be double stranded DNA with a melting temperature of 88.2°C and a corresponding molar concentration of guanine and cytosine of 45.73%. Single-step growth experiments revealed a characteristic long latent period (100 min) with an average burst size of 230 phage per infected cell (Thomson and Woods, 1974).

An infecting temperate phage has the choice of two developmental pathways. Either it can embark on the lytic cycle and lyse the cell with

release of progeny, or it can enter into a heritable symbiotic relationship with the host, known as lysogeny. Phage in the lysogenic state are known as prophages.

True lysogeny is characterized by the inheritance of the prophage by all bacterial progeny and immunity to superinfection by homologous phage (Lwoff and Gutman, 1950). Other symbiotic states known as pseudolysogeny are discussed in a later chapter.

The majority of known prophages integrate into the host chromosome but P1 and P7 prophages are normally maintained as autonomously replicating plasmids, at one or two copies per host chromosome (Yarmolinsky, 1977).

Integration of phage λ is the best understood of the integrative recombination systems and follows the Campbell model (Miller and Friedman, 1977). The integrative precursors of phage λ, P2 and P22 are circular and insertion of viral DNA occurs by reciprocal recombination at specific loci in both host and phage DNA (Calendar, Six and Kahn, 1977; Susskind and Botstein, 1978). Integration of phages λ and P22 shows a high degree of specificity for one site (Schwesinger, 1977). However, if the primary λ attachment site is deleted, λ can integrate with a low frequency at other sites (Enquist and Weisberg, 1977). Phage P2 shows strong preference for one integration site but can integrate at at least 10 distinct sites (Calendar, Six and Kahn, 1977).

Recently integration of the temperate phage Mu has been intensively studied because it has been found to be very similar to integration of insertion sequences. Mu insertion shows no site specificity and integration involves linear insertion of viral DNA at random sites. The integrative precursor is not circular and phage replication appears to be a prerequisite for integration (Bukhari et al., 1977).

Phage specific integrase (int) catalyses integrational recombination of λ, P22 and P2 and also functions in the reverse recombination that is

required for excision. For λ and P2, a second gene, xis and cox respectively, is required for efficient excision of the prophage (Campbell et al., 1977; Susskind and Botstein, 1977; Calendar, Six and Kahn, 1977).

The maintenance of lysogeny in λ is brought about by the action of the repressor molecule (CI protein), which blocks transcription of the early genes and prevents the production of the lytic control proteins (these control mechanisms are discussed in 5.4). The repressor also executes superinfection immunity by blocking transcription of the incoming phage (Ptashne, 1971). Unlike λ, P22 prophages exhibit two systems of superinfection exclusion of homologous phage, but these systems are distinct from the repressor systems which maintain the lysogenic state (Susskind and Botstein, 1978).

In a small proportion of a lysogenic population, the stable prophage existence ends, the lytic cycle ensues and phage progeny are released spontaneously. To enter the lytic cycle, phage λ and P22 genomes are excised from the host chromosome, after which they circularize and the rolling-circle mode of phage replication begins (Kaiser, 1971; Susskind and Botstein, 1978). Induction of a Mu prophage does not result in excision and replication of the genome appears to take place in situ (Toussaint, Faelen and Bukhari, 1977).

Methods for inducing some prophages artifically include treatment with UV irradiation or the use of mutagenic chemicals, such as Mitomycin C (MC) and nitrosoguanidine (NTG) (Adams, 1959).

To determine whether Achromobacter sp. 2 was inducible and thereby explain the origin of α3a and α3b, cultures were tested for induction by UV, MC and NTG. Phage could not be induced by UV, indicating that the isolation of α3b after UV irradiation was an unusual occurrence. MC and NTG induction yielded phage (α3) which were very similar to α3a and α3b

(Thomson and Woods, 1974).

It was proposed that Achromobacter sp. 2 was a cryptic lysogen. Cryptic lysogens of λ (λcrg) do not release phage (spontaneously or by artifical induction), they are not immune to superinfection and the cryptic prophage can only be detected in the lysogenic state by rescue of its genetic markers (Fischer-Fantuzzi and Calef, 1964; Marchelli et al., 1968). The cryptic (cry) nature of λ prophage appears to be due to a deleted repressor gene (Marchelli et al., 1968; Adhya and Campbell, 1970) and a defective excision function which may be influenced by an insertion element (IS2) (Zissler et al., 1977).

Achromobacter sp. 2 had an extremely low frequency of spontaneous and UV inducibility and was sensitive to superinfection, indicating defective excision and repression mechanisms. Cryptogenicity in Achromobacter did not seem to involve deletion of the repressor. It was found that although phage could not be induced by UV, phage (α3) could be isolated by MC or NTG induction. These phage formed turbid plaques on the parent strain and some of the resistant colonies isolated were shown to be lysogenic. They produced phage spontaneously, were resistant to superinfection, were UV inducible and were referred to as double lysogens. This suggested that although the cryptic prophage did not synthesize repressor, the vegetative phage was able to synthesize repressor and lysogenize the host.

It was proposed that the Achromobacter cryptic prophage was similar to that of Proteus mirabilis (Krizsanovich, 1973) and could be explained in the following way: The cryptic prophage produces a defective enzyme for excision and/or vegetative development. This defective enzyme can be reverted or suppressed following MC or NTG treatment, resulting in the induction of the prophage. The lack of functional repressor in the cryptic

prophage could be caused by physical disruption of the repressor gene by integration, as a result of the phage integration site being within the repressor gene.

Achromobacter double lysogens show superinfection immunity and UV induction. It was proposed that the repressor gene may be reconstituted by superinfecting phage lysogenizing in tandem with the cryptic prophage or at another locus which does not disrupt the repressor gene (Thomson and Woods, 1974). This split repressor model is similar to that for the split P2 int gene (Calendar, Six and Kahn, 1977).

The transducing abilites of the α phages were tested with a view to developing a genetic mapping system (Woods and Thomson, 1975).

Transduction of genetic markers can be generalized, in which case a number of markers can be transduced, or specialized, when markers adjacent to prophage attachment sites are transduced. Usually, prophages attach at preferred chromosomal sites.

The ability of P22 to mediate generalized transduction is now considered to be brought about by the non-specific packaging mechanism of the phage. Generalized transducing particles result when the host chromosome instead of the phage concatemer is used as a substrate for the sequential headful packaging mechanism. There is strong evidence that packaging of the chromosome is initiated at preferred sites and also that the P22 transducing particle does not contain any phage DNA (Susskind and Botstein, 1978).

Transducing particles containing host and phage DNA usually result from aberrant excision of an integrated prophage to form specialized transducing particles (Schwesinger, 1977).

Generalized transduction usually yields stable transductants by integration, whereas specialized transducing phage incorporate the donor

bacterial genes into the recipient genome by lysogenization and yield unstable heterogenotes. As long as the prophage is present the transductant carries the transducing material but loses it if the prophage is lost. In some rare cases, if the bacterial genes carried by the transducing phage are homologous to recipient genes, gene substitution via recombination can produce stable transductants regardless of whether the transducing phage remains (Susskind and Botstein, 1978). However, the distinction between generalized and specialized transduction is not clearly defined. There are reports of generalized transducing phages causing transduction either by integration or lysogenization, yielding stable or unstable transductants respectively. The type of integration depends on the degree of homology between the transducing DNA and the recipient DNA as well as the residual phage functions in the transducing particles (Luria et al., 1960; Chakrabarty and Gunsalus, 1969).

The four α phages (α1, α2, α3a and α3b) were all capable of generalized transduction and were able to transduce six auxotrophic markers of Achromobacter sp. 2 at similar frequencies. The transduction frequencies were highest when either α3a or α3b were used and ranged from 3.2×10^{-7} to 7.6×10^{-7}/p.f.u. adsorbed for the six markers (arg, cys A, cys B, met, pro and trp).

However, transductants were unstable. Using α3a as the transducing lysate, the average rate of segregation of trp^- in trp^+ transductants was 3.6×10^{-3}/cell/generation. Similar segregation rates were obtained with the other markers.

The phage characteristics of a number of trp^+ transductants obtained with an α3a lysate were examined after four cycles of cloning on minimal medium and were found to vary considerably. Table 1.1, reproduced from Woods and Thomson (1975) shows the phage characteristics of

20 transductants. The majority of the transductants were either sensitive or resistant to phage and did not release phage spontaneously or after UV induction (types 1 and 2). Some transductant colonies gave rise to both resistant and sensitive strains (types 3 and 6). After one cycle of cloning, erratic and spontaneous phage release occurred in some resistant and sensitive strains (types 5, 6, 7 and 8) but none of the resistant clones were UV inducible. All the released phages had host ranges different from α3a and were able to infect some strains which were resistant to α3a. Each was able to infect the respective transductants from which they were isolated.

Strains 14 and 15 (which have been used in the present study) were initially resistant to phage α3a but after 3 or 4 clonings on minimal medium became semi-sensitive (e.o.p. 10^{-1} - 10^{-2}). The decreased e.o.p. did not seem to be caused by a restriction and modification phenomenon as plaques from semi-sensitive lawns still showed the same reduced e.o.p. when reassayed on strains 14 and 15.

To account for the range of phage characteristics found in the transductants, it was suggested that the generalized transducing particles contained bacterial DNA and varying amounts of phage DNA, and complementation between the resident cryptic phage and the phage genes of the transducing particles occurred. To form transducing particles containing phage and host DNA, phage α3a may be able to integrate with equal efficiency at a number of sites or the particles may be formed by some as yet uncharacterized mechanism.

Three interesting aspects of this transduction system were:

(i) Although the transductants were unstable, usually indicative of integration by lysogenization (Morse, Lederberg & Lederberg, 1956),

Table 1.1 Phage characteristics of transductants obtained with an α3a transducing lysate at a m.o.i. of 1.

Twenty transductants (Nos. 1 to 20) were streaked on to minimal agar and duplicate samples of three clones from each transductant were cloned a further three times on to minimal agar. Phage characteristics were determined after each cycle of cloning.

Type	All 3 clones ex transductant sensitive to α3a	All 3 clones ex transductant resistant to α3a	Two clones ex transductant sensitive and one resistant to α3a	All 3 clones ex transductant initially resistant but at least one clone becoming semi-sensitive after 3rd or 4th cloning	Spontaneous and erratic phage liberation by one or more of the clones ex transductants*	U.v. induction of phage	Total no. of transductants and their identification numbers
1	+	-	-	-	-	-	4 (1-4)
2	-	+	-	-	-	-	8 (5-12)
3	-	-	+	-	-	-	1 (13)
4	-	-	-	+	-	-	2 (14, 15)
5	-	+	-	-	+	-	1 (16)
6	-	-	+	-	+$^{\phi}$	-	1 (17)
7	-	-	-	+	+	-	2 (18, 19)
8	+	-	-	-	+	+	1 (20)

*Transductants went through a minimum of one cycle of cloning and growth in broth without phage release prior to spontaneous phage liberation.

$^{\phi}$Phage liberation by resistant clone.

From: Woods and Thomson (1975).

no high frequency transducing lysates were obtained.

(ii) Segregation of trp^- in trp^+ transductants was not correlated with changes in the phage characteristics.

(iii) None of the transductants showed the characteristics of normal double lysogens. It was shown that double lysogens only formed in complete medium and not in minimal medium which was used for selection and maintenance of the transductants.

Within the scope of the present work, the most interesting feature of the Achromobacter transduction studies was the fact that transductant strains 14 and 15 were only able to support α3a growth whilst in stationary phase. As stated, these clones were initially resistant to phage α3a but after four clonings became semi-sensitive (e.o.p. 10^{-1} - 10^{-2}). Plaques did not develop on growing bacterial lawns and were not visible after overnight incubation (Woods, 1976). Plaques only developed on stationary phase lawns and were visible after incubation for 48 h. This interesting phenomenon would have passed unnoticed, had it not been for the Fleming-like approach of D.R. Woods, who re-examined old plates before they were discarded. He investigated this unusual phage growth system and found that α3a formed clear irregular plaques on these strains (in contrast with the semi-turbid plaques formed on the w.t. strain), that stationary phase phage development was extremely sensitive to, and was inhibited by aeration, and that lysates prepared on strains 14 and 15 were unable to mediate transduction (Woods, 1976). The details of his results have been outlined in the relevant chapters.

He also found that, unlike strain 14 and 15, Achromobacter sp. 2

(referred to as Achromobacter w.t. by Woods, 1976; Robb et al., 1977 and Robb et al., 1978) was unable to support stationary phase phage growth. In subsequent studies, it has been found that this particluar Achromobacter sp. 2 was an exceptional strain and in this dissertation has been referred to as Achromobacter Lp (for log phase phage development, see 6.2.1). Achromobacter w.t. refers to an Achromobacter sp. 2 strain which is able to support phage production in exponential and stationary phase.

Since productive bacteriophage development usually declines rapidly as host cells reach stationary phase (see 3.1) and no system of prolific phage production in stationary phase cells has been reported, the Achromobacter-α3a system is unique in the literature.

The differential phage growth in exponential and stationary phase Achromobacter cells may be influenced by host control mechanisms. The bacterial sporulation systems have illustrated the use of phages as probes for discerning bacterial control mechanisms during bacterial differentiation. (This has been reviewed more fully in 3.1 and 6.1).

The physiology of stationary phase gram negative cells is interesting from a number of aspects. Whereas gram positive cells have a clearly defined differentiation system (endospore formation), biochemical differentiation in gram negative cells at the end of the growth phase is not well understood (discussed in 4.1).

The present Achromobacter-α3a system may be the only example amongst gram negative bacteria in which phage development may provide an indication of changing intracellular control mechanisms during stationary phase.

Characterization of this system has been made in a broad context in order to define areas of research that may prove fruitful in the future.

CHAPTER II

OPTIMAL CONDITIONS FOR PHAGE α3a INTERACTIONS WITH ACHROMOBACTER STRAINS

2.1 INTRODUCTION

The growth of bacteriophage on stationary phase bacterial cells represents a unique virus-host system. The Achromobacter transductant colonies (strains 14 and 15, see 1.1) were of particular interest since they supported phage α3a development in stationary phase and blocked phage development in exponential phase.

Two main problems in the study of this system were:

(i) the ability of strains 14 and 15 to support phage α3a growth in stationary phase was an unstable characteristic;

(ii) the phage host-interactions were extremely sensitive to the culture conditions.

It was essential that variation due to these factors be eliminated. No basic work on this system had been done and this chapter describes experiments which were designed to optimize the conditions for reproducible phage development.

This system was first described by Woods (1976) who found that the two trp^+ transductant colonies (strains 14 and 15, see 1.1) were originally resistant to phage α3a but became semi-sensitive (e.o.p. 10^{-1} to 10^{-2}) after 4 cycles of cloning on minimal agar. Plaques did not appear on overnight lawns of these strains as observed in Achromobacter w.t. However, incubation of preformed lawns for a further 36 h resulted in the appearance of plaques. Woods showed that these plaques developed on stationary phase lawns and

that strains 14 and 15 had normal growth rates. The plaques were not due to spontaneous release or induction of phage as uninfected lawns of strains 14 and 15 did not give rise to plaques. Phage were not modified by growth on host strains 14 or 15. Clear irregular plaques were formed on stationary phase mutant backgrounds in contrast to the circular semi-turbid plaques formed on Achromobacter w.t. lawns after overnight incubation.

These results indicated that α3a was unable to develop in exponentially growing mutant cells. Since the susceptibility of a bacterium to bacteriophage infection is primarily dependent on whether or not the bacteriophage can attach to a cell wall receptor site and inject its nucleic acid, it was important to establish whether α3a could adsorb to exponential mutant cells. Woods (1976) found that adsorption of α3a to exponential and stationary phase strain 14 cells could be measured. Comparative studies on these adsorption kinetics are presented in this chapter.

Woods (1976) showed that the plaques formed on Achromobacter w.t. lawns under strict anaerobic conditions were clear with an irregular outline. They resembled the plaques which developed on strains 14 and 15 under aerobic conditions. No plaques developed on strains 14 and 15 under anaerobic conditions (even if incubated for up to 34 days) but when the plates were transferred to aerobic conditions characteristic clear irregular plaques developed after 2 days. It was suggested that phage growth on Achromobacter strains 14 and 15 required a micro-aerophilic environment and that plaques only appeared after dense growth of the background lawn had produced these conditions. This was supported by the fact that phage growth was not detected in shaking stationary phase strain 14 cultures. Cultures which were incubated without aeration supported vigorous

phage development. The effect of aeration and the requirement for specific phage growth conditions was investigated and the optimal conditions for phage development in stationary phase liquid cultures are reported.

2.2. METHODS

2.2.1 Bacterial strains

Achromobacter sp. 2 (Thomson, Woods and Welton, 1972) was used as the indicator strain for all phage assays. This strain is referred to in the text as Achromobacter w.t. Cultures were maintained on tryptone agar slants and on tryptone agar plates which were kept at room temperature and subcultured regularly.

Achromobacter strain 14 (Woods and Thomson, 1975; Woods, 1976) was maintained on agar slants, on plates and was lyophilised on Whatman No. 1 filter paper strips saturated with overnight tryptone broth cultures. These were stored in a vacuum dessicator at $4^{o}C$. All bacterial growth was at $30^{o}C$. A Gallenkamp rotary shaker was used to provide aeration (250 revs min^{-1}) unless stated otherwise.

2.2.2 Bacteriophage and high-titre preparation

Bacteriophage α3a described by Thomson and Woods (1973) was used. High titres were prepared in the following way: Overnight cultures of Achromobacter w.t. were diluted 1:20 in fresh tryptone broth and aerated for 2.5 to 3 h. Equal volumes of bacteria (c. 2×10^{8} cells ml^{-1}) and phage (c. 10^{6} p.f.u. ml^{-1}) were mixed and allowed to stand for 10 min at room temperature. Aliquots (0.2 ml) of the mixture were added to 2.5 ml tryptone sloppy agar and poured onto thick fresh tryptone agar plates. After overnight

incubation, 2 ml tris-HCl buffer was added to each plate and the sloppy agar layer was removed. The plates were washed with a further 0.5 ml tris-HCl buffer. After standing at room temperature for 2 h the bacterial debris and agar were removed by centrifugation at 11 000g for 10 min. The lysate was sterilized with 10% (v/v) toluene and stored at $4^{o}C$.

2.2.3 Concentration and purification of α3a phage

Crude lysates were purified by alternate cycles of centrifugation at 11 000g for 10 min and 94 500g for 90 min. The phage pellets were resuspended overnight at $4^{o}C$ in tris-HCl buffer.

2.2.4 Phage assay

Overnight cultures of Achromobacter w.t. were diluted 1:20 in tryptone broth and incubated with aeration for 2.5 - 3 h. The culture could be used for phage assays after standing at room temperature for up to 4 h. Phage assays were performed using the sloppy-agar overlay method of Adams (1959).

2.2.5 Phage neutralization by antiserum

Antiserum to phage α3a was prepared as described by Thomson and Woods (1973). The serum was diluted 10^{-1}, 10^{-2} and 10^{-3} in 0.85% physiological saline and 1 ml aliquots were added to 0.1 ml phage (c. 10^{7} p.f.u. ml^{-1}). Inactivation of phage was done at $37^{o}C$ and at intervals samples were withdrawn, diluted 10^{-2} to arrest the antibody-antigen reaction and assayed for p.f.u. Inactivation by antiserum was plotted as a function of time and the velocity constant for phage inactivation, k, was calculated according to the formula: $k = 2.3 \frac{D}{t} \log \frac{Po}{P}$ (Adams, 1959) where D is the serum

dilution factor, Po is the initial phage titre and P is the titre at time t.

2.2.6 Phage Adsorption

Adsorption of α3a to exponential and stationary phase Achromobacter w.t. and strain 14 cultures was examined at 30°C. Exponential cultures (2×10^8 cells ml^{-1}) were centrifuged at 3 000 g for 10 min and resuspended in tryptone broth at a concentration of 2×10^9 cells ml^{-1}. Three day old shaking cultures (2×10^9 cells ml^{-1}) were used for adsorption to stationary phase cells. Phage was added to 10 ml bacteria at a final concentration of c. 2×10^6 p.f.u. ml^{-1}. At various time intervals 10 µl aliquots were diluted into 10 ml volumes of tris-HCl buffer containing 10% v/v toluene. The samples were shaken to distribute the toluene and allowed to stand at 25°C for 10 min. They were then stored in ice awaiting assay for unadsorbed phage. The velocity constants (k) were calculated using the equation:

$$k = \frac{2.3}{Bt} \log \frac{Po}{P}$$

where B is the bacterial concentration, Po is the concentration of unadsorbed phage at time 0, and P is the concentration of unadsorbed phage at time t (Adams, 1959).

2.2.7 Identification of the α3a adsorption site

The unusual adsorption kinetics of α3a prompted further investigation into the identification of the phage adsorption site. Suggestive evidence indicated that Achromobacter mutants with defective leucine transport were deficient in phage adsorption.

Selection and characterization of leucine transport deficient mutants

Mutant selection

A modified method of Adelberg et al. (1965) was used for N-methyl-N'-nitro-N-nitrosoguanidine (NTG) mutagenesis. An overnight Achromobacter w.t. culture was diluted 10^{-1} in prewarmed tryptone broth and incubated with aeration for 90 min to obtain exponentially growing cells. Eight 1 ml samples from the culture were centrifuged at 3 000g for 10 min, and the pellets resuspended in 2.7 ml of prewarmed TM buffer (pH 6). NTG (0.3 ml) was added to each sample at a final concentration of 100 μg ml^{-1} and the mixtures were incubated for 15 min at 30°C. The treated cells were centrifuged and the NTG removed by washing the pellets in saline. The pellets were resuspended in 3 ml tryptone broth and the cultures were incubated overnight. Aliquots (0.1 ml) of the mutagenized cultures were spread onto minimal medium plates. Wells, 0.4 cm in diameter, were punched in the centre of each plate and 50 μl of the toxic leucine analogue 5'5'5' trifluoroleucine (TFL, 0.1M) was placed in each well. Plates were incubated for 48 h. Colonies which formed within the zones of inhibition surrounding the wells were stabbed and tested for resistance to TFL by a radial streak method.

Characterization of TFL mutants

Mutants were tested for:

(i) uptake of $^{C}14$-leucine as described in 4.2.5;

(ii) the ability to support phage as determined by plaque formation (see 2.2.4);

(iii) the adsorption of phage α3a (see 2.2.6) over a period of 15 min.

2.2.8 Efficiency of Plating (e.o.p.)

It had been noticed that the plaque size and time of appearance of α3a plaques on w.t. backgrounds varied. Assuming variations in plaque size and appearance were due to variations in phage adsorption, they should be eliminated by plating infected cells. On the other hand prior adsorption of a phage population should select for a poorly adsorbing phage fraction in the supernatent. If this were the case, plating of the supernatent fractions should yield greater plaque variation. To test these possibilities the following experiments were carried out:

(i) Dilutions of untreated purified α3a were plated as described (2.2.4).

(ii) Phage α3a was added (m.o.i. .01) to exponential w.t. cells (2×10^9 cells ml^{-1}) suspended in tris-HCl buffer. After 1 h, samples (0.1 ml) were added to 0.1 ml anti- α3a antiserum (k = 10) and incubated at 37°C for 8 min to inactivate unadsorbed phage. The suspensions were diluted (10^{-2}) in cold tris-HCl buffer to remove the antiserum, and 0.1 ml samples were assayed for infected cells.

(iii) After the same phage adsorption period, samples (0.1 ml) were added to 9.9 ml tris-HCl buffer containing 10% v/v toluene, which inactivates phage-host complexes. The suspensions were shaken, incubated at 25°C for 10 min, diluted into cold tris-HCl buffer and the free phage assayed.

2.2.9 Phage growth in exponential cultures

Overnight cultures were diluted 10^{-1} and incubated with aeration to a cell density of 2×10^8 cells ml^{-1}. Phage (0.1 ml) was added to 0.9 ml cells (c. 8×10^7 p.f.u. ml^{-1}) and the mixtures were incubated for 1 h prior to dilution to ensure that good adsorption took place. Samples were then diluted 10^{-2} in warm broth and two successive 10^{-2} fold dilutions were made in broth. The first dilution tube was used for determining the number of adsorbed phage and the remaining two tubes were incubated with aeration for a further 4 h before assaying for total p.f.u.

2.2.10 Experiments to determine the optimal conditions for phage growth on stationary phase cells.

The effect of aeration on phage growth

By increasing the volume of a culture in a given container the surface : volume ratio is decreased. In shaking and standing cultures increased volumes should decrease the exchange of gases with the atmosphere. The effect of increased culture volumes on phage growth was examined in stationary phase cells: Cultures (20 ml, 40 ml, 60 ml, 80 ml and 100 ml) in 100 ml flasks and 10 ml cultures in standard containers (28 ml screw-top containers) were aerated for 48 h. Phage was added (c. 7×10^3 p.f.u. ml^{-1}) to each culture. The flask cultures were divided and aeration of one set was continued whereas the other set was incubated without aeration. The cultures in standard containers were not divided and aeration was either continued or discontinued after α3a addition. Free phage were assayed after 24 and 48 h.

The effect of preincubation of host cells without aeration on subsequent phage growth

Two day old aerated cultures were dispensed (10 ml) into standard containers. These were incubated without aeration. Phage was added at various times after removal from aeration and free phage were assayed 24 h after α3a addition.

2.2.11 Standard method for phage growth on stationary phase cultures

Achromobacter w.t. (plate culture) and strain 14 (lyophilised culture) were innoculated into 600 ml tryptone broth in a 1 ℓ flask and aerated for 3 days. Aliquots (10 or 3 ml) were dispensed into standard containers or small 5 ml tubes (sloppy agar tubes) respectively. These were incubated without aeration, but with loose caps for at least 24 h before α3a was added (c. 1×10^4 p.f.u. ml^{-1}). Assays for p.f.u. were carried out at various time intervals after phage addition.

2.2.12 Screening method for determining bacterial sensitivity to phage

A method was devised to test large numbers of bacterial clones for:

(i) resistance or sensitivity to phage;

(ii) sensitivity in exponential or stationary growth phases.

Clones (c.25) were replicated onto three tryptone plates. After 8 h incubation, one plate was sprayed evenly with an aspirator containing c. 5×10^6 p.f.u. ml^{-1}. The second plate was sprayed after 48 h and the third was not treated. The plates were re-incubated after spraying and examined after various time intervals.

The validity of this method for detecting clones which were able to support stationary phase phage growth was checked by the routine

liquid culture technique. In order to test large numbers of clones, the bacterial growth and standing periods were carried out in the same standard containers (10 ml cultures).

2.3 RESULTS

2.3.1 Achromobacter w.t. stored on agar slants or plates remained sensitive to phage α3a. However, Achromobacter strain 14 was unstable and became resistant to phage when stored on plates. The use of lyophilised cultures eliminated variations due to loss of phage sensitivity.

2.3.2 High titre lysate preparation and stability

High titres of phage (5×10^{10} to 1×10^{11} p.f.u. ml^{-1}) were usually obtained. Crude lysates were unstable when stored at $4^{o}C$ in the presence of chloroform, showing a loss of infectivity of two logs over 2 - 3 weeks. Phage were more stable in the presence of toluene with a loss of 1 log of infectivity over 3 months. Purified phage resuspended in tris-HCl buffer (pH 8) was stable over a 9 month period at $4^{o}C$. Purified phage α3a was inactivated by chloroform and a 34% and 15% loss of infectivity was obtained after 20 min at $25^{o}C$ and $4^{o}C$ respectively. Vigorous shaking with chloroform increased the rate of phage inactivation. The kinetics of α3a inactivation by chloroform showed a loss of one log of p.f.u. in 4 h (Fig 2.2). No loss of phage infectivity was observed in the presence of toluene at 4 or $25^{o}C$ (Fig 2.2).

2.3.3 Phage neutralization by antiserum

The rate of inactivation of α3a by antiserum was constant over 30 min after the addition of antiserum (Fig 2.3). The neutralization

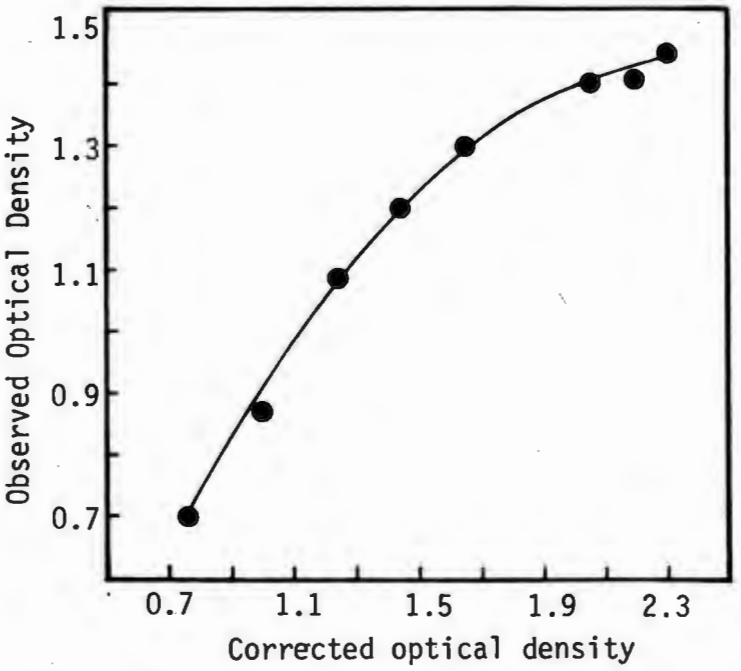


Fig 2.1a Turbidity correction for high cell density (600 nm).

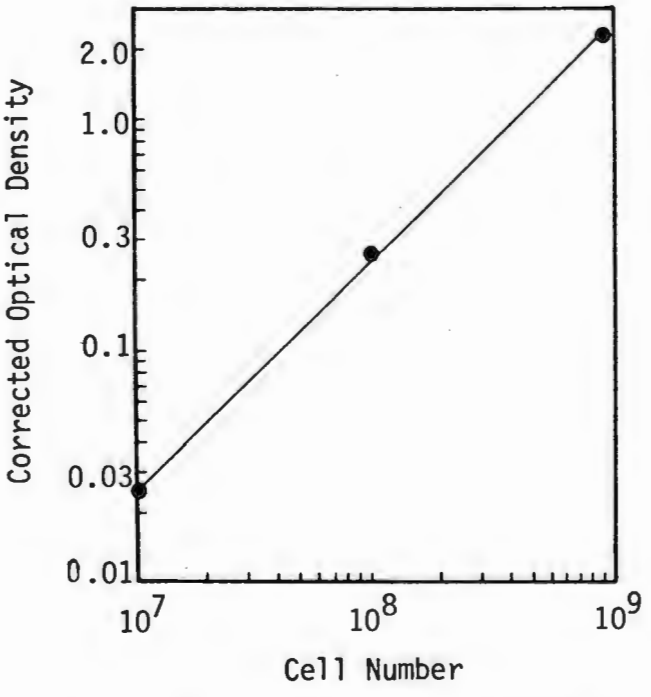


Fig 2.1b Relationship between cell number and optical density.

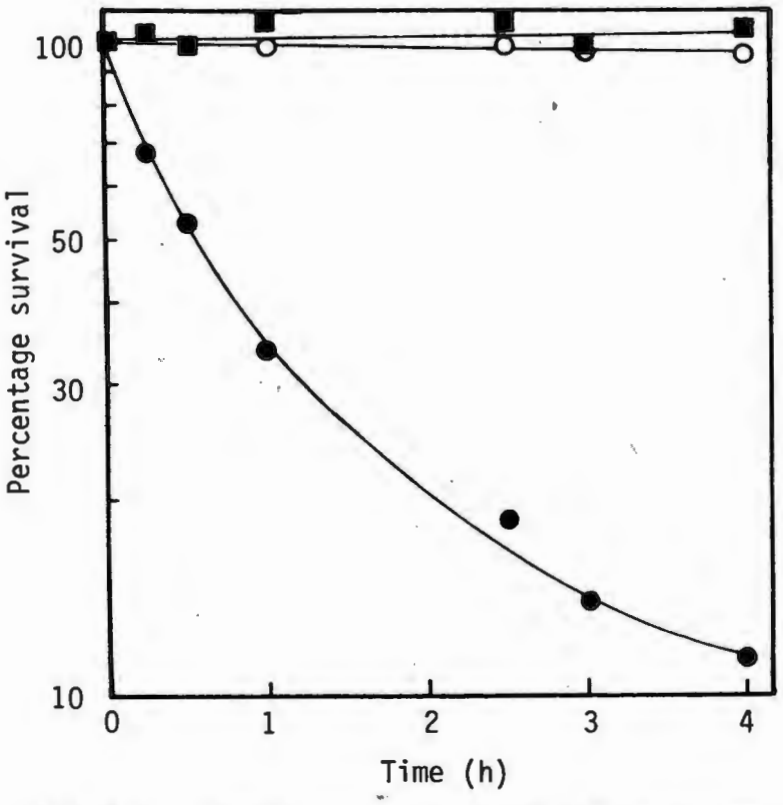


Fig 2.2 Inactivation of phage α3a by chloroform (●) and toluene (■). Control with no additions (o). Temperature 25°C.

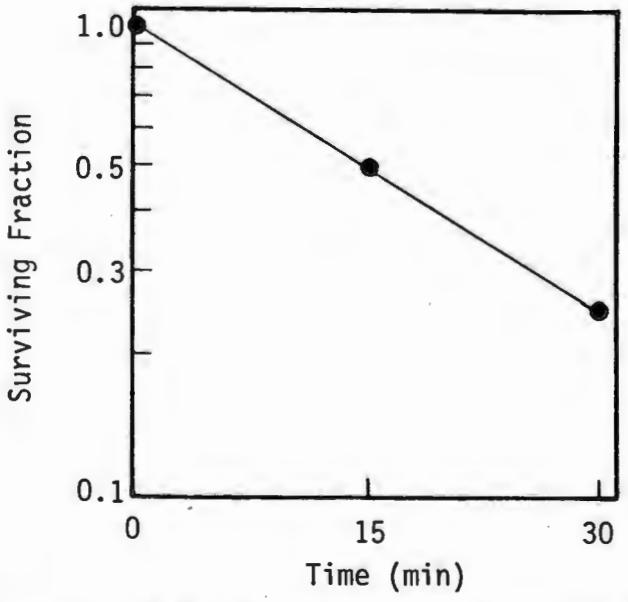


Fig 2.3 Neutralization of phage α3a by antiserum which was diluted 10^{-3}.

constant k, was 160.49 min^{-1}. The same batch of antiserum was used throughout the investigation.

2.3.4 Phage Adsorption

Although the experimental conditions were kept constant, variable phage adsorption rates were obtained for phage α3a. The velocity constants (k) for adsorption to w.t. Achromobacter cells varied from 2.8×10^{-10} to 2.6×10^{-11} ml min^{-1} in exponential cultures and from 2.03×10^{-10} to 4×10^{-12} ml min^{-1} in stationary phase cultures in different experiments. In a comparative experiment the velocity constants measured over 30 min were: 2.6×10^{-11}, 8×10^{-12}, 6×10^{-12} and 9×10^{-13} ml min^{-1} for exponential w.t. cultures, stationary phase w.t. cultures, exponential strain 14 cultures and stationary phase 14 cultures respectively. All the cultures showed an initial fast rate of adsorption followed by a slower adsorption rate (Fig. 2.4).

Wild-type cells adsorbed phage more rapidly than did strain 14 cells and exponential phase cells adsorbed phage more rapidly than stationary phase cells. The percentages of phage adsorbed over a 2 h period were: 73%, 62%, 35% and 14% for exponential w.t. cultures, stationary phase w.t. cultures, exponential strain 14 cultures and stationary phase strain 14 cultures respectively.

This decrease in adsorption was correlated with the onset of late exponential growth phase in w.t. cultures (Fig. 2.5) and α3a adsorption remained relatively constant during stationary phase.

Phage α3a adsorption to exponential Achromobacter w.t. cells was enhanced when cultures were pre-grown in the presence of 0.01M $MgSO_4$. The addition of Mg^{2+} increased adsorption from 40 to 65.5% (phage adsorbed in 30 min). This concentration of Mg^{2+} had no effect on

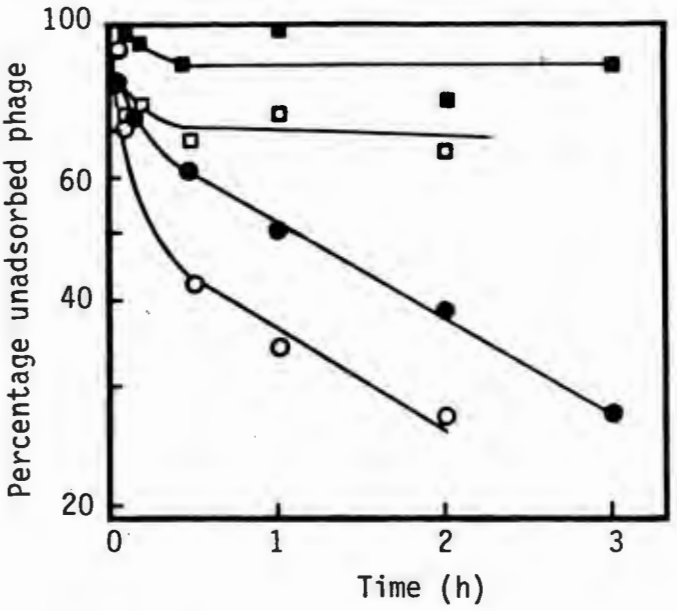


Fig. 2.4 Phage α3a adsorbtion to Achromobacter w.t. cells in exponential (o) and stationary phase (●) and to Achromobacter strain 14 cells in exponential (□) and stationary phase (■).

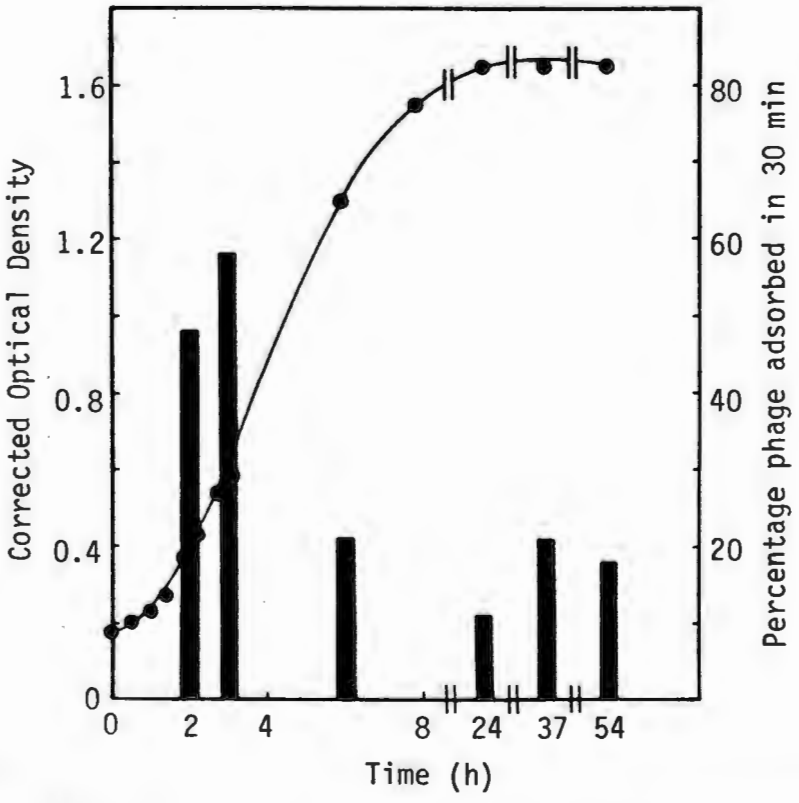


Fig. 2.5 Phage α3a adsorption to Achromobacter w.t. cells at different stages in the growth cycle. (●) corrected optical density at 600 nm.

bacterial growth rate.

2.3.5 Identity of the α3a adsorption site

Achromobacter mutants resistant to TFL were obtained from different mutagenized cultures. The growth responses of the w.t. and mutant strains to TFL are shown in Plate 2.1. The radial streaks show varying degrees of growth inhibition of Achromobacter w.t. and mutant strains 1, 5 and 6. Strains 2, 3 and 4 were not inhibited by TFL.

The transport activities of the leucine specific and the leucine/isoleucine systems in Achromobacter w.t. and TFL resistant strains are shown in Table 2.1. (Complete kinetic data showing the existence of two leucine transport systems is presented in 4.3.6).

Mutant strains 3 and 4 possessed the lowest levels of both transport systems and did not support phage growth. Intermediate mutational events produced strains 5 and 6 which retained some leucine transport activity and were semi-sensitive to phage.

Table 2.2 shows that the block in phage development in strains 3 and 4 was due to the inability of those strains to adsorb phage.

Leucine (0.5 mg ml^{-1}) had no effect on adsorption of phage to w.t. cells which had been grown in minimal medium with or without leucine (50 μg ml^{-1}).

2.3.6 Plaque type and e.o.p. on w.t. backgrounds

The plaques which appeared on Achromobacter w.t. lawns after overnight incubation varied in size from pinpricks to 2 mm diameter. After 3 - 4 days incubation the plaque size ranged from 0.5 to 4 mm. As well as increasing in size the number of plaques increased after the first day's incubation. These increases in size and number of plaques were inconsistent and did not always occur.

Table 2.1 Leucine transport by wild-type and trifluoroleucine strains, and phage resistance characteristic of the strains.

	Leucine specific		Leucine/Isoleucine		Phage growth
	cpm	%	cpm	%	
Wild type	360	100	1072	100	++
1	24	7	1043	97	-
2	0	0	450	42	-
3	0	0	0	0	-
4	60	17	40	4	-
5	100	28	10	1	+-
6	106	28	860	83	+

Table 2.2 Adsorption of bacteriophage α3a to wild-type and leucine transport defective strains of Achromobacter.

Strain	P/Po	% Adsorption
Wild-type	0.36	100
Strain 3	1.04	0
Strain 4	1.01	0

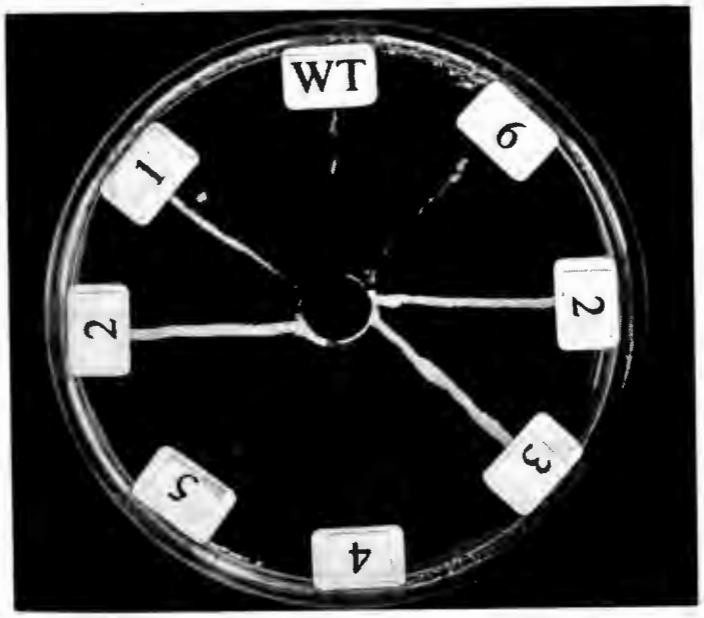


Plate 2.1. Inhibition of growth of Achromobacter w.t. and mutant strains by trifluoroleucine.

To determine whether this was due to a phage adsorption phenomenon, the plaque type and time of appearance of an untreated phage preparation was compared with:

(i) the plating of already infected cells, and

(ii) the plating of a phage fraction which remained after 95 - 97% of the phage had been adsorbed (i.e. phage which were likely to be poor adsorbers) (Table 2.3).

The results show that variation in plaque size and the time of development appeared to result from variable phage adsorption. When infected cells were plated, the plaques were more uniform in size and the increase in numbers that was observed with continued incubation was smaller. In contrast, greater variability resulted when an unadsorbed phage population was plated.

Plaque type also varied on Achromobacter w.t. lawns. In the majority of experiments plaques were semi-turbid (Plate 2.2) but occasionally clear or turbid plaques appeared although no alterations in media, growth of indicator strain, or plating technique were made. Representatives of the different plaque types were stabbed, suspended in tris-HCl buffer and reassayed. The plaque types were not heritable, indicating that the micro-environment affected plaque formation.

Concentrations of Mg^{2+} exceeding 0.016 mg ℓ^{-1} (determined by atomic adsorption spectroscopy) were required for consistent plaque formation. Plaques formed on indicator cultures pre-grown with a Mg^{2+} concentration of less than 0.016 mg ℓ^{-1} were extremely faint and uniformly turbid. Normal plaque formation was restored when the background culture was pre-grown in the presence of Mg^{2+}. Addition of 0.01M $MgSO_4$ to the sloppy agar did not result in normal plaque development, indicating the importance of growing the cultures in liquid medium containg Mg^{2+}

Table 2.3 Size and appearance of α3a plaques on Achromobacter w.t. lawns.

Phage plated	Plaque count after overnight incubation	Plaque size after overnight incubation	No. of extra plaques after 2 - 3 days incubation	% Increase
Untreated phage preparation	369	Variable <0.5 mm - 2 mm	7	1.9
	405		8	2.0
	343		23	6.7
	341		27	8.0
	352		32	9.0
	362		28	7.7
	410		17	4.1
	340		8	2.3
	290		44	15.1
Infected cells	167	Uniform c. 2mm	0	0
	180		0	0
	240		8	3.3
	224		2	0.9
	240		4	1.6
Free phage fraction (remaining after adsorption of 95% of phage population)	55	Very variable <0.5 mm - 1.5 mm (Majority less than 1 mm)	20	36.4
	49		15	30.6
	23		22	95.6
	176		74	42.0
	166		79	47.6
	196		46	23.5

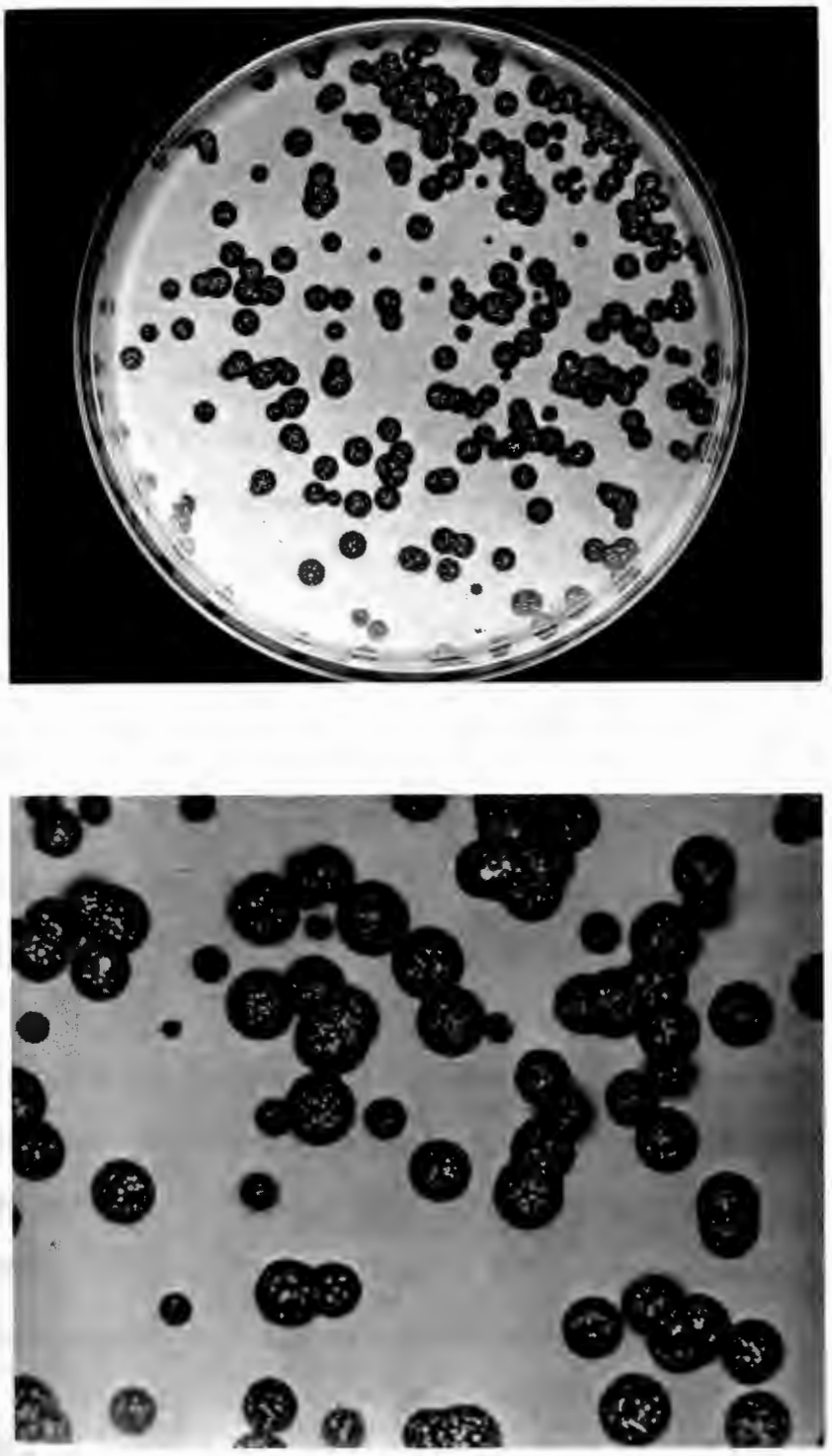


Plate 2.2. Semi-turbid plaques of phage α3a on Achromobacter w.t. lawns.

at a sufficient concentration.

Although plaque type and number varied after overnight development, there was no variation in the final e.o.p. when Achromobacter w.t. lawns from the same or different clones were used for phage assays.

2.3.7 Plaque type and e.o.p. on strain 14 backgrounds

The very few plaques that were present on Achromobacter strain 14 lawns after overnight incubation were small (c. 1 mm), irregular and only just visible (Table 2.4). After a further 2 to 3 days incubation, new plaques appeared in great excess of those observed after overnight incubation. These were large (2 to 3 mm) and clear with irregular outlines. An unusual feature of the plaque development was that plaques were concentrated around the periphery of the plate with very few in the centre (Plate 2.3).

The e.o.p. of phage α3a on backgrounds of strain 14 (clones obtained from three stock cultures) was poor and variable, ranging from 3.3×10^{-2} to 1×10^{-4} relative to plating on w.t. backgrounds (Table 2.4). The number of plaques appearing after overnight incubation relative to the total number recorded after 2-3 days was also variable and has been expressed as a percentage of the total plaques which developed. No correlation between this percentage and the final e.o.p. could be found. Similar variations in e.o.p. were observed when a number of backgrounds from a single culture were used.

2.3.8 Phage growth in exponential cultures

Phage α3a growth was measured in two exponential cultures of Achromobacter w.t. and in duplicate cultures of strain 14 from two different stocks (a and b).

Phage were adsorbed to w.t., strain 14a and strain 14b cultures to a similar extent (95%, 84% and 85% respectively, measured after 1 h.

Table 2.4 The e.o.p. of phage α3a on Achromobacter strain 14 backgrounds.

Clone	STOCK CULTURE 1		STOCK CULTURE 2		STOCK CULTURE 3	
	e.o.p.*	Overnight$^{\phi}$ plaques - % of Total	e.o.p.	Overnight plaques - % of Total	e.o.p.	Overnight plaques - % of Total
1	2.6×10^{-3}	0	2.6×10^{-3}	2.2	3.3×10^{-3}	3.0
2	3.0×10^{-3}	0.33	3.3×10^{-3}	2.0	2.6×10^{-3}	0.9
3	5.6×10^{-3}	1.05	3.4×10^{-3}	3.0	1.0×10^{-4}	16.6
4	6.6×10^{-3}	1.0	1.3×10^{-3}	0.25	1.0×10^{-4}	0.25
5	1.6×10^{-2}	1.0	2.3×10^{-2}	2.8	1.3×10^{-3}	5.6
6	1.6×10^{-2}	1.0	1.5×10^{-2}	0.35	1.0×10^{-3}	2.0
7	3.3×10^{-3}	1.1	6.6×10^{-3}	1.0	6.6×10^{-4}	8.5
8	6.6×10^{-3}	2.5	4.3×10^{-3}	2.3	2.0×10^{-3}	0.16
9	5.0×10^{-3}	13.3	7.6×10^{-3}	1.3	2.3×10^{-3}	0.14
10	1.5×10^{-3}	4.0	3.3×10^{-2}	0.2		

* e.o.p. of α3a after 2 - 3 days incubation relative to plating on Achromobacter w.t.

ϕ The number of plaques which appeared after overnight incubation expressed as a % of the total number of plaques after 2 - 3 days incubation.

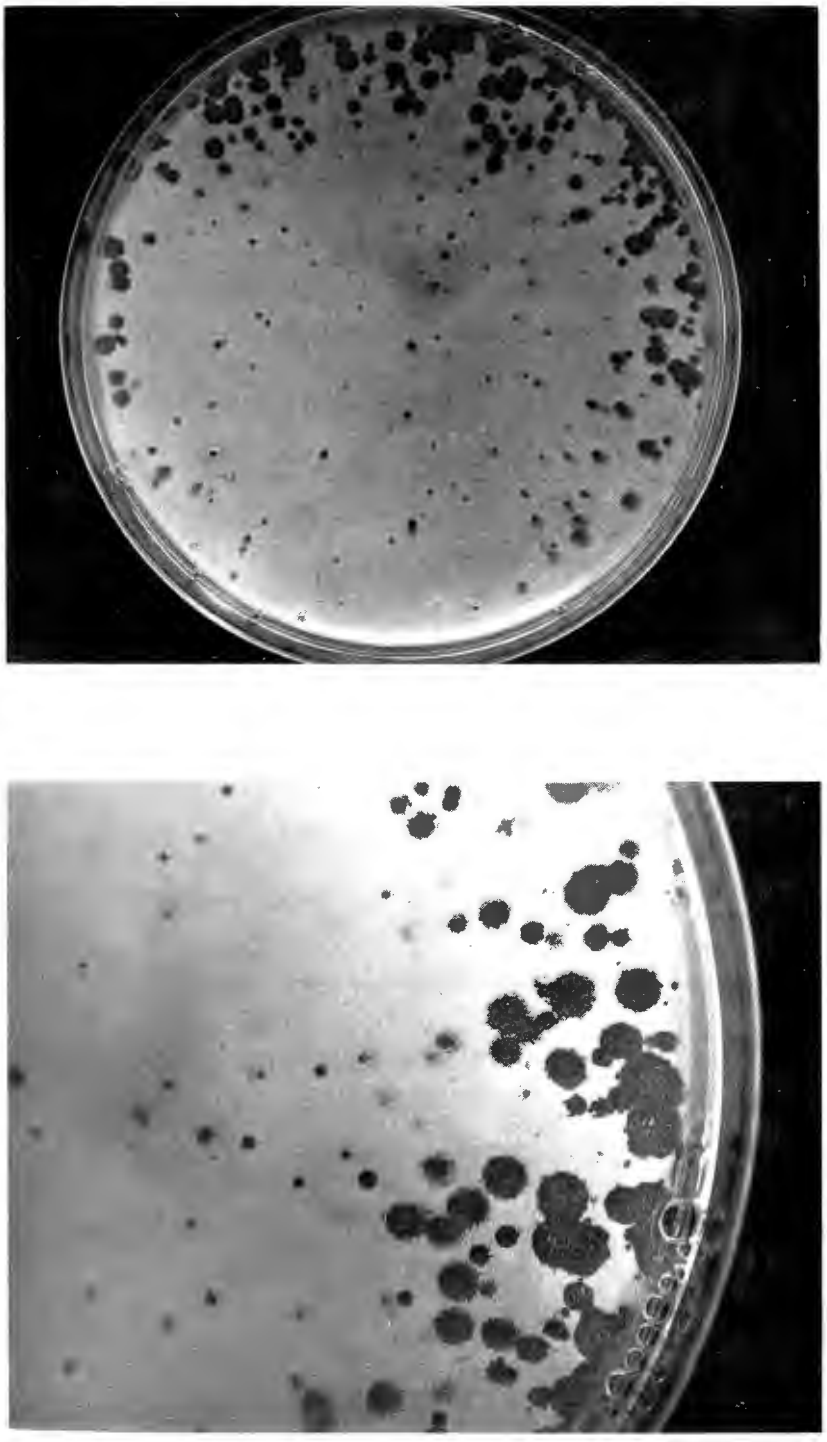


Plate 2.3. Irregular plaques of phage α3a on Achromobacter strain 14 lawns.

Table 2.5 Phage α3a development in exponential cultures of Achromobacter w.t. and strain 14.

Culture	Fold increase in p.f.u. in 5 h
w.t.	9.6×10^1
	1.1×10^2
14a	0.5
	0.25
14b	0.31
	0.1

In the 5 h period after phage addition increases of 1.1×10^2 and 9.6×10^1 p.f.u. ml^{-1} were obtained in w.t. cultures (Table 2.5).

No phage increase was observed in strain 14 cultures. After 5 h plaque assays showed a net loss in total p.f.u. ml^{-1} ranging from 50 to 90% of the input.

2.3.9 Phage growth in stationary phase cultures

The degree of aeration affected phage development. Table 2.6a shows the effect increasing culture volume on subsequent phage development in stationary phase cultures. Under conditions of maximum aeration (Exp. no. 1 and 8 for strain 14 and w.t. cultures respectively) there was a decrease in free phage after 24 h. As aeration decreased phage development after 24 h increased (Exp. no. 2 and 9). The 30, 40 and 50 ml cultures in flasks and the 10 ml cultures in standard containers showed comparable increases in free phage ranging from 4.5×10^2 to 1×10^4 p.f.u. ml^{-1}. This suggested that in all these cultures favourable phage growth conditions had developed.

The degree of aeration prior to standing the stationary phase cultures affected subsequent phage development. Phage growth in standard containers showed increases of only 2.6 and 3.5 fold in w.t. and strain 14 cultures respectively if the cultures were vigorously aerated prior to standing (Exp. no. 7 and 14). In contrast, phage increases of 1.4×10^3 and 2.8×10^3 fold in w.t. and strain 14 cultures respectively, were obtained if poor aeration was supplied prior to standing (Exp. no. 6 and 13).

No phage increases were observed in any of the shaking cultures (Exp. no. 15 and 16). It should be noted that although phage growth did not occur, 74 and 99.9% of the free phage were adsorbed (24 h after phage addition) in strain 14 and w.t. shaking cultures, respectively.

Table 2.6b shows that allowing the cultures to stand without

Table 2.6a The effect of aeration on phage α3a growth in Achromobacter stationary phase cultures. Cultures were aerated for 2 days and α3a (1×10^4 p.f.u. ml^{-1}) was added when cultures were removed from aeration. The culture containers were 100 ml flasks (F) or standard containers (SC) (28 ml screw top containers).

Culture	Experiment No.	Treatment		Fold increase in free p.f.u. ml^{-1}	
		Aeration volume (ml) and container	Standing volume and container	Time after phage addition	
				24 h	48 h
Strain 14	1	20 F	10 F	0.26	
	2	40 "	20 "	4.0×10^1	
	3	60 "	30 "	1.0×10^4	
	4	80 "	40 "	1.0×10^3	
	5	100 "	50 "	4.5×10^2	
	6	10 SC	10 SC	1.4×10^3	
	7	20 F	10 SC	2.6	1.4×10^3
w.t.	8	20 F	10 F	0.01	
	9	40 "	20 "	2.6×10^2	
	10	60 "	30 "	5.5×10^2	
	11	80 "	40 "	1.5×10^3	
	12	100 "	50 "	8×10^2	
	13	10 SC	10 SC	2.8×10^3	
	14	20 F	10 SC	3.5	8×10^2
Strain 14	15	10-100 F	Aeration continued	av.0.26	
w.t.	16	10-100 F	Aeration continued	av.0.01	

Table 2.6b Stimulation of phage growth in Achromobacter strain 14 by preincubation of stationary phase cultures without aeration prior to phage addition. Cultures were aerated for 2 days in 100 ml flasks. Samples (10 ml) were dispensed into standard containers and allowed to stand without aeration for various times before phage addition.

Culture	Treatment		Fold increase in free p.f.u. after 24 h
	Aeration volume (ml)	Time of standing (days) before phage addition	
Strain 14	60	0	0.55
		1	4.5×10^3
		2	5.5×10^3
	20	0	0.26
		1	1.3×10^1
		2	2.5×10^3

aeration before phage addition resulted in good phage development (increases of c. 3 x 10^3 p.f.u. ml^{-1}) in 24 h. Achromobacter strain 14 cultures which were 60% of the flask volume during the shaking period required only 1 day of standing in a standard container to attain a physiological state that favoured phage growth. Cultures which had been vigorously aerated (20% of flask volume) required 2 days of standing to produce comparable phage increases. Apparently cultures had to be preconditioned under poor aeration conditions for efficient phage development.

2.3.10 Standard method for phage development in stationary phase cells

In spite of the development of a reliable routine method for α3a growth in stationary phase cells, identical cultures showed considerable variation in phage yield. Fig. 2.6a shows the relative increase in total p.f.u. 12, 18 and 24 hours after phage addition to identical stationary phase strain 14 cultures and illustrates this variability.

Since phage development was affected by the aeration conditions, the effect of tight or loose caps during phage development in stationary phase cultures in standard containers was examined (Fig. 2.6b). There was no marked effect.

Above a concentration of 0.016 mg ℓ^{-1} $MgSO_4$ the presence of 0.01 mM or 0.1 mM $MgSO_4$ in stationary phase cultures did not appear to affect phage yields.

2.3.11 Screening method for bacterial sensitivity to phage

Colonies which were sprayed with a 5 x 10^6 p.f.u. ml^{-1} phage suspension at 8 h were examined 16 and 48 h after spraying. After 16 h w.t. colonies were severely nibbled at the edges of the colony and the periphery developed an opalescent appearance. Very little nibbling occurred with strain 14 colonies. After 48 h, Achromobacter

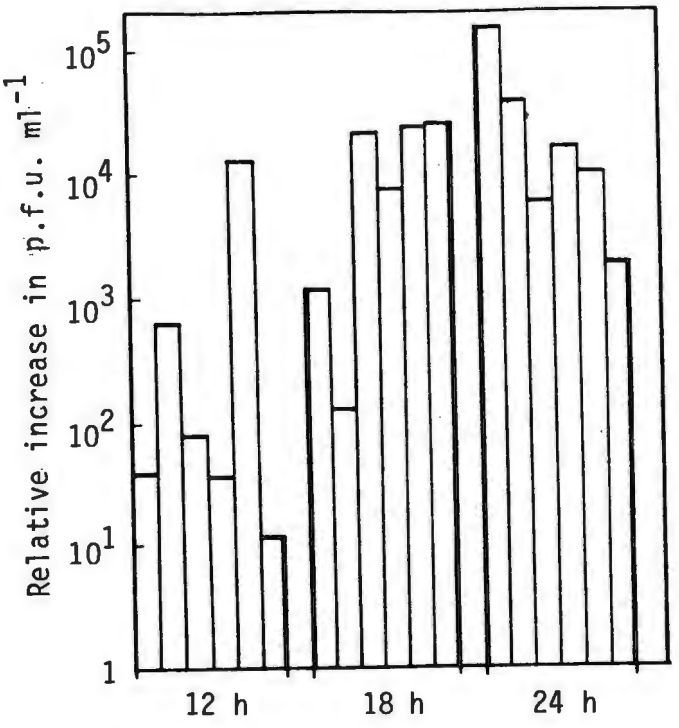


Fig 2.6a Phage increases recorded for individual cultures at different times after phage addition. (Each bar represents a separate Achromobacter strain 14 culture.)

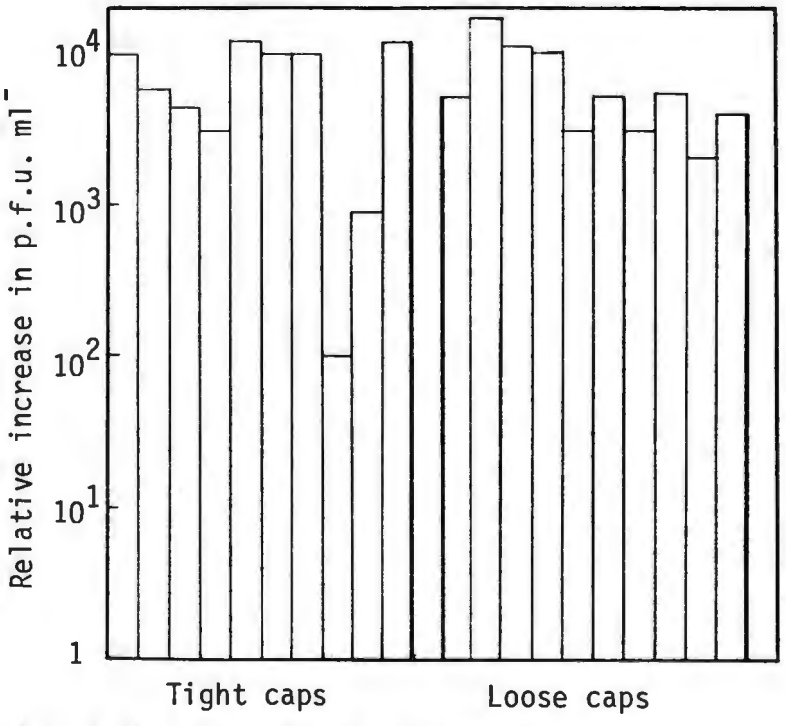


Fig 2.6b The effect of tight and loose caps on phage α3a development in stationary phase standing cultures. (Each bar represents a separate culture).

w.t. colonies had larger areas of lysis extending inwards from the periphery. The strain 14 colonies developed plaque-like pits in the centre of the colonies (stationary cells). In contrast with the Achromobacter w.t. colonies there was only a limited amount of nibbling at the edges (growing cells).

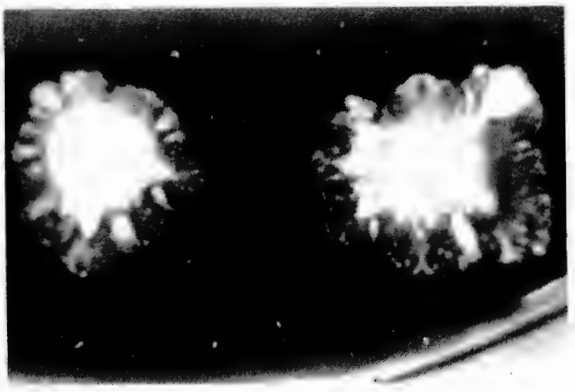
Older colonies (w.t. and strain 14) which were sprayed at 48 h were examined 21 h and 54 h after spraying. At 21 h colonies showed peripheral lysis which increased after 54 h. Central pitting was visible after 54 h (Plate no. 2.4). The appearance of central pitting correlated well with the ability to support phage growth in liquid stationary phase cultures (Table 2.7) but exceptions (e.g. Clone A4 and Clone C2) did occur.

2.4 DISCUSSION

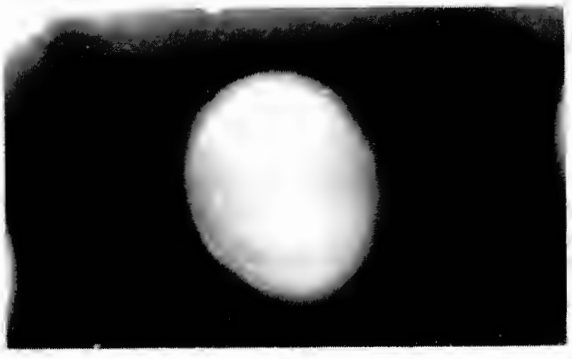
Unpurified lysates of α3a were unstable. Purified phage preparations were inactivated by chloroform but were unaffected by toluene. Although most bacteriophages are insensitive to chloroform, some B. subtilis phages (Brodetsky and Romig, 1965) and B. megaterium phages ϕT (Hendry and Fitz-James, 1973) and CK-1 (Cassity and Kolodziej, 1979) have been found to be inactivated by chloroform. It is of interest that ϕT and CK-1 resemble α3a morphologically, both possessing iscosahedral heads and long non-contractile tails.

Toluene was found to be suitable for the sterilization of phage preparations and for the inactivation of host-phage complexes for α3a adsorption studies.

Many structures exposed on the bacterial cell surface can act as specific receptor sites for phage (Lindberg, 1973). T3, T4 and T7 phages



(a)



(b)

Plate 2.4. Lysis of single Achromobacter colonies by phage α3a. (a) Sensitive strain 14 colonies 54 h after spraying with phage. (b) Resistant colony 54 h after spraying with phage.

Table 2.7 The relationship between e.o.p., the appearance of central plaques after spraying 48 h colonies with a phage suspension, and increases in phage titres in stationary phase liquid cultures. Individual clones from 3 Achromobacter strain 14 stock cultures 1, 2 and 3 were used.

Stock culture	Clone number	e.o.p.*	Central$^{\phi}$ plaques	Increase in p.f.u. in 24 h
1	1	8×10^{-3}	+	2.5×10^{4}
	2	1.6×10^{-2}	+	4×10^{3}
	3	Resistant	-	-
	4	2.6×10^{-3}	+	-
2	1	6.6×10^{-3}	+	2×10^{4}
	2	1.6×10^{-2}	+	2×10^{3}
	3	Resistant	-	-
	4	3.3×10^{-2}	+	3.5×10^{4}
3	1	2.6×10^{-3}	+	2.3
	2	6.6×10^{-3}	+	-
	3	1.5×10^{-2}	+	3×10^{4}
	4	1.6×10^{-2}	+	4×10^{4}

*e.o.p. after 2 - 3 days incubation

$^{\phi}$central plaques 54 h after spraying

adsorb to lipopolysaccharides whereas T6 and T2 adsorb to lipoproteins (Weidel, 1958). Bacterial appendages such as sex pili serve as receptors for filamentous 1 DNA phages (Marvin and Hohn, 1969) and 1 RNA phages (Bradley, 1972; Crawford and Gesteland, 1964). Recruitment of membrane transport receptors into the process of bacteriophage adsorption is well known. Mutations inhibiting λ adsorption to E. coli map in one cistron, lam B (Thirion and Hofnung, 1972). The lam B gene, which is believed to be the structural gene for the λ receptor is located in one of the operons (mal B) concerned with the maltose permease system (Hofnung, 1974; Hofnung, Hatfield and Schwartz, 1974). Another example is the ton B gene of E. coli which governs the phage T1 receptor site and the transport of iron (Wang and Newton, 1969).

The adsorption of α3a to Achromobacter cells is apparently correlated to the leucine uptake system, since mutants which have lost the ability to transport leucine become totally resistant to α3a infection and are also unable to adsorb phage. Revertants of these mutants are being isolated and characterized. This is the first report to implicate an amino acid transport system in phage adsorption.

The variations in α3a adsorption which were observed could be due to the extreme sensitivity of phage development to the Mg^{2+} concentration. The requirement for monovalent or divalent cations in different phage-host interactions varies considerably (Stent, 1963). Cations often promote the initial attachment of phage to the host cell and are probably required to neutralize the negative surface charges of the phage and bacterial cell (Garen and Puck, 1951). The ionic requirements for injection of phage nucleic acid are very specific. Calcium ions are required by T5 (Lanni, 1968), φμ4 (Shafia and

Thomson, 1964), SP5, SP6, SP7, SP8 and SP13 (Brodetsky and Romig, 1965) for penetration of phage DNA and Mg^{2+} for λ DNA injection (MacKay and Bode, 1976a and b). Zinc ions, although they promote the initial attachment of T1 phage to E. coli (as do Mg^{2+} and Ca^{2+}), inhibit the second irreversible adsorption step (Garen and Puck, 1951).

Hershey et al. (1943) studied the effect of electrolytes on phage P9H plaque morphology. Extremely small plaques were formed in the absence of Na^{+}, Ca^{2+} depressed the size and number of plaques and Li^{2+} increased the plaque size. Plaque formation by $\phi\mu 4$ was totally inhibited in the absence of Ca^{2+} (Shafia and Thompson, 1964).

Magnesium ions may play a role in the initial attachment of phage α3a to the host cell since they do increase the rate of adsorption. However, it is likely that Mg^{2+} is required at a post adsorption step (possibly DNA injection) because:

(i) plaque formation is inhibited at Mg^{2+} concentrations of < 0.016 mg ℓ^{-1};

(ii) this inhibition is not alleviated by adding Mg^{2+} at the time of plating but Mg^{2+} must interact with the host cells during growth prior to plating with phage. This suggests that Mg^{2+} is not merely required to neutralize the electrostatic negative charges of the phage and host cell.

Plaque size and morphology are usually characteristic of particular phage-bacterium combinations (Adams, 1959). In systems where bacteriophage adsorption is slow, a single plate will show plaques of varying size since phage particles which adsorb late in the development of the bacterial lawn will develop into small plaques or fail to develop (Sajik, 1954; Hendry and Fitz-James, 1973).

The variable plaque size on Achromobacter w.t. lawns can also be attributed to adsorption of phage since plating of already infected w.t. cells produced plaques which were uniform in size. However, increased variation in plaque size was observed when 95% of a phage population were adsorbed to w.t. cells and the remaining 5% of phage which were unadsorbed were plated. This 5% probably represented a class of phage which adsorbed poorly to host cells and therefore the plaque size showed increased variability. Schlesinger (1965) observed that bacteriophage populations were composed of several classes of widely varying absorbabilities. Consistent with this, Roa and Scandella (1976) found that there was heterogeneity in the λ phage population in terms of DNA injection. Some phages eject all or most of their DNA and others none or little.

Plaque development does not continue indefinitely since it is dependent on the active metabolism of the host and plaques usually reach a maximum size after 8 - 12 h (Adams, 1959). The Achromobacter-α3a phage system is unique since plaque development continues for 3-5 days on w.t. backgrounds and large increases in plaque size are observed. This implies that phage which adsorb late in exponential phase of the background lawn can still produce plaques as they are able to develop on stationary phase lawns.

Adsorption of α3a to Achromobacter strain 14 was consistently not as rapid as adsorption to Achromobacter w.t. Thomson and Woods (1974) showed that Achromobacter sp. 2 (w.t.) was a cryptic lysogen which did not release phage spontaneously nor was it resistant to superinfection. By superinfection with homologous phage, double lysogens were obtained (Woods and Thomson, 1975) which did not adsorb phage (lysogenic conversion) and liberated phage spontaneously. Thus, the introduction of additional phage DNA complemented the resident phage DNA, immunity

of the lysogen was restored and all the genes essential for phage production became available. Achromobacter strain 14 was a transductant and therefore it is possible that with the introduction of additional phage DNA through the transduction event (see 1.1), "partial" lysogenic conversion conferring resistance to phage adsorption may have occurred. Lysogeny in some systems is known to have an effect on phage adsorption. Salmonella group E_1 strains which were lysogenic for phage ε15 or ε34 lost the ability to adsorp phage (Uetake et al., 1955; Barksdale and Arden, 1974). Palva (1979) analysed changes in the E. coli outer membrane caused through lysogenic conversion by lambdoid phage PA-2. Lysogenization by PA-2 led to the production of a new outer membrane protein, protein 2. Protein 2, probably phage coded, partially replaced protein 1, a major outer membrane protein which forms pores for small hydrophilic molecules and is the receptor for PA-2. This immunity is distinct from superinfection immunity which acts intracellularly as a result of phage coded repressor production (Ptashne, 1967). For example Shigella dysenteriae lysogenic for phage P2, adsorbs but is resistant to lysis by phages P1, T2, T4, T5 and T6. Such lysogens are however sensitive to T1, T3 and T7, illustrating the specificity of repression (Bertani, 1953). Because α3a double lysogens are subject to lysogenic conversion by the phages it is not known whether they produce repressor. Since they are stable and can be induced, it is postulated that they are under repressor control (J. Thomson, Ph.D. thesis).

Phage α3a adsorption was also affected by the age of the host culture. Exponential phase Achromobacter w.t. and strain 14 adsorbed phage more readily than did stationary phase cells. Similar results were reported by Delbrück (1940a) and Shafia and Thomson (1964) for

other phage systems. This implies that the inhibition of phage development in exponential phase strain 14 occurs after the initial stages of infection are completed. It is of interest to note that assays for total phage in exponential strain 14 cultures show a net decrease of 50 - 90% of phage input after 5 h. It seems likely that phage are being adsorbed but are not being expressed. The appearance of a few faint plaques on strain 14 lawns after overnight incubation suggest that a very small proportion of the phage added (c. 0.01%) are able to propagate, albeit inefficiently on exponential strain 14 cells, or that there are areas of the plate where cells reach stationary phase early. However, no phage increases are observed in exponential liquid cultures. Approximately 1% of phage added produce plaques (e.o.p. 10^{-2}) on the stationary phase lawns, i.e. 2 - 3 days of incubation. The reduced e.o.p. may at least in part be attributed to poor phage adsorption (Woods, 1976).

The e.o.p. of α3a varied as much as 5×10^{1} on lawns from different strain 14 clones and on a number of plates using the same clone. This is an unusual feature and is consistent with the suggestion of Woods (1976) that a condition, other than the constituents of the medium, namely the limited exchange of gases is a prerequisite for phage development in stationary phase Achromobacter cells.

The study of α3a development in Achromobacter stationary phase cultures was initially extremely difficult since phage growth required stringent conditions of aeration before and after phage addition. Variable conditions produced inconsistent phage development. These problems have largely been overcome and a standard procedure for obtaining optimum conditions has been developed.

In Achromobacter w.t. and strain 14 cultures, optimal phage growth conditions developed in non-aerated (standing) cultures of a specific

surface area : volume ratio which afforded limited interchange of gases. No increases in p.f.u. were observed in cultures which were aerated by shaking. However, there was a net decrease in free phage indicating that the phage were being adsorbed. Consistent with the data obtained for the phage adsorption rates, this decrease was far greater in Achromobacter w.t. cultures than in Achromobacter strain 14 cultures.

It appears that there is a specific bacterial state induced under limited aeration of Achromobacter stationary phase cells. This state is a prerequisite for phage development since cultures must stand for at least 24 h before phage addition for optimal phage growth. Increased aeration prior to standing caused a delay in phage development, and vigorously aerated cultures required a longer standing period prior to phage addition to achieve optimal phage growth.

Although growth conditions were optimal, phage yields still showed considerable variation which was not affected by using tight or loose caps on the standing cultures or by varying the Mg^{2+} concentration, providing it was above 0.016 mg ℓ^{-1}.

Woods (1976) did not report α3a growth on stationary phase Achromobacter w.t. cells but it has been found that phage development can occur. As found with strain 14, phage development in stationary phase Achromobacter w.t. is subject to control by the aeration conditions before and after phage addition. Phage propagation requires that the bacteria are in a specific state which is induced under limited aeration. Variants of the w.t. strain which do not support phage growth in stationary phase do occur and one such variant has been used for experiments described in 6.2.

CHAPTER III

THE KINETICS OF PHAGE DEVELOPMENT IN STATIONARY PHASE ACHROMOBACTER

3.1 INTRODUCTION

The kinetics of phage α3a development in stationary phase Achromobacter cultures have been determined since prolonged productive phage development into stationary phase is an unusual feature and has not been previously reported. Furthermore the study of this phage development system may be useful in probing aspects of stationary phase metabolism.

Adsorption of bacteriophage to a host cell is followed by an eclipse period (Doermann, 1953) during which no mature phage can be found in artificially lysed cells. The minimum time period between phage adsorption and lysis of the first cells in the culture with release of phage progeny is the latent period. This time interval and the average burst size (the mean yield of phage particles per infected bacterium) can be measured by a single step growth experiment (Ellis and Delbrück, 1939).

The duration of the latent period and burst size depend on the phage and host strains and also the environmental conditions (Archibald, Elwood and Thomas, 1978). Widely different and distinct latent periods are obtained with different phages growing on the same host and even with closely related phages such as T2, T4 and T6 (Adams, 1959).

The latent period and the burst size are strongly affected by the physiological state of the host cell. Ellis and Delbrück (1939)

showed that the latent period for phage development increased as the division time of the host increased. This is not true of all phage-host systems; Delbrück (reported in Adams, 1959) later showed that the latent periods for phages T1, T2 and T7 were the same in synthetic medium as in broth, although the synthetic medium supported much slower bacterial growth. Furthermore, Delbrück (1940b) in comparing phage development in rapidly dividing cells and in a 24 h culture, reported that the latent period doubled in the latter and the burst size was drastically reduced to an eighth of that in rapidly dividing cells (20 compared to 170). Thus, a decline in bacterial metabolism resulted in a decline in phage development. Hëden (1951) found that the phage yields per cell were maximal at the time of cell division when the cells were largest and the RNA content per cell was highest.

The single burst experiment was used by Delbrück (1945) to study the fluctuation in phage burst sizes from individual host cells. The wide distribution of burst sizes (ranging from below 50 to 1 000) could not be accounted for by variations in the size of the bacteria alone.

Phage genes also affect the burst size. An interesting situation was found in phage P2 (Bertani et al., 1978), where particle assembly was naturally deficient due to the properties of one of the F proteins (tail proteins), with the result that a fraction of the particles remained tailess. A phage mutant (lg) with larger burst sizes and no difference in the latent period corrected this natural shortcoming of P2.

At the end of the latent period lysis of the host cell results in the release of phage progeny. Doermann (1948) discovered that lysis of bacteria infected with a T-even phage could be delayed by superinfection with additional phage. Lysis inhibition resulted in extremely high phage

yields, as many as 1 000 per infected cell. This suggested that lysis, rather than exhaustion of the required components for phage development, interrupts phage growth.

The mechanism of host cell lysis in T4 and λ infected cells appears to be analagous. In T4 infected E. coli, the cell wall is degraded by the muramidase activity of T4 coded lysozyme. Under normal conditions T4 infected bacteria lyse at a characteristic time after infection, each infected cell releasing a few hundred progeny phage. Josslin (1970) found that Su^- E. coli mutants infected with amber t phage mutants failed to lyse and continued to manufacture phage beyond the latent period even though lysozyme was present. A new gene, gene t, was defined as being required for the cessation of phage infected cell metabolism at the characteristic lysis time. The T4 lysis mechanism was shown to be a sequential process of at least two steps. It was proposed that the t gene product degrades the cytoplasmic membrane, metabolism ceases and the lysozyme (coded for by gene e) which is compartmentalised in the cytoplasmic membrane is released, and gains access to its substrate in the host cell wall.

Lysis in λ infected cells is brought about by the action of endolysin which has an endopeptidase activity (Taylor, 1971). Two classes of ts λ mutants deficient in lysis of the host cell were distinguished by Harris et al. (1967). One class mapped in the R cistron and produced thermolabile endolysin or no detectable endolysin. The other class had high levels of endolysin, long latent periods (100 - 150 min) and unusually high phage yields (1 000 phage per cell). Mount et al. (1968) and Reader and Siminovitch (1971a) mapped these mutants in the S cistron which lies adjacent to the R cistron. Its function appeared to alter the cytoplasmic membrane in such a way that intracellular endolysin degraded the cell wall. Reader and Siminovitch (1971b) found that the expression of the S cistron had three effects on the host

cell:

(i) cessation of respiration;

(ii) alteration in the cytoplasmic membrane such that its effectiveness as an osmotic and mechanical barrier was reduced, and

(iii) hydrolysis of phospholipids to free fatty acids.

This is similar to the proposed T4 t gene function (Josslin, 1970) where phage directed alteration of the cytoplasmic membrane prior to the action of lysozyme is a necessary event in lysis. This is supported by the fact that long before lysis cells contain sufficient lysozyme to lyse the cells. Thus, the S and t gene products are positive regulators of host cell lysis by λ and T4 respectively. Unlike T4, lysis by λ is also controlled in a negative manner by an inhibitor of lysis (Campbell and Rolfe, 1975). Evidence suggests that this inhibitor may be the λ rex gene product.

The cessation of phage synthesis and subsequent lysis is not controlled exclusively by the phage but also involves an interaction with a host component. Sphaeroplasts of Aerobacter aerogenes infected with T4 produced an average of 1 000 phage per infected cell compared with 185 in E. coli sphaeroplasts (Wais and Goldberg, 1973). This was due to the failure of infected A. aerognes sphaeroplasts to lyse at the normal time.

The RNA phage MS_2, which is very dependent on host cell synthesis, has been used to probe changes in E. coli regulatory mechanisms from exponential to resting phase cells (Propst, Ricciuti and Haywood, 1972). It was found that viral progeny were produced in stationary phase cells but viruses were not released, viz. there was no cell lysis. Evidence suggested that a short-lived host-controlled protein, essential for bacteriophage release, was not made or activated in stationary phase cells.

The most studied system of phage development in non-growing cells has been in sporulating Bacillus subtilis strains. During sporulation a bacterium undergoes a sequence of biochemical and morphological changes under genetic control. It has been shown that certain bacteriophage-host interactions may serve as probes for the investigation of cell differentiation (Yehle and Doi, 1967; Sonenshein and Roscoe, 1969; Ito et al., 1973; Kawamura and Ito, 1974; Osburne and Sonenshein, 1976). When sporulating Bacillus cells are infected by certain phages, the phage genomes are incorporated (trapped) into the spores without production of mature progeny, whereas phage multiplication and subsequent host lysis takes place in exponentially growing cells. Expression of the incorporated phage genome occurs only after the germination and outgrowth of the spore (Yehle and Doi, 1967; Sonenshein and Roscoe, 1969; Ito et al., 1973).

Sonenshein and Roscoe (1969) infected B. subtilis cultures with the virulent phage ϕe at various times during growth and sporulation and the average burst sizes for each time were determined. The phage yield was highest (c. 60 p.f.u. per cell) in mid-exponential growth phase and then fell to approximately 1 in 8 - 10 h cultures (5 - 7 h after the end of exponential growth). Phage ϕe genomes were maximally trapped when cells were infected 5 - 6 h after the end of exponential growth (Sonenshein and Roscoe, 1969; Yehle and Doi, 1967).

Ito et al. (1973) investigated the growth characteristics of two small B. subtilis phages, ϕ29 and ϕ15 during growth and sporulation of the host cells. Results similar to those observed for phage ϕe infection were obtained. Average burst sizes of about 700 and 300 were obtained in mid-exponential growth for ϕ29 and ϕ15 respectively, but the decline in burst size was far more rapid than for ϕe. Phage ϕ29 could not propagate and lyse host cells 1 h after the end of exponential

growth phase and trapping was maximal at this time (Kawamura and Ito, 1974). The average burst size of temperate phage ϕ105 in B. subtilis fell to less than one, 4 h after the end of exponential growth, indicating that it behaves like the virulent phages of B. subtilis in failing to infect sporulating cells productively.

There is a critical stage during sporulation when DNA replication and transcription of the infecting phage genome cease. These times may not coincide and vary from phage to phage. Phage DNA synthesis in ϕ29 and ϕe infected cells was blocked 1.5 and 3 h after the end of exponential phase, respectively. However, phage specific mRNA continued for a further 0.5 and 1 h in ϕ29 and ϕe infected cells, respectively (Kawamura and Ito, 1974).

The precise reason(s) why the sporulation process interferes differentially with viral gene expression is not understood. Possible mechanisms have been discussed in 6.1.

The Achromobacter - phage α3a interactions represent a system where very old cells support extremely productive phage development. The fact that phage production is totally inhibited in aerated stationary phase cells and in exponential cultures of Achromobacter strain 14 is particularly interesting. Most of the work in this chapter has been devoted to phage development in this strain.

3.2 METHODS

The standard method for bacterial growth and phage development in stationary phase cultures (see 2.2.11) was used, unless otherwise stated.

3.2.1 Age of cultures supporting stationary phase phage growth

Two cultures of Achromobacter strain 14 were grown for 3 days with aeration by shaking (c. 2×10^9 cells ml^{-1}). One culture was dispensed into 10 ml volumes in standard containers which were allowed to stand without aeration for a further 16 days. Phage α3a was added (1×10^4 p.f.u. ml^{-1}) at intervals to different 10 ml cultures and the p.f.u. were assayed after 24 hours. To the second culture, phage α3a was added (1×10^4 p.f.u. ml^{-1}) and aeration was continued. At different time intervals over the subsequent 16 days, 10 ml samples were removed and allowed to stand for 24 h before assaying for phage.

3.2.2 NTG Mutagenesis

Dilution of stationary phase Achromobacter strain 14 infected cells inhibited phage development (see 3.3.5). It was thought that dilution into a stationary phase bacterial mutant which did not adsorb phage may provide the microenvironment suitable for continued phage development.

Achromobacter phage resistant mutants which did not adsorb phage were obtained by NTG mutagenesis. The method outlined in 2.2.7 was employed. NTG was used at a final concentration of 300 μg ml^{-1} and the culture volume used was 10 ml. After removal of the NTG by centrifugation and resuspension in tryptone broth, the culture was incubated for 3 h after which it was divided into two 5 ml amounts. Fresh tryptone broth (5 ml) was added to both cultures and α3a (6×10^8 p.f.u. ml^{-1}) was added to one. After overnight growth appropriate dilutions of the treated cultures were made and spread onto tryptone plates which had been pretreated with phage α3a.

Treatment involved spreading 0.2 ml of phage (6×10^6 p.f.u. ml^{-1}) onto the plates and allowing them to dry for at least 10 min. After 30 h those colonies which showed no visible phage lysis were picked and tested for:

(i) the presence of phage α3a;
(ii) adsorption of phage α3a (see 2.2.6), and
(iii) the ability to support α3a growth (see 2.2.4 and 2.2.11).

3.2.3 Preparation of used old medium

A 3-day-old aerated culture of Achromobacter strain 14 was centrifuged to remove the cells. The supernatant was filtered through a millipore filter (0.45 μm) and stored at 4°C (old medium 1) or was reinnoculated with strain 14. After 2 days of aeration the medium was resterilized (old medium 2). Two further cycles of growth and sterilization were undertaken to exhaust the nutrients in the medium. The ability of the used old media to reinitiate bacterial growth was measured by innoculation with 4-day-old cells.

3.2.4 Phage development in diluted stationary phase cultures

The single step growth experiment of Ellis and Delbrück (1939) relies on large dilutions of the bacterial-phage mixture after a period to allow for phage adsorption. The dilutions prevent further phage adsorption and enable the study of phage development in a known number of infected cells. Phage α3a development was measured in diluted stationary phase cultures.

Phage α3a was added (c. 1×10^4 or c. 1×10^7 p.f.u. ml^{-1}) to Achromobacter stationary phase cultures (10 ml in standard containers) which had been preconditioned for phage growth by

standing without aeration for at least 24 h. At intervals after phage addition the cultures were diluted 10^{-1} and/or 10^{-2} into (i) identical stationary phase cultures of Achromobacter mutants which did not adsorb phage, (ii) into used old medium, (iii) into fresh tryptone broth or (iv) into tris-HCl buffer. The diluted cultures were incubated without aeration and assayed for total phage after 24 and 48 h. An undiluted control culture was also assayed for total phage.

3.2.5 The kinetics of phage development in undiluted stationary phase cultures

Phage α3a was added (c. 1×10^4 p.f.u. ml^{-1}) to 4-day-old preconditioned stationary phase cultures (standing 24 h) in standard containers. Increases in phage titre were monitored by sampling from one culture over a period of time or by sampling at different times from identical cultures which were then discarded.

3.2.6 Reversible phage adsorption

Phage (1×10^8 p.f.u. ml^{-1}) was added to 4-day-old preconditioned standing stationary phase w.t. and strain 14 cultures. After 30 min for phage adsorption the number of free phage was determined (2.2.6). Cultures were then diluted 10^{-3} in tris-HCl buffer and allowed to stand without aeration. Assays for free phage were carried out at the time of dilution and at time intervals over the following 2 h. Reversible adsorption would result in an increase in the free phage titre.

3.2.7 Single burst experiment

Burst sizes of individual cells were determined by a modification of the method of Ellis and Delbrück (1939). To examine the burst sizes of α3a in stationary phase cells, phage α3a (m.o.i. 10^{-5}) was added to

a 4-day-old preconditioned culture (2×10^9 cells ml^{-1}) of Achromobacter strain 14 and reincubated without aeration for 16 h. A sample was diluted 10-fold into α3a antiserum (k = 5) and free phage were inactivated by 7 min incubation at 37°C. After a further 10^{-2} dilution in tris-HCl buffer, samples were plated to obtain the number of infected cells. Further 10^{-4}, 10^{-5} and 10^{-6} dilutions were made into prewarmed tryptone broth or tris-HCl (since the exact titre of phage after 16 h was not known, a wide range of dilutions had to be carried out). Samples of these dilutions (0.5 ml) were then dispensed into tubes and incubated for 18 h. The entire contents of each sample was assayed for p.f.u.

To obtain single bursts in exponential phase w.t. cells, an overnight w.t. culture was diluted 1:20 into tryptone broth and aerated for 2.5 h (2×10^8 cells ml^{-1}) prior to the addition of phage α3a (m.o.i. 0.5). After 15 min for adsorption the free phage were inactivated by antiserum and the infected bacteria were diluted to give less than one infected cell per 0.5 ml sample. The burst sizes were assayed after 3 h incubation.

By determining the number of plates with no plaques (indicating that no infected centres were in those samples), the number of sample tubes which were likely to contain more than one infective centre was calculated using the Poisson formula (Adams, 1959; Ellis and Delbrück, 1939):

$$P(r) = \frac{n^r e^{-n}}{r!}$$

P(r) is the proportion of samples containing r particles when the average number of particles per sample is n. For the single burst method the value of n was as low as possible so that the proportion of

samples containing two or more infected centres was small. The distribution of particles among a number of samples follows this formula if the average number of particles (n) per sample is 10 or less.

3.3 RESULTS

3.3.1 Age of cultures supporting stationary phase phage growth

The ability of standing and shaking stationary phase Achromobacter strain 14 cultures to support phage growth after extremely long incubation periods was determined (Fig. 3.1). After dispensing the 10 ml samples into the standard containers, these standing cultures retained the ability to support phage growth for at least a further 16 days. When phage was added at the time of dispensing, a 7-fold increase in titre was observed after 24 h. When cultures were allowed to stand 1 and 2 days before phage addition, the phage yields after 24 h increased by factors of 3×10^3 and 7×10^4 respectively. Nineteen day old cultures were able to support a 10^3-fold increase in phage titre.

In a second experiment phage α3a was added to 3-day aerated cultures which were continuously aerated for 21 days. Samples (10 ml) from these cultures were removed and assayed for phage after standing for 24 h. These infected cells initially supported phage growth but lost this ability after approximately 13 days (Fig. 3.1). Although shaking cells gradually lost the ability to support phage growth the input phage titre did not decrease after 21 days.

3.3.2 Isolation of phage resistant mutants

The addition of phage α3a to NTG treated cultures during the subsequent growth period was a positive selection for phage resistant

clones. After overnight incubation the titres of the treated cultures with and without added phage were 2×10^6 and 3×10^8 cells ml^{-1} respectively. Thirty clones which showed no lysis on the phage treated plate were tested as described. The phage characteristics of 3 selected clones were compared with Achromobacter w.t. and strain 14 clones (Table 3.1).

Table 3.1 Comparison of phage characteristics of Achromobacter w.t., strain 14 and phage resistant mutants.

Clone	Presence of phage as determined by spot test	% Adsorbed phage in 30 min.	Increase of phage in stationary phase cultures (after 24 h)
Clone 6	-	0	0
" 7	-	14.8	0
" 8	-	11	0
w.t. 1	-	29	$> 10^3$
w.t. 2	-	41	$> 10^3$
st. 14 1	-	35	$> 10^3$
st. 14 2	-	27	$> 10^3$

Phage resistant clone no. 6 was used for further experiments.

3.3.2 Properties of used old medium

Old used medium was prepared for use in studying the phage growth cycle in stationary phase cells. Single step burst experiments require that the infected culture is diluted after phage adsorption to minimise readsorption of phage. Since a stationary phase system was

being studied, a medium was required which simulated the stationary phase culture medium and supported limited bacterial growth.

Old media which had supported one cycle of bacterial growth (old^1) still supported substantial bacterial growth when re-innoculated (Fig. 3.2). Even old^2, old^3 and old^4 media (see 3.2.3) supported limited bacterial growth upon reinnoculation, and although there was no absolute cessation of bacterial growth, they were the most appropriate for the dilution of stationary phase infected cultures.

3.3.4 Phage development in diluted stationary phase cultures

Dilution of phage - bacterial mixtures 30 min after phage addition to stationary phase Achromobacter strain 14 cultures severely inhibited phage production (Fig. 3.3). Increases no greater than 10^1 were obtained after 48 h, whether the dilution was into identical cultures of a mutant which did not adsorb phage (and should simulate the microenvironment of stationary phase cultures) or into used old medium, which did not permit the reinitiation of rapid exponential bacterial growth. Addition of thioglycollate (a reducing agent) to the old medium did not improve phage growth. A phage increase of 1×10^5 p.f.u. ml^{-1} was observed in the undiluted stationary phase culture.

Thus it was impossible to use a dilution system for the determination of the latent period and burst size of α3a in stationary phase Achromobacter strain 14 cells.

3.3.5 Inhibition of phage development by sampling

Phage α3a was added to three identical 10 ml stationary phase cultures of Achromobacter strain 14 and incubation without aeration was continued. The growth of phage was monitored in each of the three

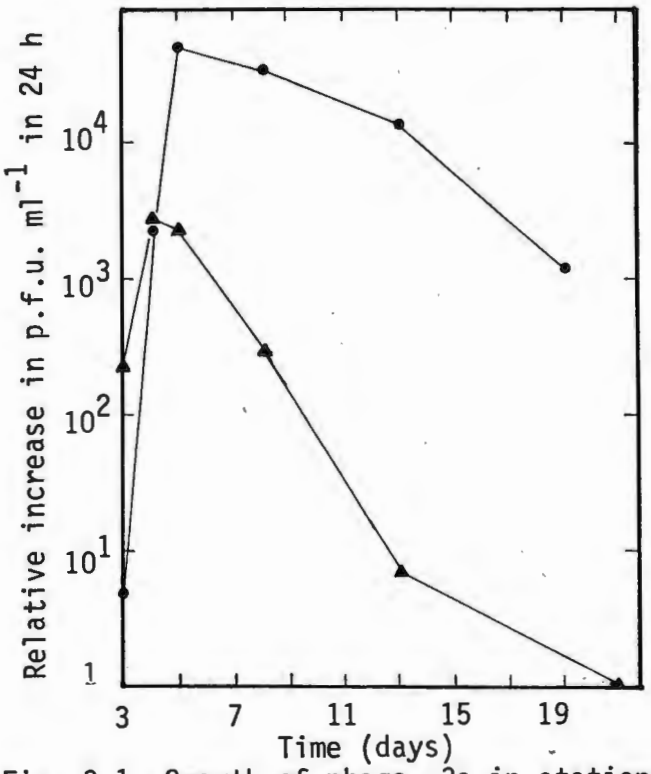


Fig. 3.1 Growth of phage α3a in stationary phase cultures of Achromobacter strain 14. Phage was added to standing cultures of various ages and assayed after 24 h (●) or to a shaking culture from which samples were removed and allowed to stand without aeration before assaying (▲) Refer 3.2.1.

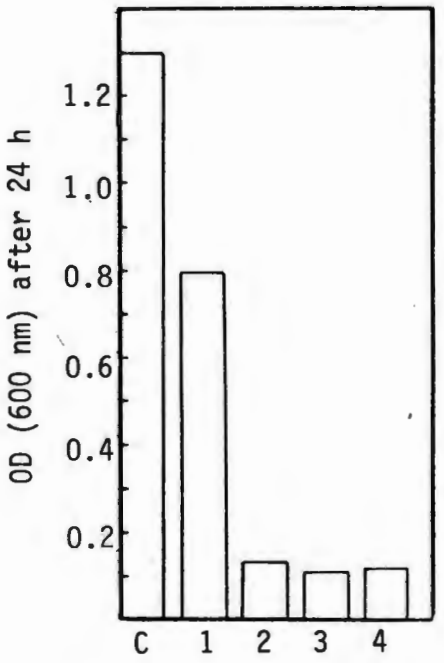


Fig. 3.2 Growth of Achromobacter strain 14 in used old media which had been subject to 1, 2, 3 or 4 cycles of bacterial growth and sterilization. Control (C) measures growth in tryptone broth. OD at time 0 was 0.03 and cultures incubated with aeration.

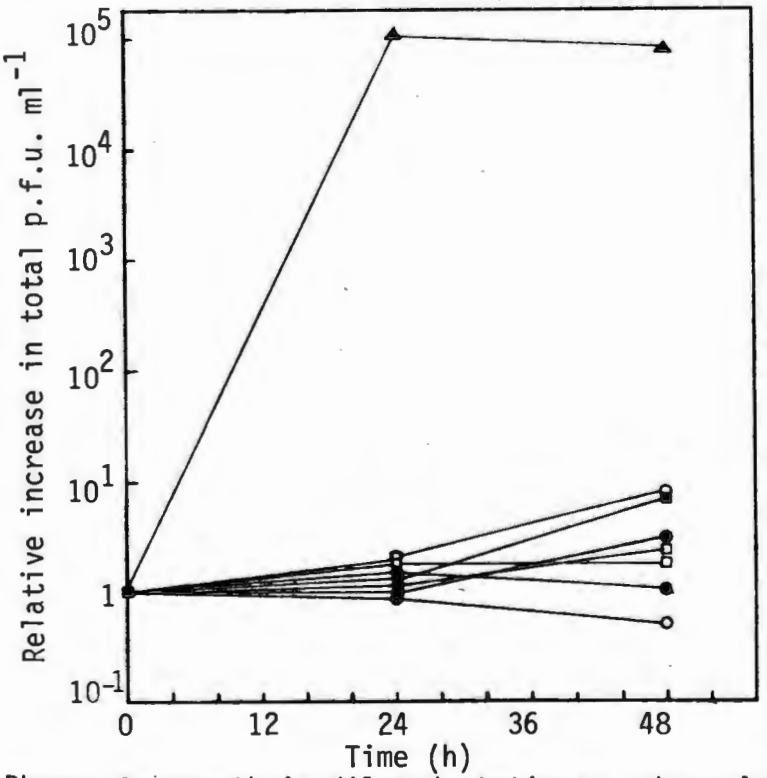


Fig. 3.3 Phage α3a growth in diluted stationary phase Achromobacter strain 14 cultures. After 30 min for phage adsorption, cultures were diluted 10^{-1} (■, □) and 10^{-2} (●, o) into phage resistant cultures and used old medium respectively. Control culture (▲) was not diluted.

cultures by sampling at time intervals over 26 h. The cultures were not shaken or moved during sampling since aeration was known to affect phage growth. They were merely opened and a 0.1 ml sample was removed. During the first 12 to 13 h when samples were removed at intervals of 1 - 2 h a decrease in the total number of phage was observed (Fig. 3.4). After that, the cultures were not assayed for 6 - 7 h and thereafter at intervals of 2 - 3 h. During this period the total number of phage increased by 2.1×10^3, 2.2×10^2 and 2.2×10^2 in the three identical cultures.

In a second experiment sampling from three identical cultures only commenced 8 to 10 h after the addition of phage (Fig. 3.5). The phage titre increased by 3.5 and 2.2 fold after 10 h and 1.1 fold after 8 h in the different cultures. Thereafter regular sampling (1 to 2 hourly) was continued and phage titres dropped by 90, 82 and 50%, in the three cultures at 22, 21 and 22 h respectively. After 24 h the sampling of cultures was discontinued until 54 h when phage titres were found to have increased by 1×10^3 to 2.8×10^4 fold.

Control cultures which were only sampled 24 h after phage addition showed increases in phage titre of 1×10^4 to 2.8×10^4 fold.

3.3.6 Stationary phase phage growth kinetics

The problem of phage inhibition by sampling (3.3.5) was overcome by preparing a large number of identical 10 ml cultures, assaying two 10 ml cultures for p.f.u. at each sampling time and then discarding the sampled cultures.

The kinetics of phage α3a growth on stationary phase Achromobacter strain 14 cells were studied by the addition of phage (c. 1×10^4 p.f.u. ml^{-1}) to 10 ml cultures that had been aerated for 3 days and allowed to stand for different time intervals prior to phage addition (Fig. 3.6).

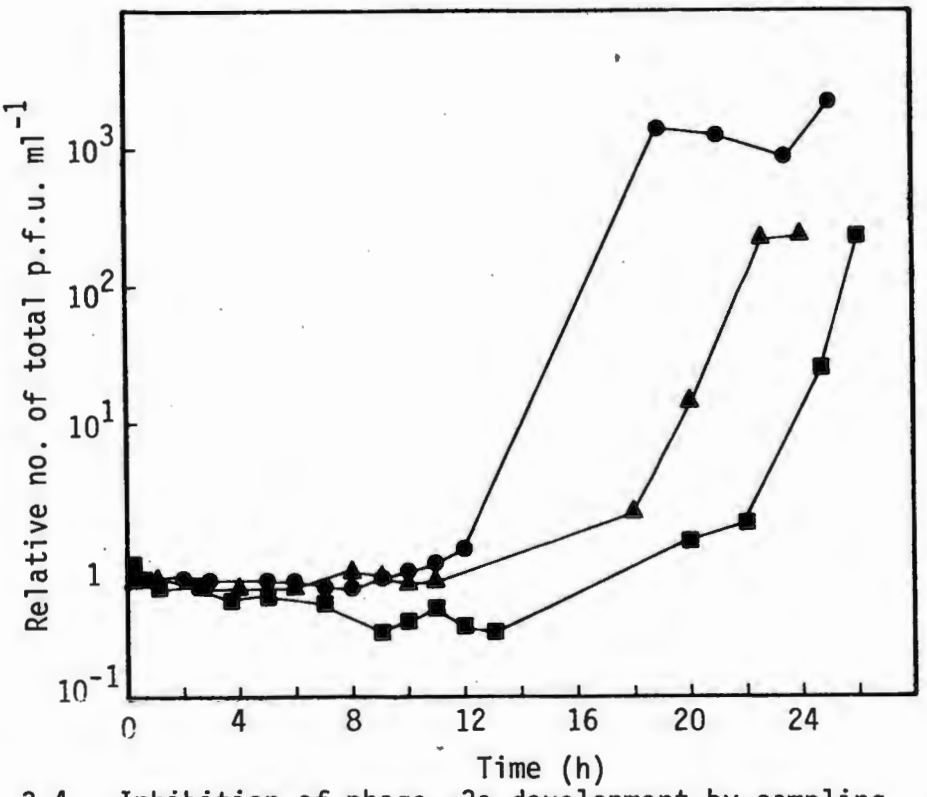


Fig. 3.4 Inhibition of phage α3a development by sampling. Phage α3a was added (1 x 10^4 p.f.u.) to 3 identical preconditioned standing stationary phase cultures of Achromobacter strain 14 (●,■ and ▲). At times indicated cultures were opened, 0.1 ml samples removed and assayed for total p.f.u.

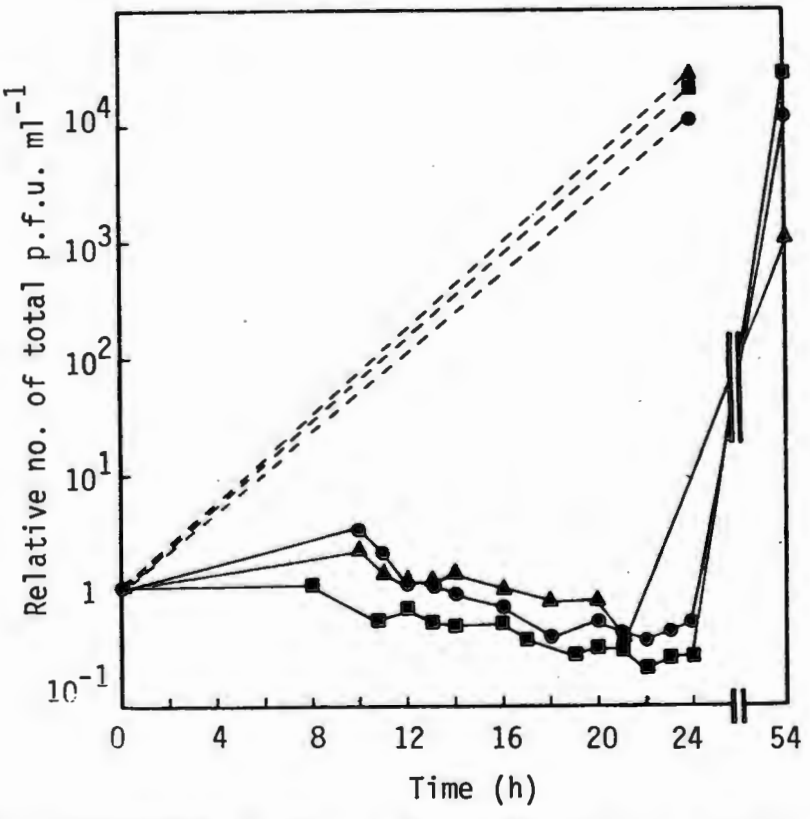


Fig. 3.5 Inhibition of phage α3a development by sampling. See Fig. 3.4 and 3.3.5 for legend. Sampling commenced 8 - 10 h after phage addition and was discontinued from 24 - 54 h in 3 identical cultures (●—●, ■—■ , ▲—▲). Another three identical cultures (●---●, ■---■ , ▲---▲) were sampled only once, 24 h after phage addition.

Under optimal conditions the minimum length of time before phage production (which has been defined as the latent period, Robb, Woods and Robb, 1978) was 6 - 9 h. Optimal conditions involved standing 3 day aerated cultures for at least one day before the addition of phage. There was a very long latent period (18 h) in cultures which were not incubated without aeration before the addition of phage.

To determine whether this long latent period (6 - 9 h) was peculiar to stationary phase phage development on strain 14 or was a characteristic feature of Achromobacter strains in general, the kinetics of phage growth in stationary phase w.t. cultures were examined. The results indicated that the long latent period was peculiar to phage growth on Achromobacter strain 14. The latent period for phage α3a cells in stationary phase Achromobacter w.t. cells was less than 2 h (Fig. 3.7).

3.3.7 Inhibition of phage development by dilution during the growth cycle

Dilution of infected stationary phase Achromobacter strain 14 cultures inhibited phage growth (Fig. 3.3). However, once the 6 - 9 h latent period had ended, the infected cultures could be diluted and variable increases in p.f.u. were observed. Dilutions (10^{-2}) into identical phage resistant, non-adsorbing cultures during the latent period inhibited increases in p.f.u. entirely (Fig. 3.8). At 12, 15 and 18 h (when phage development was well underway) dilution did not result in complete inhibition of phage development and phage titres increased by 3.6×10^2, 5.2×10^2 and 2.6×10^2 p.f.u. ml^{-1} respectively.

A similar pattern of development was observed when cultures were diluted into used old medium (Fig. 3.9). Phage development continued if a 15 h infected culture was diluted into autoclaved old medium, indicating that no specific extracellular thermolabile component was required for phage development (Fig. 3.10c).

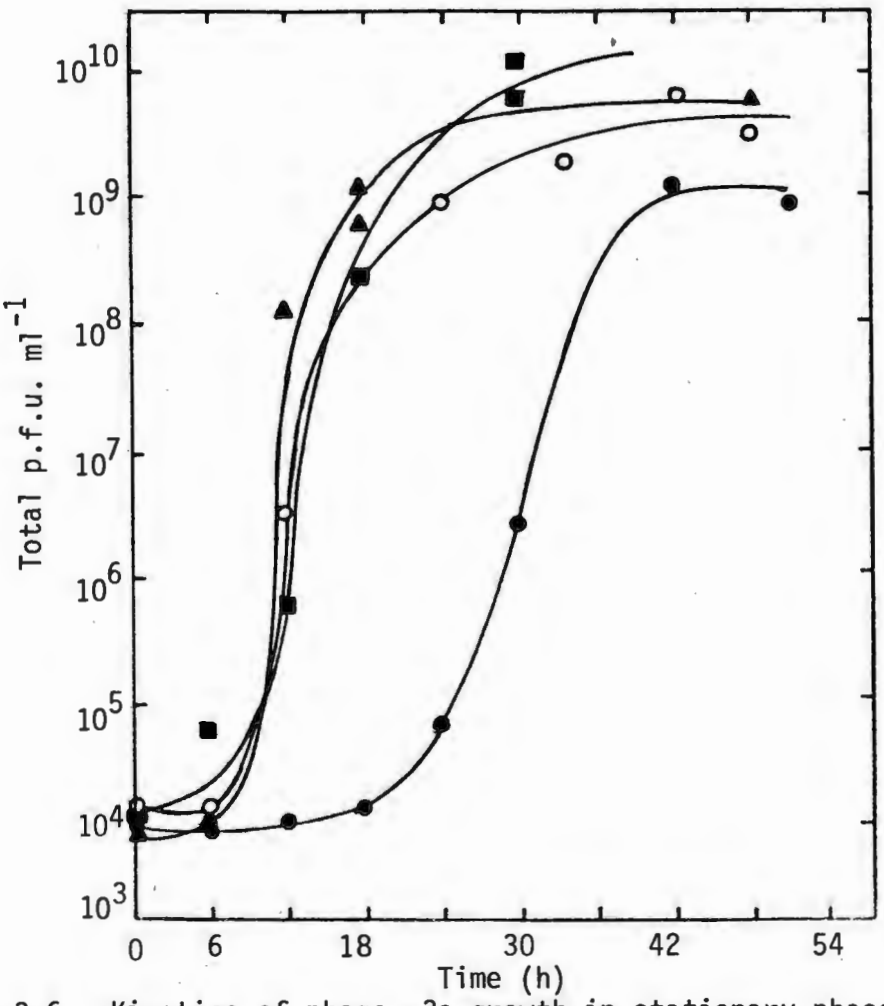


Fig. 3.6 Kinetics of phage α3a growth in stationary phase Achromobacter strain 14 cultures. Phage was added (1 x 10^4 p.f.u. ml^{-1}) to cultures which had been aerated for 3 days and allowed to stand for 0 days (●), 1 day (o), 1.5 days (■) and 2 days (▲) and identical samples were assayed at the times shown.

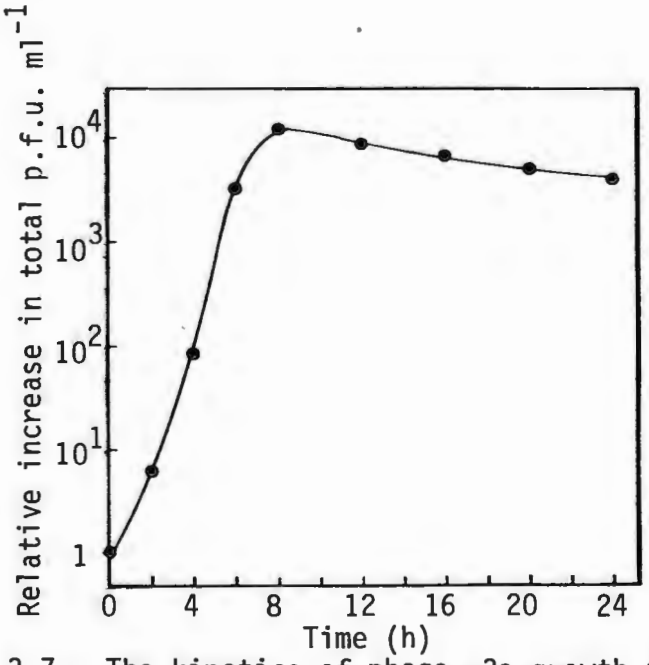


Fig. 3.7 The kinetics of phage α3a growth in stationary phase Achromobacter w.t. Phage was added to 4-day-old cultures which had been aerated for 3 days and standing for 1 day.

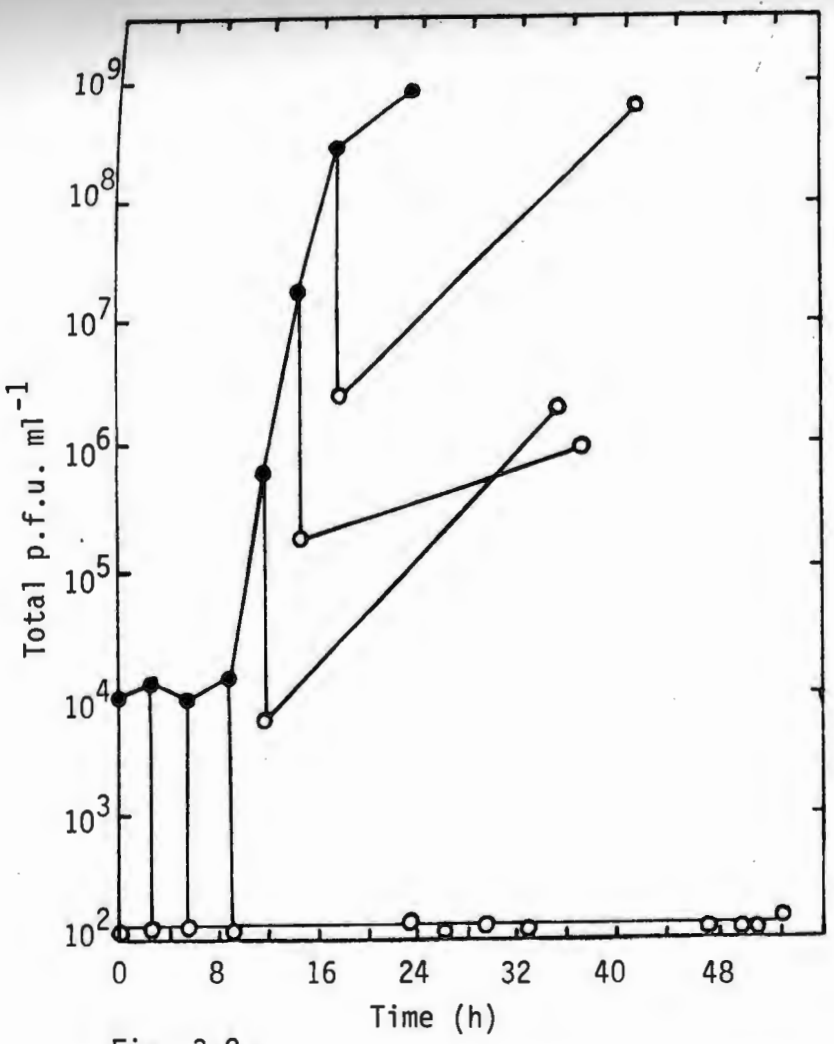


Fig. 3.8

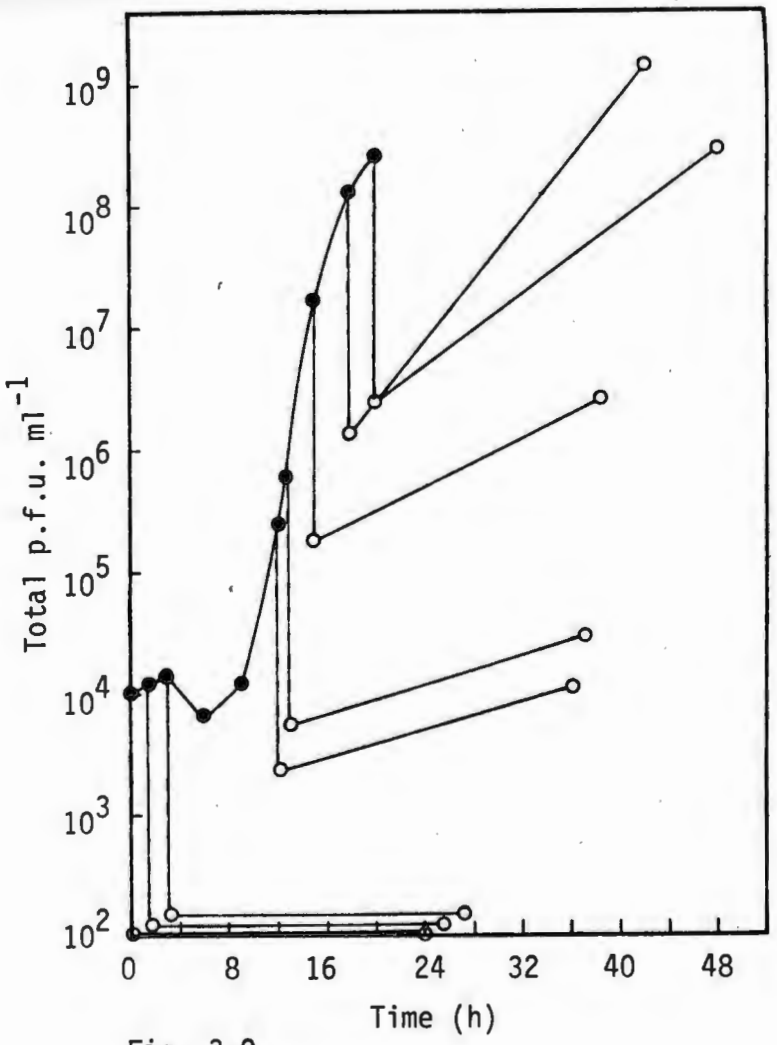


Fig. 3.9

Inhibition of phage α3a development during the latent period. Phage was added to standing stationary phase Achromobacter strain 14 cultures and phage development monitored (●—●). At time intervals after phage addition samples were diluted* 10^{-2} (●—○) and phage titres assayed at the time of dilution and after incubation without aeration (○—○).

* Samples were diluted into stationary phase phage resistant cultures (Fig. 3.8) and into used old media (Fig. 3.9).

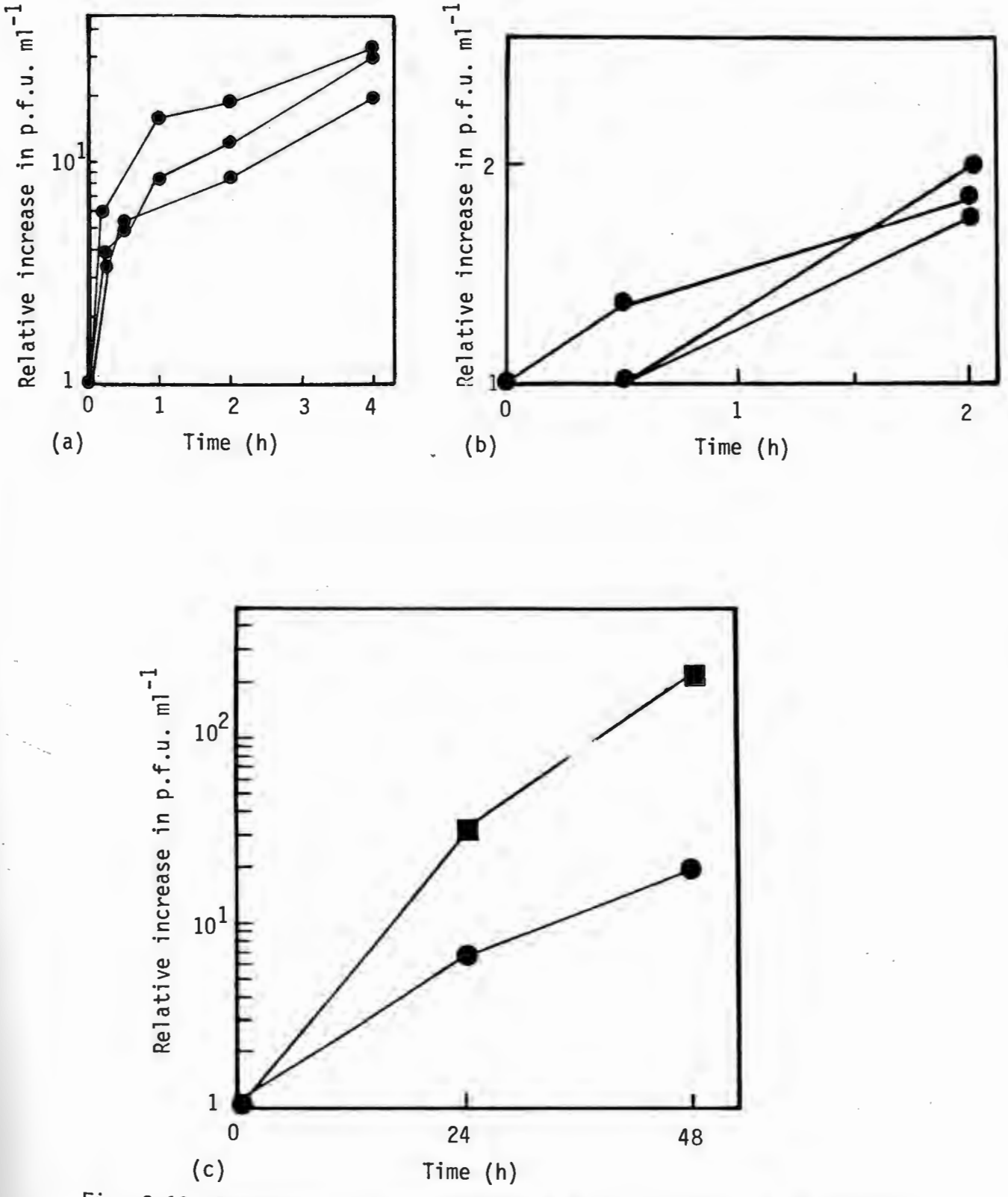


Fig. 3.10 Increases in phage α3a titre after dilution of an infected stationary phase Achromobacter strain 14 culture 15 - 16 h after phage addition. Dilutions were made into: (10a) tryptone broth (●), (10b) tris-HCl buffer (●) and (10c) autoclaved phage resistant cultures (■) and autoclaved used old media (●). Identical symbols show results from different cultures.

Infected cultures diluted 16 h after phage addition were able to increase in titre even if diluted into fresh tryptone broth (which reinitiated bacterial growth, Fig. 10a) or into tris-HCl buffer. The increases observed after dilution into tris-HCl were small (2-fold in 2 h) but consistent (Fig. 10b).

The development of α3a in Achromobacter w.t. stationary phase cultures was not inhibited by dilution 10 min. or 12 h after phage addition (Fig. 3.11).

3.3.8 Reversible phage adsorption

Coupled with the relatively slow adsorption of phage α3a to strain 14 cells, reversible adsorption could account for cessation in phage growth caused by dilution of infected stationary phase cells. However, measurement of free phage after dilution into tris-HCl buffer showed that phage adsorption to stationary phase Achromobacter w.t. and strain 14 was not reversible over a 2 h period (Fig. 3.12). The % of phage adsorbed after 30 min corresponded with the adsorption kinetics of α3a to w.t. and strain 14 cultures.

3.3.9 Determination of burst sizes by single burst experiments

Stationary phase Achromobacter strain 14

Burst sizes of α3a in stationary phase Achromobacter strain 14 cells were obtained by allowing phage development to commence for 16 h, removing free phage and then diluting infected cells into tryptone broth or tris-HCl buffer (3.2.7).

The total number of infected cells in all the samples (as calculated by Poisson's formula) was 38 (Table 3.2). The known number of cells which did not burst was 9 (plates with only 1 plaque).

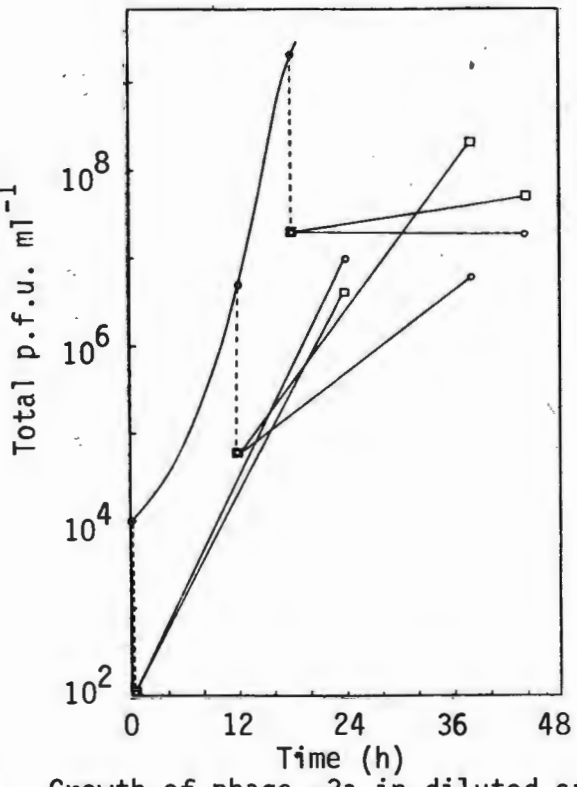


Fig. 3.11 Growth of phage α3a in diluted stationary phase Achromobacter w.t. cultures. Phage was added to standing cultures and phage development monitored (●—●). At time intervals after addition samples were diluted 10^{-2} into used old media (●---□) and into stationary phase phage resistant cultures (●---o). Phage titres were assayed in the diluted cultures after incubation without aeration.

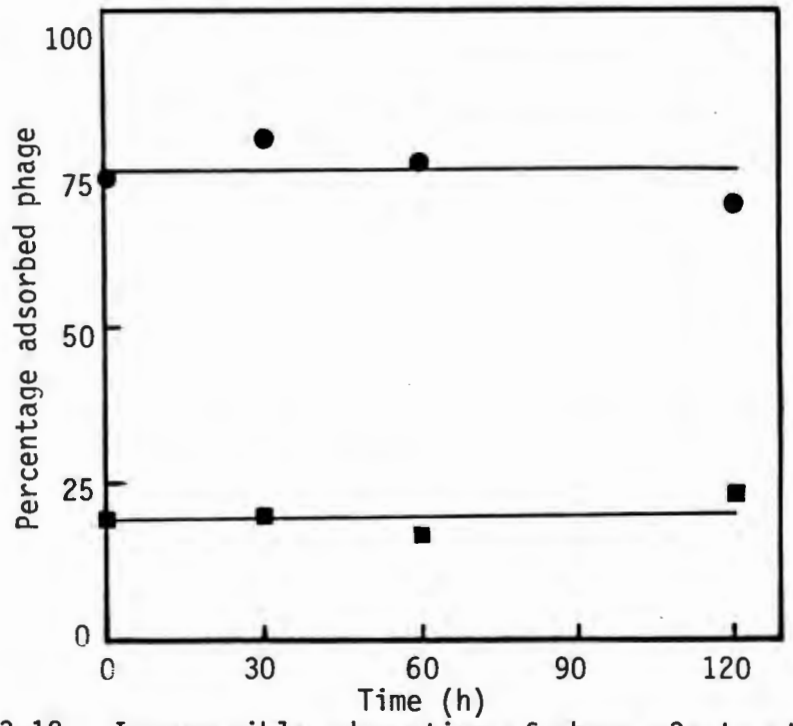


Fig. 3.12 Irreversible adsorption of phage α3a to stationary phase Achromobacter w.t. (●) and strain 14 (■). Phage were allowed to adsorb for 30 min and then diluted 10^{-3} in tris-HCl buffer. Adsorbed phage were measured for 2 h following dilution.

Therefore 29 infected cells were in the samples showing bursts. It is not known how many of these burst, but assuming all burst the average burst size was 709. If only one infected cell per sample burst then the average burst size would be 1154. The actual average burst size is likely to be an intermediate value but it is known that the average burst size could not have been less than 700. In tris-HCl buffer, although the number of infected cells which did not burst was greater (60% of total infected cells), the average burst size obtained was not less than 600.

Stationary phase Achromobacter w.t.

Bursts from infected stationary phase Achromobacter w.t. cells were measured by carrying out the single burst experiment 3.5 h after addition of phage α3a to standing cultures. All the dilutions were made into tris-HCl buffer since dilution into broth would reinitiate bacterial growth and phage α3a is able to develop on growing w.t. cells.

Dilution into tris-HCl buffer resulted in a large number of infected cells which did not burst until after plating (Table 3.2). The known number of cells which did not burst was 25 (from the plates with only 1 or 2 plaques). The total number of infected cells in all the samples was 66, therefore 41 infected cells were in the 17 samples which showed bursts. Assuming that all these infected cells burst (which was unlikely) the average burst size would be 81. If it is assumed that each burst shown was the result of one infected cell bursting, i.e. 17 altogether, the average burst size would be 196. It is not possible to know the exact number of cells which did burst but the experiment shows that the average burst size could not have been greater than 200.

Table 3.2 Single cell bursts of phage α3a in Achromobacter cells.

	Stationary phase strain 14 cells diluted in tryptone broth	Stationary phase w.t. cells diluted in tris-HCl buffer	Exponential w.t. cells diluted in broth
Total number of plates	48	48	50
Plates with no plaques	21	12	12
Plates with 1 plaque	9	13	1
Plates with 2 plaques	-	6	-
Plates showing bursts	18	17	37
	2000 2000	294 412	397 209 169
	1168 55	238 339	280 193 106
	46 1012	176 407	148 590 257
	8 1215	47 33	258 68 245
	1760 952	95 4	174 419 243
	1700 48	24 227	491 78 165
	1900 2000	31 40	608 418 236
	1700 1800	280 579	352 317 164
	13 1400	102	429 82 86
			184 96 384
			123 196 134
			850 280 198
			618
Total number of plaques	20777	3328	10245
n value	0.83	1.39	1.43
Total number of infected cells	38	66	67
Average burst size *	709-1154	81-196	153

* The maximum and minimum burst sizes have been calculated since a proportion of infected stationary phase cells were unable to burst (see 3.3.9).

Exponential phase Achromobacter w.t.

The average burst size of α3a in exponential cells was 153 per infected cell (Table 3.2). Of the total number of 67 infected cells, the known number which did not burst was one.

3.3.10 Reduction of burst sizes from stationary phase cells caused by the addition of chloramphenicol and nalidixic acid

After initiation of phage α3a development in stationary phase cells, dilution into fresh broth does not prevent further phage development. This is shown by the difference in phage development after dilution into tris-HCl and broth which indicates that although the cells reinitiate exponential growth, phage development can continue (3.3.7 and 3.3.9). To analyse this development with respect to phage DNA and protein synthesis, single burst experiments were carried out as described (3.2.7) with the following modifications:

(i) After inactivation of free phage for 7 min by antiserum, the number of infected cells was determined. The cell-anti-serum mixture was then stored at $4^{o}C$ overnight to ensure that all infected cells at the end of the lytic cycle would burst. The liberated phage were inactivated by the antiserum and the infected cell population consisted largely of cells which required further development for lysis by phage. The number of infected cells dropped from 2.0×10^{6} to 3.6×10^{5} p.f.u. ml^{-1} overnight, i.e. 82% of infected cells were able to burst at $4^{o}C$ without further phage development.

(ii) After overnight incubation at 4°C the mixtures were diluted as described into broth with no additions, tris-HCl and into broth containing chloramphenicol (cml) or nalidixic acid (nal) at their minimum inhibitory concentrations under these conditions (0.625 and 0.0795 μg ml^{-1}, respectively). Nal inhibits DNA synthesis and cml inhibits protein synthesis. Samples of each (0.3 ml) were incubated at 30°C for 18 h without aeration by shaking.

Under these conditions, the cells in broth were in exponential growth phase (determined by plate counts) for 26 h but there was no growth in the cml and nal treated cultures during the 18 h. The inhibitory effect of cml and nal on bacterial growth was reversible by dilution of the antibiotics.

The number of infected cells bursting and the burst sizes obtained in the presence of the drugs were compared to those values found in broth with no additions (Table 3.3). From the results it appears that phage DNA and protein synthesis continued after dilution into broth. If no further phage DNA or protein synthesis took place after reinitiation of bacterial growth, the number of cells bursting and the average burst size in broth, broth + nal and broth + cml should be similar. However, although the number of infected cells showing bursts was similar, the average burst sizes were adversely affected by the antibiotics. Burst sizes obtained were 462, 138 and 65 in broth, broth + nal and broth + cml respectively. This indicates that in broth (without additions) continued DNA and protein synthesis results in larger bursts. The average burst size obtained in tris-HCl is not comparable to the others since the observed n value was very low and this average was obtained from a total of only 8 plates (see Table 3.3*).

The burst size of 462 in broth with no additions was not as large as that obtained when infected cells which had reached the end of the lytic cycle were not eliminated by storing overnight at 4°C (see 3.3.9).

Table 3.3 The effect of nal and cml on single cell bursts of α3a in Achromobacter strain 14 (see 3.3.10).

Infected stationary phase cells diluted into:	Total no. of plates	No. of plates with plaques	n value[x]	Total no. of plaques	No. of[ϕ] infected cells	Average burst size
Broth with no additions	48	34	1.23	27270	59	462
Broth + cml	50	34	1.14	3486	54	65
Broth + nal	50	35	1.20	8002	58	138
Tris-HCl buffer	30	8	0.31	1376	9.3	148

[x] This n value represents the observed n value. Since all dilutions into broth, broth + nal, broth + cml and Tris-HCl were made from the same infected cell-antiserum mixture, the actual n values in each case should be identical. The observed n value is therefore an index of the number of infected cells which have not produced viable phage progeny but have been lost entirely - possibly due to cell lysis without phage morphogenesis.

[ϕ] This represents the maximum no. of infected cells which could have burst, as calculated from the observed n value, and excludes the number of infected cells which did not burst, as shown by 1 or 2 plaques on a single plate.

3.4 DISCUSSION

Achromobacter strain 14 is capable of supporting prolific phage development in cultures as old as 19 days. In other systems studied, phage development declines very shortly after the bacterial culture reaches the end of exponential growth (see 3.1). Some features of this unusual phage development have been characterized.

The classical one step growth experiment of Ellis and Delbrück (1939) could not be used to determine the latent period and burst size of α3a in stationary phase Achromobacter strain 14. The experiment requires that reinfection is minimized (after allowing phage to adsorb) during the period of observation by diluting the phage-bacterial mixture to such an extent that the rate of phage adsorption becomes extremely small. By assaying the number of infected cells at the time of the dilution, the average burst size can be calculated from the number of p.f.u. at the end of the rise period. Thus, in a single experiment the latent period and the average burst size are realised.

The growth of phage in stationary phase cultures has been shown to be very sensitive to the microenvironment and aeration inhibited phage development (2.3.9). It seemed feasible that dilution of infected cells into a medium which did not reinitiate bacterial growth would provide a suitable system for carrying out a one step growth experiment. After a period to allow phage adsorption, stationary phase infected cultures were diluted into identical phage resistant cultures (which did not adsorb phage) and into used old medium. However, despite efforts to simulate the microenvironment of a stationary phase culture, any dilution of the infected culture

inhibited increases in phage titre. This inhibition did not appear to be caused by a drop in the number of infected cells due to reversible adsorption.

The conditions for phage development were so stringent that sampling from a single undiluted stationary phase Achromobacter strain 14 culture prevented further increases in p.f.u. In one experiment where the first sample was taken 10 h after addition of phage, the total number of phage dropped by approximately 90% over the next 10-12 h of regular sampling. This drop in phage titre may indicate that phage were adsorbing under these unfavourable conditions but were unable to form plaques on plating. However, the inhibition of phage development by mere sampling is not understood.

Due to these difficulties, the latent period and the burst size of α3a in stationary phase Achromobacter strain 14 were determined by independent experiments. The latent period was obtained by adding phage to a large number of standing stationary phase cultures, assaying from duplicate cultures at any one time and then discarding the sampled cultures. Under optimal conditions (i.e. using cultures which had been allowed to stand for at least 24 h before phage addition) there was a long and variable latent period of 6 - 9 h. This latent period was increased to 18 h if the culture was not allowed to stand for 24 h prior to phage infection. This may indicate that a host component essential for phage development is synthesised during the standing period, or that repression of phage is relieved by the standing conditions (Robb, Woods and Robb, 1978).

A single burst experiment to determine the burst sizes from individual infected cells was possible because cultures could be

diluted with further increases in p.f.u. once the 6 - 9 h latent period was over and phage production was underway. Increases in phage titre were observed if dilutions were made into old medium, phage resistant cultures, tryptone broth or tris-HCl buffer. Since tris-HCl buffer provides no nutrition, increases in p.f.u. observed after dilution into buffer appear to represent those phage which had completed their infective cycle and were committed to lysing the host cell with release of phage progeny. In comparison to dilution into tris-HCl buffer, the phage increases observed when dilution was into tryptone broth were far greater (between 8 and 2 x 10^1 fold increase in broth compared with < 2 fold in buffer). This suggested that further phage development was taking place, and later experiments indicated that phage DNA and protein synthesis could continue, even though exponential bacterial growth had been reinitiated. It appears that at the time of dilution (15 - 18 h after phage addition) there were 4 states in which the phage could exist:

(i) as free phage;

(ii) as an infected cell which was committed to lysis in any type of diluent;

(iii) as an infected cell in which the lytic cycle was underway but needed further nutrition for completion;

(iv) as an infected cell which would not burst if removed from the stringent conditions of the stationary phase culture and may or may not manifest itself as a plaque when plated.

The single burst experiment for α3a in stationary phase Achromobacter strain 14 was carried out by diluting infected cells into tris-HCl buffer and into tryptone broth. The minimum average burst sizes in both diluents were similar (709 in broth; 600 in tris-HCl) although more infected cells burst in the broth than in buffer. The bursts obtained in broth were likely to result from cells in state (ii) and (iii) whereas bursts in tris-HCl resulted from cells in state (ii). A significant proportion of the stationary phase infected cells failed to burst in the liquid medium but produced a single plaque on the indicator plate. This suggests that phage development at the time of dilution had not proceeded past the critical stage which required the environmental conditions of the stationary phase culture (state iv). However, these conditions were restored on the indicator plate and the infected cell was able to burst and produce a plaque. The average burst size of greater than 600 in stationary phase cells was far larger than the average burst size of 153 obtained in exponential w.t. cells using a similar procedure. Thus, infection of old cells with phage α3a resulted in a longer latent period with larger bursts compared to those in exponential cells. Delbrück (1940b) also found longer latent periods in old cells but burst sizes were drastically reduced.

These characteristics are pecular to phage development in Achromobacter strain 14. Phage α3a growth in stationary phase Achromobacter w.t. cells differed from growth in strain 14 cells in the following respects:

(i) Dilution of infected w.t. cultures did not inhibit further increases in p.f.u.

(ii) There was no long (6 - 9 h) latent period in w.t. infected cells. Increases in phage were observed after 2 h.

(iii) The average burst size (as determined by the single burst experiment with dilutions into tris-HCl buffer) was less than 200 compared to bursts of greater than 600 in strain 14 cells.

In other bacterial-virus systems, inhibition of host cell lysis results in continued phage production and consequently very large bursts (Doermann, 1948; Harris et al., 1967; Josslin, 1970; Mount et al., 1968; Reader and Siminovitch, 1971 a & b). The long latent period and large bursts observed in stationary phase Achromobacter strain 14 cells may be due to inhibition of cell lysis. Supporting this is the fact that smaller bursts were obtained in stationary and exponential w.t. cells where the latent periods were reduced. Cell lysis by α3a may require a product analogous to the T4 gene t product (Josslin, 1970) or the λ S cistron product (Reader and Siminovitch, 1971 b) for alteration of the cytoplasmic membrane which is a prerequisite for lysis. It is tempting to speculate that in strain 14 cells (which are presumed to contain additional α3a genes) the accumulation of such a product is curtailed. The late genes of the λ genome consist of the genes for the structural components of the head (genes A - F), the genes for the tail components (genes Z - J) and the S and R genes which are concerned with lysis. The late genes are not under repressor control. They are transcribed in a single transcriptional unit and are positively regulated by the Q gene product, which acts as an anti-terminator at a site between

Q and S (Echols and Murialdo, 1978). There is also evidence for post-transcriptional regulation of the late genes (Ray and Pearson, 1974). In view of what is known of the intricacies of regulation of the λ genome (Echols and Murialdo, 1978) and the interactions with the host cell even at the stage of phage head morphogenesis (Murialdo and Becker, 1978), there are numerous possibilities for altered regulatory mechanisms in strain 14.

Phage α3a development is not initiated in exponential phase strain 14 cells. A comparison of the burst sizes obtained after dilution of infected stationary phase cells into broth, broth + nal and broth + cml was undertaken to determine whether phage DNA and protein synthesis could continue if phage development had been initiated in stationary phase. The results indicate that they can. Although the growth of the infected cells was reinitiated by dilution into fresh broth, these experiments showed that in the absence of protein and DNA synthesis the burst sizes were markedly reduced. In the presence of the drugs, the developing phage appear to be able to undergo assembly and are able to lyse the cells (as shown by the high percentage of cells bursting). It seems likely that the reduced burst sizes may be caused by a shortage of phage DNA and structural proteins. Chloramphenicol treated cells showed lower bursts than nal treated cells. This could be explained if development of α3a is similar to that of λ, where DNA synthesis begins earlier than the production of the tail and head proteins (Echols and Murialdo, 1978). Thus, on dilution of the infected cells, the pools of DNA would be greater than the pools of structural protein. A hypothetical model for phage development in strain 14 based on further results (5.4) encompasses control mechanisms to explain how phage DNA and protein synthesis can continue in exponential cells.

An important feature of phage development in Achromobacter w.t. cells is that the latent periods and burst sizes in exponential and stationary phase cells are similar. This contradicts the most frequently cited reason for a decline in phage production in stationary phase cells, namely that a decline in the metabolic activity inhibits phage growth. It is likely that the majority of phages respond to bacterial regulatory mechanisms that depress the metabolism of stationary phase cells. Phage α3a may belong to a class of phages which override these regulatory mechanisms. This development in stationary phase cells also reflects the ability of phage α3a to activate the quiescent DNA, RNA and protein synthesising machinery of the infected cell.

CHAPTER IV

CHARACTERISTICS OF AERATED AND NON-AERATED STATIONARY PHASE ACHROMOBACTER CULTURES

4.1 INTRODUCTION

The bacterial growth cycle has been divided into three stages. The first stage, the lag phase, is a period when the cells are adapting to growth in the new environment and there is a slow rate of bacterial growth. The length of the lag phase depends on the organisms used and the culture conditions (Mandelstam, 1968). At the end of the lag phase the cells are in a balanced state of growth and all cell constituents increase proportionately over the same period of time. During this stage, known as exponential growth phase, the cells grow and divide at a steady rate. This phase of growth is terminated gradually when the cells begin to exhaust the available nutrients or when unfavourable levels of toxic substances accumulate, or the pH becomes unsuitable for growth (Dean and Henshelwood, 1966). Resting phase or stationary phase are terms which have been used to describe the state of the bacterial cells once exponential growth has terminated. In a synthetic medium, growth of *E. coli* stops abruptly when the carbon or nitrogen source is limiting. This is an example of a drastic 'shift down', where cells are transferred from a given growth condition to one where growth is extremely slow or non-existent. Under such conditions, DNA synthesis and subsequent cell division can occur for a relatively short period whereas the RNA and protein content, as well as the optical density remain constant (Schaechter, 1961). This results in the production of very small

cells. During starvation each cell can complete its cycle of DNA synthesis but cannot initiate a new cycle (Mandelstam, 1968). Thus, the cells in a stationary culture are synchronous with regard to their DNA replication cycle. In nutrient broth the cessation of exponential growth can be considered as a series of gradual 'shift downs'. Cessation of growth occurs gradually as many different nutrients are being depleted in sequence. The cells also decrease in size as they approach stationary phase (Mandelstam, 1968).

Stationary or resting phase cells are not, as the term implies, inactive. Many bacterial species produce specific antibiotics or extracellular enzymes in stationary phase but not in exponential phase (Hopwood and Merrick, 1977; Priest, 1977). In Bacillus and Clostridium species gross morphological and biochemical changes such as the formation of endospores take place in stationary phase cells without a net increase in protein (Hanson et al., 1970). Stationary phase cells also have the capacity to adapt to changes in the environment. For example, the enzyme β-galactosidase, which is required for the utilization of lactose, was induced in starved cells in the absence of net protein synthesis (Mandelstam, 1957). It is apparent that without some mechanism to enable stationary phase cells to respond to, and produce the required enzymes for the maximum utilization of the growth medium, the stationary phase state would be permanent even if cells were added to a rich growth medium.

The flexibility of stationary phase cells is made possible by the ability of these cells to degrade intracellular macromolecules to their constituent amino acids or nucleotides and then reutilize these breakdown products for synthesis. This process is known as turnover and is of importance to the cell when the re-synthesised

molecules differ from those which were degraded, since this is a means whereby the cell can respond to alterations in the environment. This process has been referred to as biochemical differentiation (Mandelstam, 1960).

Estimates of protein turnover in non-growing cells vary with the strain of organism used, the condition of starvation and the amino acid used. Turnover rates of 4 to 20% per h for non-growing E. coli cells have been reported (Pine, 1965; Mandelstam, 1960) and the rate of protein synthesis equals the rate of protein degradation (Mandelstam, 1957 and 1958). Protein synthesis and degradation (caused by the action of protease) are both subject to regulation (Mandelstam, 1968) and these processes can be studied separately. In E. coli, the addition of azide or chloramphenicol immediately stops synthesis but permits the release of labelled amino acids from degradation to continue at a steady rate for some time (Mandelstam, 1960).

Mandelstam (1957 and 1958) compared the stability of proteins in growing and non-growing cells. An E. coli strain requiring threonine and leucine was heavily labelled with ^{14}C-leucine during growth. To measure protein degradation in a non-growing suspension excess ^{14}C-leucine was removed and the cells were incubated with glucose and an excess of unlabelled leucine. To obtain a growing suspension threonine and ammonium salts were added to the above. The release of ^{14}C-leucine from the cells was a measure of protein degradation. The total loss of label from the growing cells was 4% in 3 h. This loss occurred largely in the first 60 min when the cells were still in lag phase. In the non-growing cells protein degradation continued linearly at a rate of 5% per hour.

From these results it was thought that many of the cellular proteins that were broken down in starving cells were conserved during growth.

However, it has since been found that only the relatively stable proteins are conserved (Pine, 1972). Pine (1970) and Nath and Koch (1971) showed that extremely rapid degradation of an unstable fraction of the bacterial protein in E. coli could occur during growth and starvation. However, this degradation is a relatively minor process compared to the degradation which is induced only by starvation.

Certain nucleic acids are stable in growing bacterial populations but become unstable when growth ceases. Of the nucleic acids only mRNA and two terminal nucleotides of tRNA are metabolically unstable during growth of E. coli (Ben-Hamida and Shlessinger, 1966). In starved cells however, there is extensive degradation of RNA (Mandelstam, 1960). Most of the degraded RNA is derived from rRNA and the majority of nucleotide material is reutilized for RNA synthesis. No turnover of rRNA was found in rapidly growing cells (Nierlich, 1978). The degradation of RNA in non-growing E. coli cells has been attributed to a preexisting nuclease activity rather than to the induced formation of a nuclease when growth ceases. Supporting this were the observations that stable rRNA became unstable very soon after starvation conditions were imposed and the degradation was not dependent on protein synthesis (Ben-Hamida and Shlessinger, 1966).

Koch (reported in Nierlich, 1978) studied the regulation of growth of E. coli in chemostat-grown cultures. He found that slowly growing cells possessed ribosomes in far greater numbers than were required for the rate of protein synthesis and that rRNA synthesis was in excess of rRNA accumulation. This was evidence for rRNA turnover and also that slowly growing cells have the reserve capacity for rRNA synthesis. In slowly growing cells there is a pool of non-functional ribosomes and activation of these enables the cells to respond to a nutritionally rich environment by an immediate increase in protein

synthesis (Nierlich, 1978; Koch and Deppe, 1971). This is consistent with the *in vitro* protein synthesis studies of Daneo Moore *et al.* (1966). They found that the *in vitro* protein synthesis activity of extracts from *Streptococcus faecalis* and *E. coli* increased with culture age and remained high in stationary phase, indicating that these cells had the potential for extensive protein synthesis. It seems paradoxical that maximum protein synthetic activity occurs during stationary phase when there is no net protein synthesis in intact cells. There is also evidence that slowly growing cells have an excess of RNA polymerase but the mechanism(s) by which this and the excess ribosomes are held in a non-functional state is not understood (Nierlich, 1978).

Thus, the controls of slowly growing or stationary phase cells appear to have evolved to provide rapid adaptability, whereas those of rapidly growing cells have evolved to provide efficiency.

The regulation of bacterial growth with respect to control of macromolecular synthesis (DNA, RNA and protein) has been extensively researched in exponentially growing cultures. It is now generally accepted that control of RNA components (particularly rRNA and tRNA) of the protein synthesising machinery is central to the regulation of growth in rapidly growing bacteria (Nierlich, 1978). Studies on the control of the synthesis of ribosomes, tRNA and other components required for protein synthesis have shown that the 16 S, 23 S and 5 S rRNA's are cotranscribed (Pace, 1973; Nierlich, 1978). The synthesis of rRNA and tRNA appears to be coordinated and at least six species of tRNA are cotranscribed with rRNA species (Nierlich, 1978). Starvation of certain bacterial strains for a required amino acid (or a variety of amino acids) results in a severe restriction on the amount of stable

RNA (rRNA and tRNA) accumulating. In contrast, the production of other mRNA species (e.g. phage ϕ80 mRNA) is unaffected. Within seconds of the onset of starvation there is a rapid accumulation of the guanosine nucleotide ppGpp (Travers, 1976). The synthesis of rRNA *in vivo* is inversely correlated with concentrations of ppGpp (Cashel, 1975), and the synthesis *in vitro* has been shown to be inhibited by ppGpp (Nierlich, 1978). This shut down on rRNA synthesis mediated by ppGpp is known as stringent control. Mutants unable to accumulate ppGpp fail to shut off rRNA synthesis and are said to show relaxed control. Evidence suggests that ppGpp acts by specifically inhibiting the formation of RNA polymerase - rRNA promoter complexes. Thus, the regulation is brought about by transcriptional control (Travers, 1976).

Although ppGpp plays a major role in transcriptional specificity, there is strong evidence for coupling mechanisms between transcription and translation (Chakrabarti and Gorini, 1975) and the involvement of other elements in the control of transcription. Components of the translation machinery have been shown to affect the specificity of RNA polymerase. Pongs and Ulbrich (1976) showed that fmet tRNA specifically binds to RNA polymerase and affects its transcriptional specificity *in vitro*. Elongation factor TuTs increases the affinity of RNA polymerase for *E. coli* rRNA promotors and simultaneously increases the sensitivity of transcription to ppGpp (Travers, 1976). Recently Zurawski, Elseviers, Stauffer and Yanofsky (1978) have shown that transcriptional termination at the attenuator of the *E. coli* tryptophan operon is regulated by translation of the trp leader sequence. This control is mediated by changes in the levels of trp $tRNA^{trp}$ and there is emerging evidence that this may be a transcriptional control mechanism in many bacterial species.

The transcription of rRNA genes and the synthesis of ribosomal proteins are coordinated but they are subject to independent transcriptional control (Dennis, 1977; Gansing, 1977). Other components for protein synthesis such as EF-Tu (elongation factor) and some amino-acyl tRNA synthetases show similar patterns of regulation to rRNA, but the control mechanisms, although they may be coupled in some way, are thought to be distinct (Nierlich, 1978). In growing cells, the total rate of RNA synthesis is undoubtedly an important determinant of rRNA synthesis and it is likely that the concentrations of RNA polymerase, ATP, GTP and ppGpp affect this rate.

What are the control mechanisms for macromolecular synthesis in stationary phase cells? Slowly growing or stationary phase cells have an excess of ribosomes (Koch and Deppe, 1971) and tRNA. For example, in growing E. coli cells tRNA constitutes between 12 - 18% of the total cellular RNA, but in slowly growing cells tRNA levels increase to 20 - 25% of the RNA (Norris and Koch, 1972; Skjold, Juarez and Hedgoth, 1973). RNA polymerase in stationary phase cells is also in excess of that required for the rate of protein synthesis (Koch and Deppe, 1971). Thus, it is unlikely that the rates of RNA and protein synthesis are controlled by the relative concentrations of these components in stationary cells as they are in rapidly growing cells. It is likely that factors controlling the activation of the function of the ribosomes, tRNA and RNA polymerase are central to the regulation of macromolecular synthesis in stationary phase cells. As yet, these are unknown mechanisms (Nierlich, 1978).

The Achromobacter strains used in this study have the capacity for extensive phage development in stationary phase. However, growth of phage α3a never occurs in shaking stationary phase cultures of

Achromobacter w.t. or strain 14, but favourable conditions for optimal phage growth develop if cultures have been allowed to stand without aeration for 24 h.

Woods (1976) found that RNA synthesis was inhibited in shaking cultures but was reinitiated under standing conditions. These results implied that important cellular regulatory mechanisms were being affected by the aeration conditions.

In an effort to determine the possible reason(s) for phage inhibition in shaking cultures a number of other physiological and morphological characteristics of shaking and standing cultures have been examined. It was also thought that such a study might indicate how phage development is possible in stationary phase cells.

4.2 METHODS

In all experiments unless stated otherwise cultures were grown in tryptone broth with aeration for 3 days. The culture was then dividied and aeration of one half was continued (shaking culture) while the other half was incubated aerobically without shaking (standing culture) for 1 or 2 days.

4.2.1 The effect of aeration on phage adsorption

Phage adsorption was measured (see 2.2.6) in duplicate sets of Achromobacter strain 14 cultures of different volumes (10, 25, 50 and 100 ml volumes in 100 ml flasks and 10 ml in standard containers) which had been standing or shaking. The conditions of aeration were maintained during phage adsorption.

4.2.2 RNA Synthesis

RNA synthesis was determined by the incorporation of ^{3}H-uracil into trichloroacetic acid (TCA)-precipitable material by the method of Eichenlaub and Winkler (1974). Samples of standing and shaking cultures were adjusted to the same turbidity (OD 1.2) with old medium (see 3.2.3). One ml of the culture was added to 4 ml of prewarmed old medium containing ^{3}H-uracil (2 μg ml^{-1}, 0.4 μCi ml^{-1}) and uracil (2 μg ml^{-1}). Uracil incorporation was measured over 1 h. At intervals 0.5 ml samples were removed and added to 0.5 ml cold 10% (w/v) TCA containing 1 mg ml^{-1} uracil. After 30 min on ice the samples were collected on 2.5 cm GF-C filters (Whatman) and washed with 2 x 10 ml cold 5% (w/v) TCA and 10 ml cold 1% (v/v) acetic acid. The filters were dried in a 50^{o}C oven, added to 10 ml scintillation fluid in vials and counted in a Beckman LS 310T scintillation counter.

4.2.3 DNA Synthesis

DNA synthesis was determined by the incorporation of ^{3}H-adenine into NaOH hydrolysed TCA-precipitable material (Friesen, 1968) as the Achromobacter strains did not incorporate ^{3}H-thymidine.

Cold adenine (2 μg ml^{-1}) and ^{3}H-adenine (1 μCi ml^{-1}) were added to standing cultures (3 ml in sloppy tubes) and shaking cultures (3 ml in 50 ml flasks). At intervals samples (0.4 ml) were withdrawn and added to an equal volume of 1N NaOH on ice. After a few minutes the samples were covered with parafilm (to prevent evaporation) and incubated overnight at 37^{o}C to hydrolyse the RNA. The following morning, the samples were cooled and neutralized by the addition of 26 μℓ acetic acid. And equal volume (0.8 ml) of 10% TCA containing 1 mg ml^{-1} adenine was added to precipitate the DNA. After 30 min on ice the procedure for filtering and counting (as described, 4.2.2) was carried out.

For comparative purposes, DNA synthesis was also measured in exponential phase cells. An overnight culture of Achromobacter strain 14 was diluted 1:20 in tryptone broth and aerated for 2 h. DNA synthesis was measured in a 5 ml culture (OD 0.3) in a 50 ml flask. Aeration was continued throughout the experiment and the increase in OD was measured in a parallel culture.

4.2.4 Protein synthesis

Protein synthesis was measured by the incorporation of ^{14}C-leucine into TCA precipitable material. Unlabelled leucine (10 μg ml^{-1}) and ^{14}C-leucine (1 μCi ml^{-1}) were added to standing cultures (3 ml in sloppy tubes) and to shaking cultures (3 ml in 50 ml flasks). For assaying leucine incorporation in standing cultures label was added to a number of identical cultures at time 0. At each time interval, samples were taken from a different culture which was then discarded. This was to ensure that the aeration conditions of the standing cultures were not altered during sampling. At intervals, samples were withdrawn and added to an equal volume of 10% TCA containing 1 mg ml^{-1} leucine on ice. After at least 30 min the samples were filtered and counted as described (4.2.2).

Protein synthesis was also measured in exponential cultures using the method outlined in 7.2.1. The optical density was measured in a parallel culture. After 30 min incorporation (protein synthesis was linear for at least 1 h) the radioactivity per OD unit was compared to those obtained in stationary phase cultures.

4.2.5 Leucine Uptake

Leucine uptake was measured in old shaking and standing Achromobacter strain 14 cultures as an index of cellular energy levels.

The transport of branched chain amino acids in E. coli is mediated by several genetically and kinetically distinct systems (Wood, 1975). One system, the leucine specific (leu system) has a high affinity for leucine (leu) and fails to recognize isoleucine (ileu). The other two leucine uptake systems (LIV I and LIV II, known together as LIV) are completely inhibited by ileu and valine. LIV I is a high affinity transport system and LIV II a low affinity system. The LIV I and leu specific binding proteins are separate binding proteins found in the periplasmic space but both require the livH gene product for activity. The LIV II system is membrane bound and its activity is completely independent of the other systems (Anderson and Oxender, 1978).

Assay for leucine transport

The cells were washed twice by centrifugation and resuspension in phosphate buffer (pH 6.9) at room temperature. The cell density was adjusted turbidometrically to 1 x 10^9 cells ml^{-1}. The leu specific and LIV (leu, ileu and valine) systems were assayed essentially as described by Rahmanian et al. (1973). Cells (50 µl) were added to 50 µl of double strength transport buffer and held for 60s before starting the reaction by the addition of 50 µl ^{14}C-leu (final concentration 10 µM). All assay mixtures contained chloramphenicol (50 µg ml^{-1}), glucose (0.5% w/v) as the energy source and 0.4 M NaCl (transport was severely inhibited at lower NaCl concentrations). The reactions took place at 20°C and were stopped at 15, 30, 45, 60 and 120s by filtration of 100 µl samples and washing with buffer on 0.45 µm membranes (Gelman Instrument Co.). Each nanomole of leu was represented by 209,000 cpm. The Leu and LIV systems were distinguished as follows. Both systems were

assayed together in the absence of ileu and the leu specific system was then measured in parallel by leucine uptake in the presence of 200 μM ileu which completely inhibits the LIV system in E. coli (Rahmanian et al., 1973). The LIV system was determined by difference.

The protein content of the cultures was determined by the method of Lowry et al. (1951).

4.2.6 Cellular levels of ATP

Cellular ATP was measured by the Luciferin-luciferase assay, counting the flashes of light emitted from mixtures of cell extracts and firefly lantern extracts (Foulds, 1971).

A comparison of ATP levels in exponential phase and stationary phase standing and shaking Achromobacter strain 14 cultures was made. Samples of the cultures (3 ml) were pelleted and resuspended in distilled water (6 ml). ATP was extracted from these samples by heating at 100°C for 10 min. The dried firefly lantern extracts (Sigma) were rehydrated to a concentration of 10 mg ml^{-1} in 0.05 M KH_2AsO_4 (pH 7.4) and 0.02 M $MgSO_4$. The lantern extracts were kept on ice during the experiment.

Assay for ATP

The sample (0.3 ml) was mixed with 4.7 ml distilled water and 2.4 ml of buffer (0.05 M KH_2AsO_4, 0.02 M $MgSO_4$, 0.001 M ethylene-diaminetetraacetic acid, ph 7.4) in a scintillation vial at room temperature. At time zero, 0.5 ml of the firefly lantern mixture was added and mixed into the contents of the vial. The vial was immediately lowered into the counting chamber of the scintillation counter set with a wide window (6 sec lag). Each sample was counted for 6 sec. The amount of ATP in the sample was determined from counts

obtained with known concentrations of ATP. The protein concentrations of the samples were determined by the method of Lowry et al. (1951) with bovine serum albumin used as the standard.

4.2.7 UV Inactivation

Standing and shaking Achromobacter strain 14 cultures were diluted 10^{-2} in cold phosphate buffer (2 - 5 x 10^7 cells ml^{-1}). The source of irradiation was a Hanovia lamp (2537Å) used at a distance of 22 cm. The lamp was calibrated using a U.V.P. UV Meter J-225 and a YSI Kettering 6.5A radiometer calibrated against an Eppley 12-junction bismuth silver thermopile and was found to deliver 1.15 Jm^{-2} sec^{-1}.

Cell samples (7 ml) in sterile petri dishes were kept at 0^oC during irradiation to prevent photoreactivation due to the visible light emitted by the lamp and were agitated gently. Irradiation was carried out in the dark and post irradiation manipulations in subdued light. Samples were irradiated for various time intervals, appropriate dilutions made and then plated for viable counts on well dried tryptone plates which were incubated in the dark.

4.2.8 Heat Inactivation

Standing and shaking stationary phase cultures were diluted in phosphate buffer to a final concentration of c. 3 x 10^7 cells ml^{-1}. Samples (2 ml) in sloppy agar tubes were placed in a 60^oC waterbath and aliquots were removed at various times over 30 min. Appropriate dilutions were made in phosphate buffer and the cells plated on well dried tryptone agar plates.

4.2.9 Cell Morphology

A comparison of the morphology of shaking and standing stationary phase cells was undertaken using several methods of examination.

Phase Contrast Microscopy

Samples of cell cultures were examined without treatment using a Zeiss Photomicroscope III fitted with phase contrast and dark background optics.

Electron Microscopy

Preparation for the Scanning Electron Microscope (SEM)

Cell samples were either fixed in 5% glutaraldehyde (phosphate buffered) followed by suction onto polycarbonate filters of 0.2 μm pore size (Nucleopore Corporation, Pleasanton, California) or were transferred to the nucleopore filters and then fixed in glutaraldehyde (overnight at $4^{o}C$). Small sections (2 mm) of the filter were then cut and dehydrated through an alcohol and amyl acetate in absolute ethanol series (Cross, Allanson, Davies and Howard-Williams, 1977). Critical point drying was carried out in liquid carbon dioxide in a Polaron E3000 critical point drying apparatus (Anderson, 1951). Dried samples were subsequently mounted on specimen stubs and coated with palladium/gold alloy (62 : 38) in an Edwards 306 vacuum coating unit and viewed with a JEOL JSM U3 scanning electron microscope. Representative areas were photographed.

Metal shadowing

A drop of bacterial suspension (in broth) was placed on a carbon coated 400 mesh, 3 mm copper E.M. grid and air dried. Gold/

palladium alloy (62 : 38) was evaporated onto the grid surface from a low angle ($\pm$ 15^{o}) in an Edwards 306 vacuum coater. The coated samples were observed in an Hitachi H U 11B transmission electron microscope. Representative bacteria were photographed.

4.3 RESULTS

4.3.1 Bacterial viability

After 3 days of aeration, part of an Achromobacter strain 14 culture was removed from aeration while aeration of the rest was continued. The viability (as determined by cell counts) of these two cultures differed (Fig. 4.1). Shaking cultures showed an immediate and near linear decline in viable count from 2.7 x 10^{9} to 1.5 x 10^{7} cells ml^{-1} after 18 days (0.55% survival). The viability of standing cultures was characterized by a shoulder from 3 to 7 days with only a very small decrease in viable count. After 18 days the viable count had dropped to 2 x 10^{8} cells ml^{-1} (8.33% survival).

4.3.2 Phage Adsorption

Phage α3a adsorption to Achromobacter strain 14 cells was not affected by shaking of the cultures or by the degree of aeration (Fig. 4.2). The % adsorption in standing and shaking cultures varied from 32 to 70% in 30 min but could not be correlated with differences in aeration.

4.3.3 RNA Synthesis

The incorporation of ^{3}H-uracil into TCA precipitable material (Fig. 4.3 a and b) showed that in both Achromobacter w.t. and

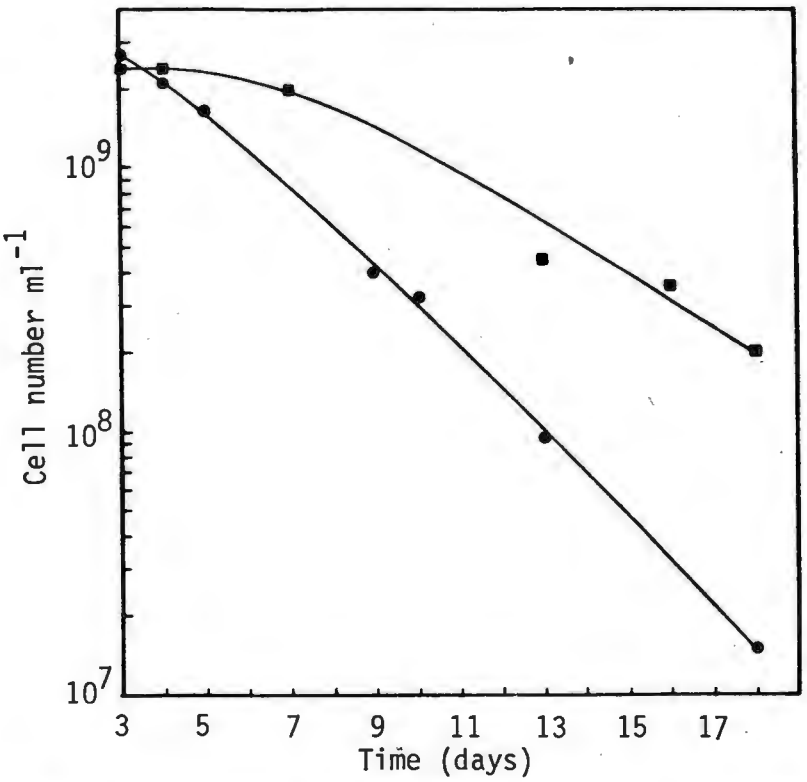


Fig. 4.1 Viability of shaking (●) and standing (■) stationary phase Achromobacter strain 14 cultures.

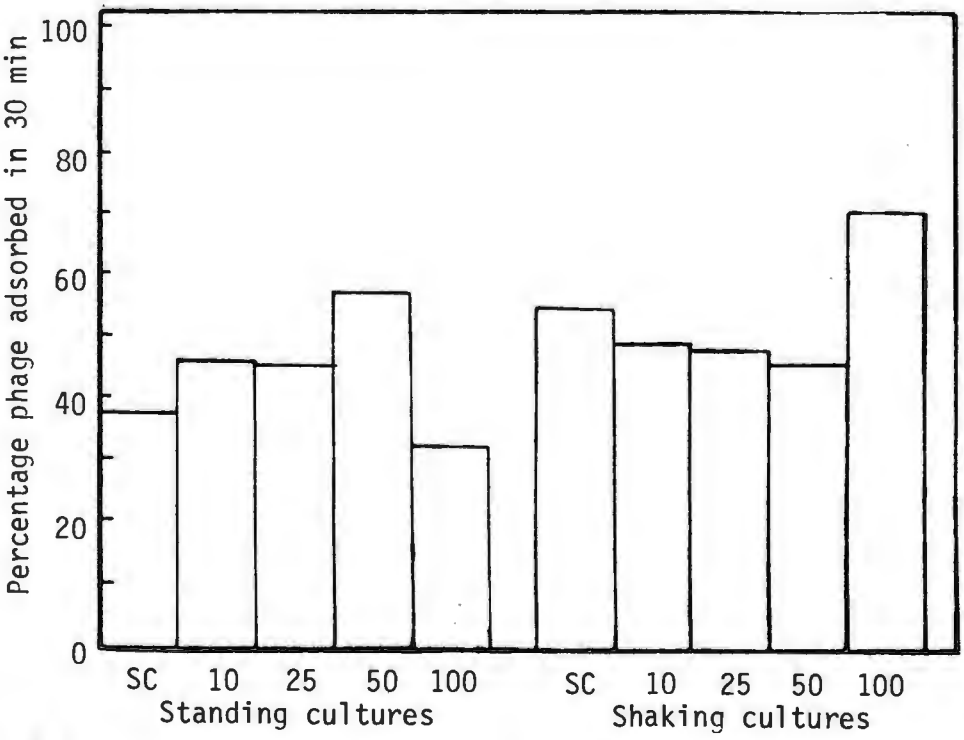


Fig. 4.2 Adsorption of phage α3a in standing and shaking Achromobacter strain 14 cultures of different volumes: 10 ml in a standard container (SC) and 10, 25, 50 and 100 ml in 100 ml flasks.

strain 14 cultures RNA synthesis was severely inhibited by aeration of stationary phase cultures (as reported by Woods, 1976, for strain 14). Incorporation of ^{3}H-uracil into DNA was negligible as shown by control experiments where samples were treated with 0.5 M NaOH 30 min before TCA precipitation.

4.3.4 DNA Synthesis

There was no detectable DNA synthesis in standing stationary phase Achromobacter strain 14 cultures (Woods, 1976). However, in shaking cultures a small but detectable amount of ^{3}H adenine was incorporated into DNA over a 60 min period (Fig. 4.4a).

These levels were compared to levels obtained in Achromobacter strain 14 in exponential growth phase (Fig. 4.4b). Corrections for the optical density of the suspensions were made. The levels of incorporation in the stationary phase culture were negligible compared to those in the growing culture. The actual counts show that DNA synthesis in the old shaking culture was only 0.88% of that in the exponential culture at 60 min.

4.3.5 Protein synthesis

The incorporation of ^{14}C-leucine into protein was measured in standing and shaking stationary phase cultures of Achromobacter strain 14. The standing cultures had been standing for 24 h before the leucine incorporation was undertaken whereas aeration of the shaking culture had continued. Protein synthesis was drastically reduced in the standing cultures compared to the synthesis in aerated cultures (Fig. 4.5). In all experiments (repeated 7 times) the protein synthesis in standing cultures did not exceed 4% of the level of protein synthesis in shaking cultures.

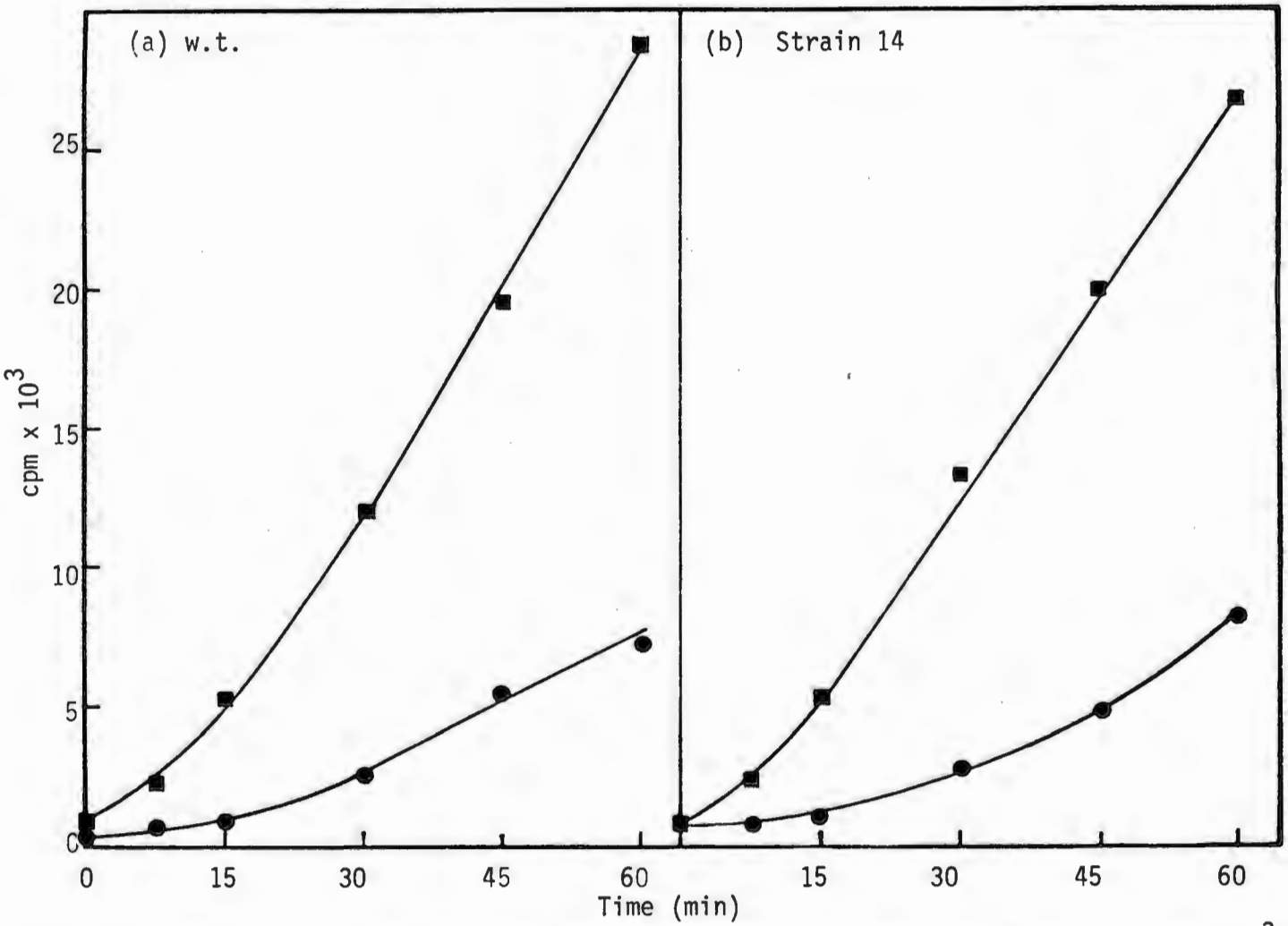


Fig. 4.3 RNA Synthesis in stationary phase Achromobacter cultures. Incorporation of ^{3}H-uracil into TCA precipitable material in standing (■) and shaking (●) cultures.

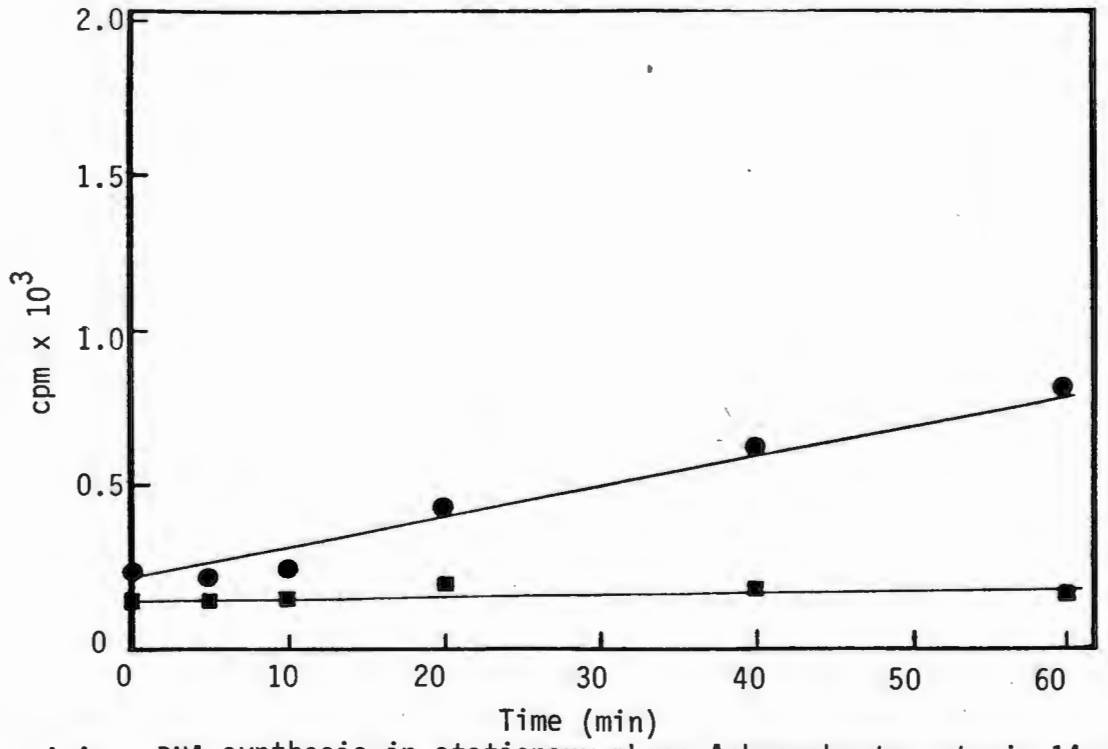


Fig. 4.4a. DNA synthesis in stationary phase Achromobacter strain 14 cultures. Incorporation of ^{3}H-adenine into TCA-precipitable material in standing (■) and shaking (●) cultures.

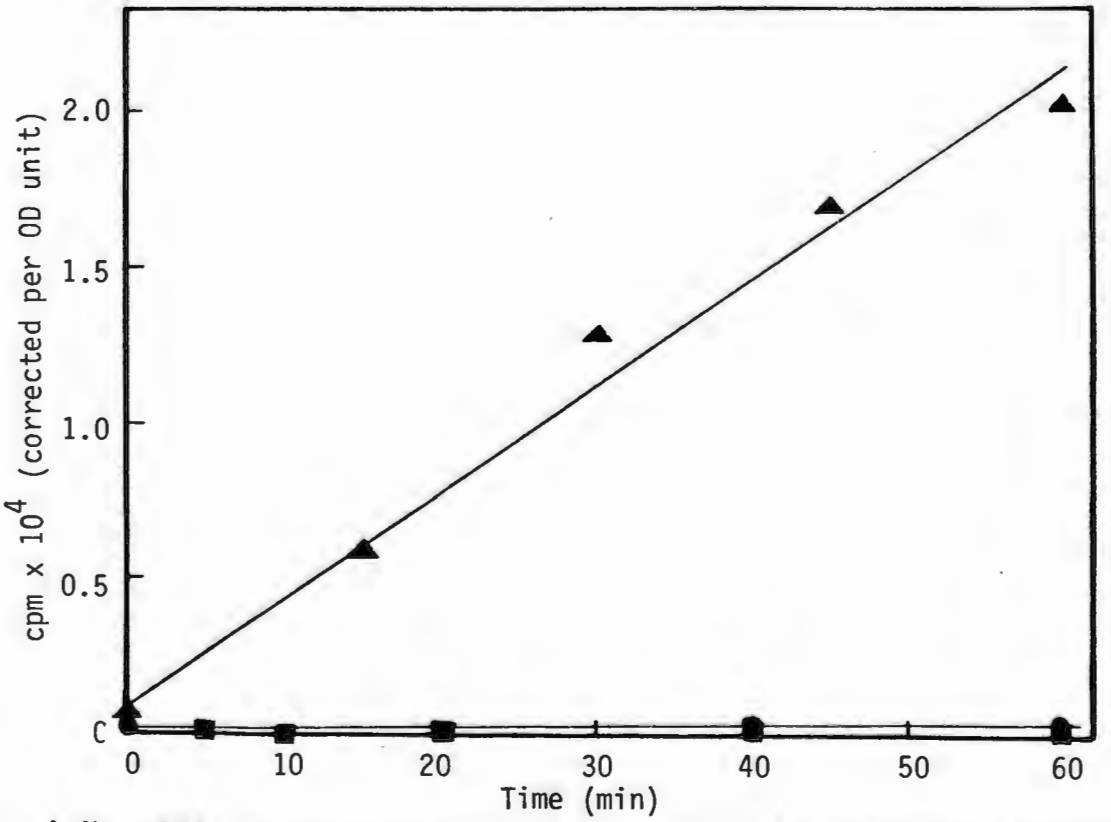


Fig. 4.4b DNA Synthesis in Achromobacter strain 14 cultures. Incorporation of ^{3}H-adenine into TCA precipitable material in exponential phase (▲) and stationary phase standing (■) and shaking cultures (●).

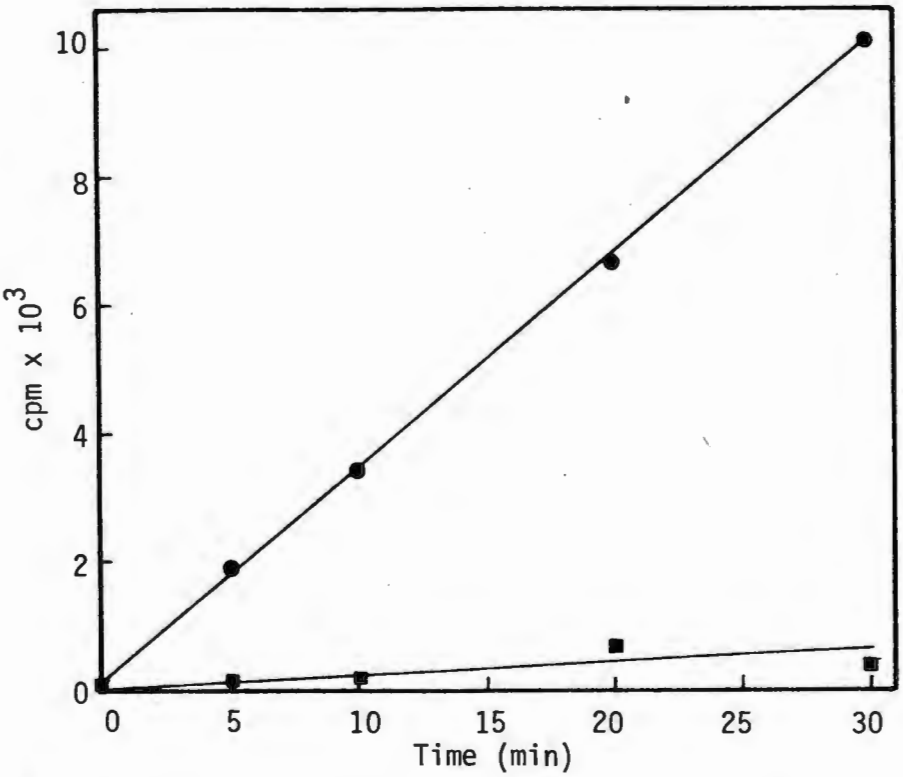


Fig. 4.5 Protein synthesis in stationary phase Achromobacter strain 14 cultures. Incorporation of ^{14}C-leucine into TCA-precipitable material in standing (■) and shaking (●) cultures.

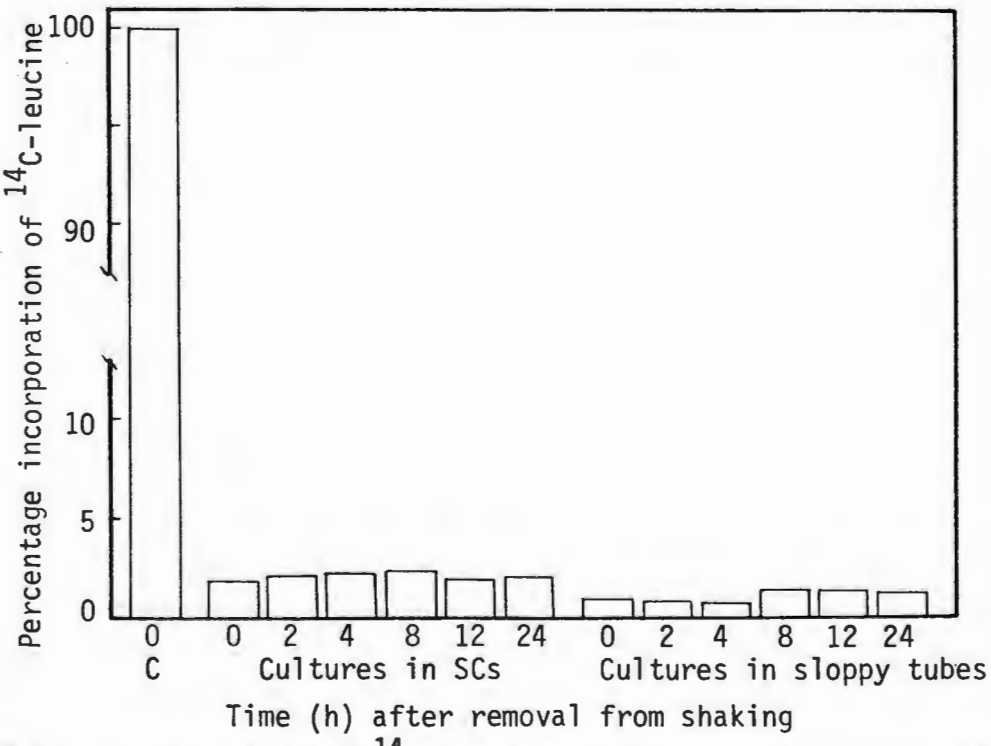


Fig. 4.6 Incorporation of ^{14}C-leucine in Achromobacter strain 14 stationary phase standing cultures at various times after removal from shaking. Incorporation measured over 30 min. Shaking of control culture (C) was continuous.

In order to determine whether this low level of incorporation was attained gradually during the 24 h standing period, the following experiment was carried out: A 4-day-old shaking culture was dispensed into standard containers (10 ml) and into sloppy tubes (3 ml) which were stood at 30°C. Incorporation of ^{14}C-leucine was measured over a 30 min period in samples which had been standing for 0, 2, 4, 8, 12 and 24 h.

Incorporation of leucine at time 0 (i.e. immediately after dispensing) showed that there was an immediate reduction in protein synthesis on standing (Fig. 4.6). From 0 to 24 h of standing there was no significant difference in the levels of protein synthesis. From an average of the radioactivity measured at different times over the 24 h standing period it was calculated that incorporation in sloppy tube cultures was reduced to 1% of the level obtained at 0 h in shaking cultures. In standard container cultures there was a reduction to 2% of the protein synthesis level in the shaking cultures.

A comparison with protein synthesis levels in exponential phase was made. Correcting for optical density, protein synthesis in shaking stationary phase culture was 38.5% of the level obtained in exponential cultures at 30 min. In standing cultures the level was 0.5% of that in exponential cultures.

4.3.6 Leucine Transport

The kinetics of leucine uptake were initially studied in exponential Achromobacter w.t. cells which had been grown in minimal medium. Uptake of leucine over a 30s period was measured with increasing concentrations of isoleucine in the assay mixture (Fig. 4.7). The kinetics of inhibition of leucine transport were similar to those

of E. coli. The major component of the transport system represented 90% of the total leucine uptake. This was inhibited by isoleucine and is a shared system for leucine and isoleucine uptake, analogous to the E. coli LIV system (Wood, 1975). The residual 10% of transport activity represented the minor leu specific system which probably has a high affinity for leucine. In Achromobacter, growth of the cells in a medium containing 50 mg of leucine ml^{-1} induced the major transport system (Fig. 4.7), whereas in E. coli it was repressed by growth in leucine (Wood, 1975). The shared transport system has a Km of approximately 2 μM leucine (Fig. 4.8) which is similar to that in the E. coli system (Wood, 1975).

Preliminary experiments showed that leucine uptake in exponential Achromobacter cells was slightly stimulated by growth of the bacteria in tryptone broth compared to growth in minimal medium (which is the usual procedure in uptake experiments). Incorporation was expressed as nMoles/OD unit/min and the values obtained for tryptone and minimal medium grown cells were 0.06815 and 0.05532 nMoles/OD/min respectively. It is likely that this was due to induction of the transport systems by leucine present in the tryptone broth.

Levels of active transport of leucine into stationary phase Achromobacter strain 14 cells were low in shaking cultures compared to the levels obtained in standing cultures (Fig. 4.9). At 120s cells from the standing culture had transported approximately 4-fold more ^{14}C-leucine across the membrane than cells from the shaking culture. In the absence of isoleucine this transport is brought about by the leu specific and LIV transport systems. In the presence of isoleucine in the assay mixture, leucine uptake was reduced to approximately 10% of the uptake measured without isoleucine in cells from both standing and shaking cultures. Protein assays showed that both

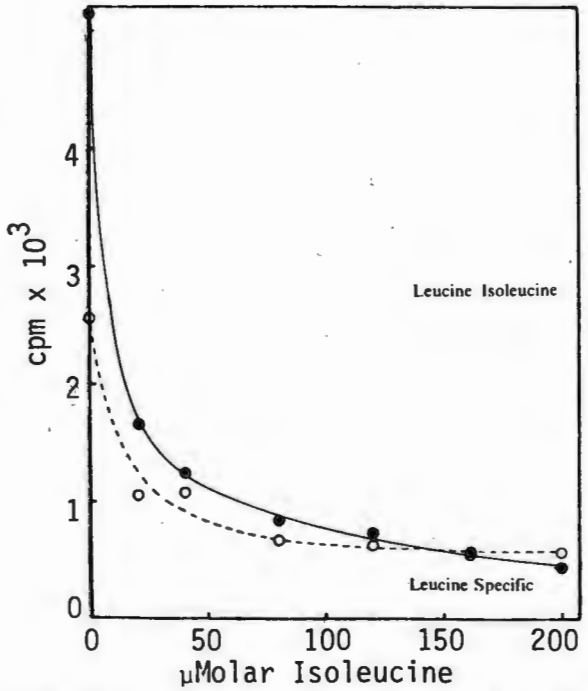


Fig. 4.7 The kinetics of leucine uptake of Achromobacter w.t. in the presence of increasing isoleucine levels. Uptake of ^{14}C-leucine by cells grown in the absence of leucine (o) and in the presence of 5 mg ml^{-1} leucine (●).

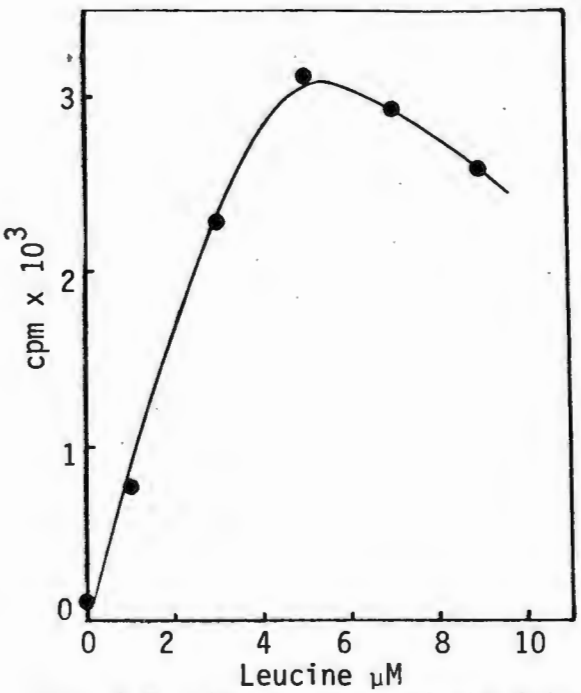


Fig. 4.8 The response of the Achromobacter major leucine transport system to increasing concentrations of leucine. Determined by the uptake of ^{14}C-leucine.

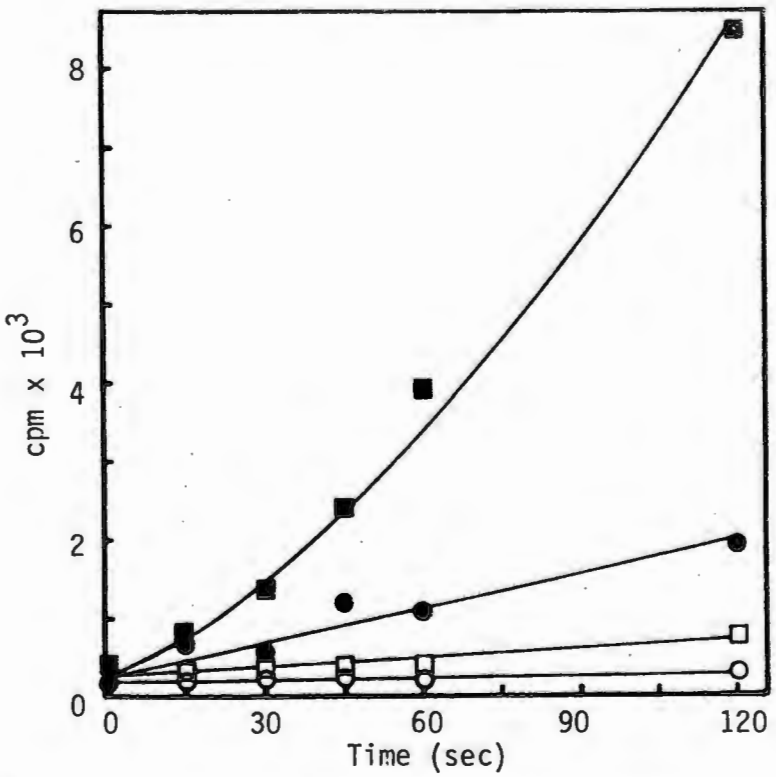


Fig. 4.9 Leucine uptake by Achromobacter strain 14 stationary phase cells. Measured in standing (■) and shaking (●) cells without ileu in the assay mixture and in the presence of ileu (200 µM) (□ and o, respectively).

standing and shaking cultures contained 500 μg ml^{-1} protein.

4.3.7 Cellular ATP Levels

The Luciferin-luciferase assay used to determine intracellular levels of ATP in Achromobacter strain 14 cultures was extremely sensitive and gave a linear response from 2.5 pMoles to 10 pMoles of ATP (Fig. 4.10).

The cellular ATP levels have been expressed as pMoles ATP mg^{-1} protein. The results obtained showed that the levels of intracellular ATP in stationary phase cells were affected by the conditions of aeration. ATP levels in shaking cultures were approximately 50% of those obtained in corresponding standing cultures (Table 4.1).

Table 4.1 Intracellular ATP levels in Achromobacter strain 14 cultures.

Culture	Intracellular ATP pMoles mg^{-1} protein	% of Exponential ATP level
Exponential	54.4	100%
Stationary standing	43.89	80%
Stationary shaking	23.25	42.7%

ATP levels in stationary phase standing cultures were 80% of those obtained in exponential cultures.

It should be noted that the concentrations of protein in standing and shaking cultures were identical (440 μg ml^{-1}).

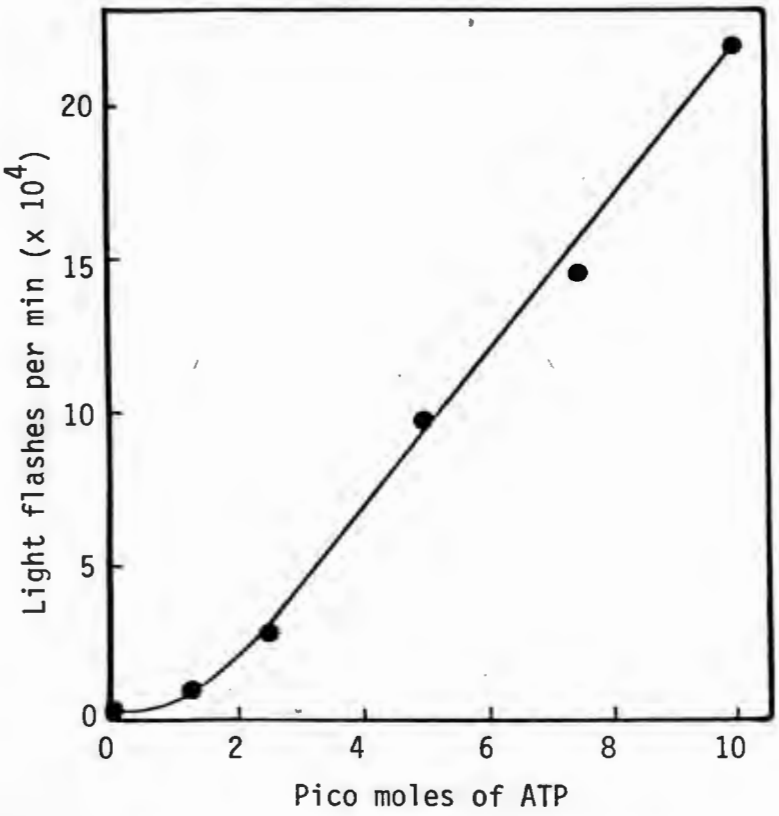


Fig. 4.10 Calibration for the Luciferin-luciferase ATP assay.

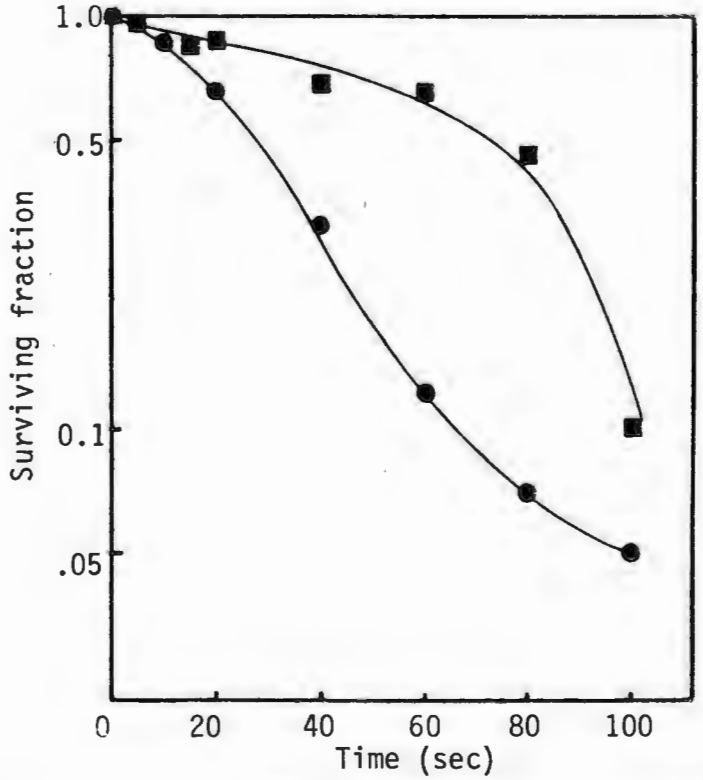


Fig. 4.11 Inactivation of Achromobacter strain 14 standing (■) and shaking (●) stationary phase cultures by UV irradiation.

4.3.8 UV Inactivation

Ultraviolet irradiation (2537 Å) was more effective in killing stationary phase Achromobacter strain 14 bacteria in cultures which had been aerated by shaking, than bacteria in cultures which had been incubated without shaking for 24 h (Fig. 4.11).

UV inactivation of standing cultures was characterized by a shoulder and 44% of the bacteria had been killed at 60s as compared with 99.9% of cells in a corresponding shaking culture. After this time, the rate of killing of standing cultures increased and 99.9% of cells were killed at 100s.

4.3.9 Heat Inactivation

Achromobacter strain 14 stationary phase shaking cultures were more sensitive to heat than standing cultures. After 30 min at 60°C the viable count for stationary phase standing cultures dropped from 1.7×10^7 to 6.3×10^3 cells ml^{-1}. Inactivation of shaking cells was greater, with a drop in viable count from 1.8×10^7 to less than 1×10^2 cells ml^{-1}.

4.3.10 Cell Morphology

Exponential and stationary phase Achromobacter strain 14 cells were examined by phase contrast microscopy.

Exponential cells were large motile rods and many dividing cells were present (Plate 4.1a). Shaking stationary phase cells were in comparison, small and non-motile (Plate 4.1b). After standing for 24 h these cells appeared to increase in size, became motile and tended to form long filaments of cells linked end to end (Plate 4.1c). An interesting observation was that standing cultures examined 12 h after the addition of phage were even larger in size and no long filaments were observed (Plate 4.1d).

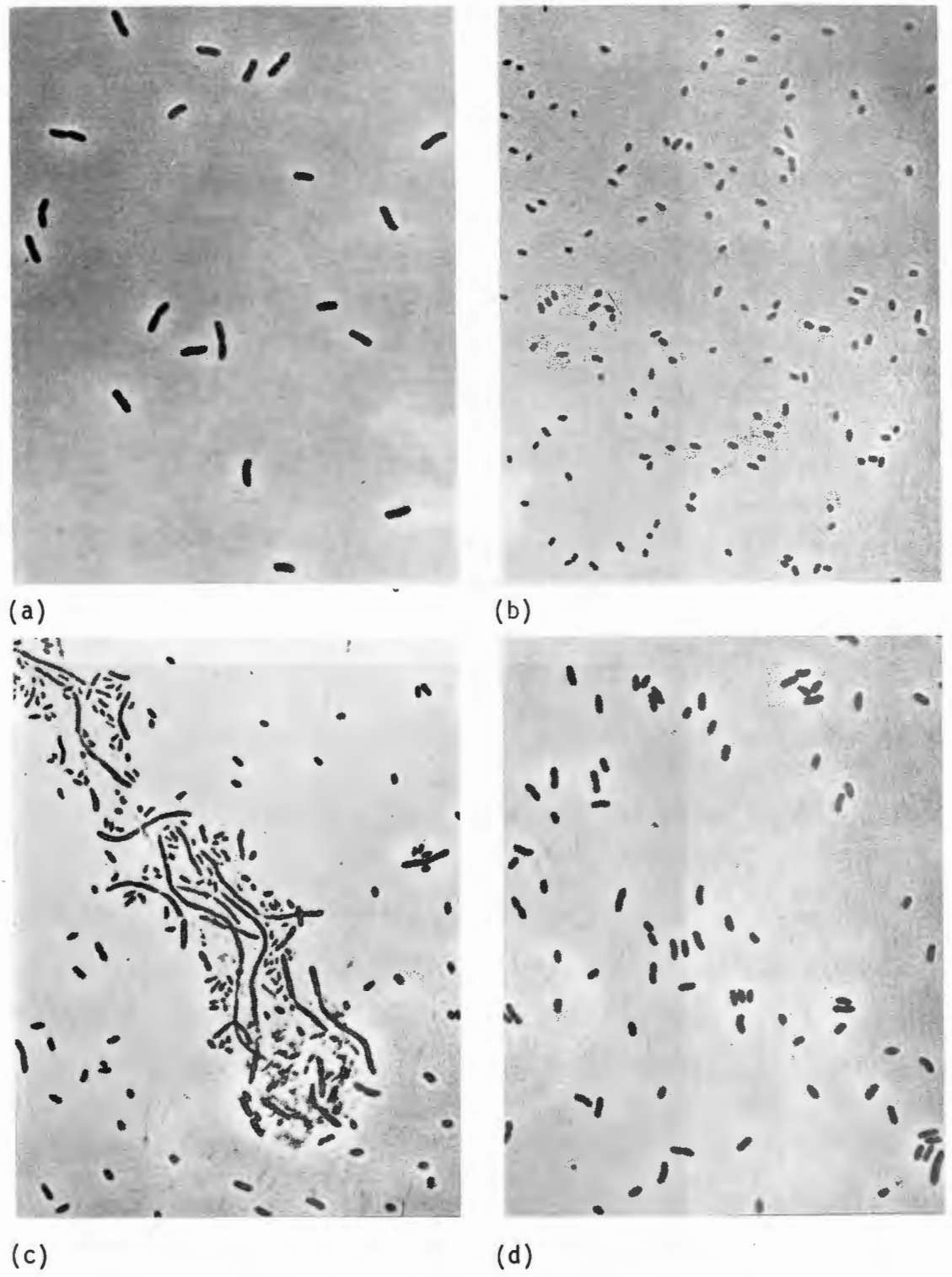


Plate 4.1. Achromobacter strain 14 cells. (a) Exponential phase cells, (b) shaking stationary phase cells, (c) standing stationary phase cells, (d) standing stationary phase cells 12 h after addition of phage α3a. Examination by phase contrast microscopy, magnification 1000 x.

(a)

(b)

1 μm

Plate 4.2. Electron micrographs of metal shadowed stationary phase Achromobacter strain 14 cells. (a) Shaking culture, (b) standing culture.

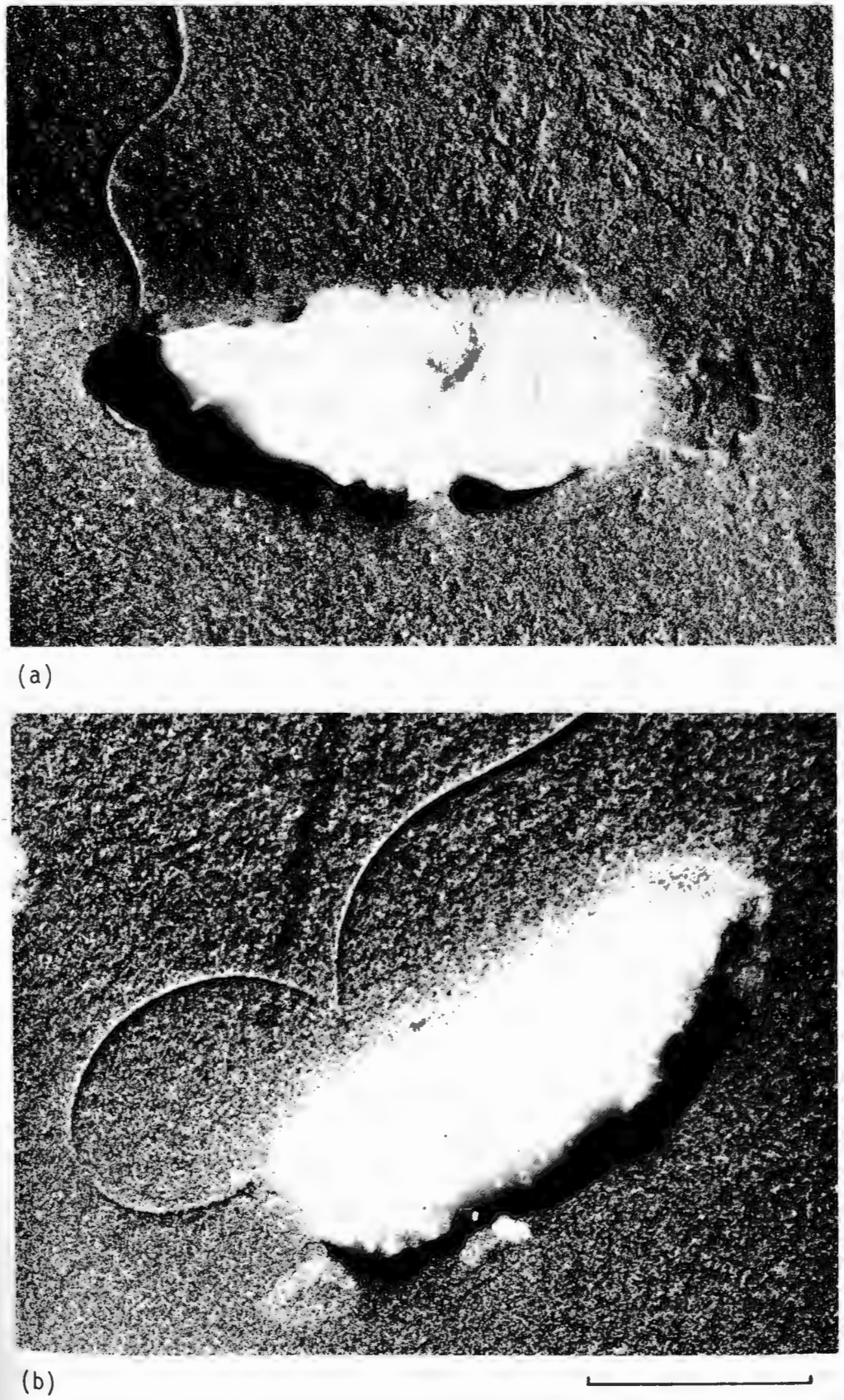


Plate 4.3. Electron micrographs of metal shadowed stationary phase Achromobacter strain 14 cells. (a) 1.5 h and (b) 3 h after removal from shaking.

In electron micrographs of metal shadowed bacteria, shaking stationary phase cells appeared as small (from 1.1 μm - 1.5 μm in length and c.0.7 μm in width) collapsed cells with no flagella (Plate 4.2a). The collapsed appearance may be an artefact brought about by the processing of the cells. After standing for 24 h, these small cells developed into larger cells (c. 3 μm in length and 1.25 μm in width) which possessed flagella (Plate 4.2b). Plates 4.3a and b show that cells which had been removed from shaking for 1½ and 3 h respectively had increased in size and possessed flagella.

Scanning electron micrographs (Plates 4.4 and 4.5) show stationary phase shaking cultures (a), standing cultures (b) and standing cultures 16 h after the addition of phage (c). The increased size (particularly in width) and the filament formation of the standing culture are shown clearly by this method of examination. The abundance of flagella produced a cobweb effect in the standing culture electron micrographs There was (as observed by phase contrast microscopy) an increase in size and an absence of filaments in the standing cultures which had been treated with phage (4.4 and 4.5c).

DISCUSSION

A comparative study of stationary phase Achromobacter strain 14 cells has revealed that gross changes in the physiology and morphology of the cells are brought about by removal from aeration (summarized in Table 4.2). These studies were primarily undertaken in an effort to determine the reason(s) why phage α3a development was inhibited by aeration of stationary phase cells. Throughout this discussion 'standing cells' refer to 4 - 5 day old cells which have been standing for 24 h,

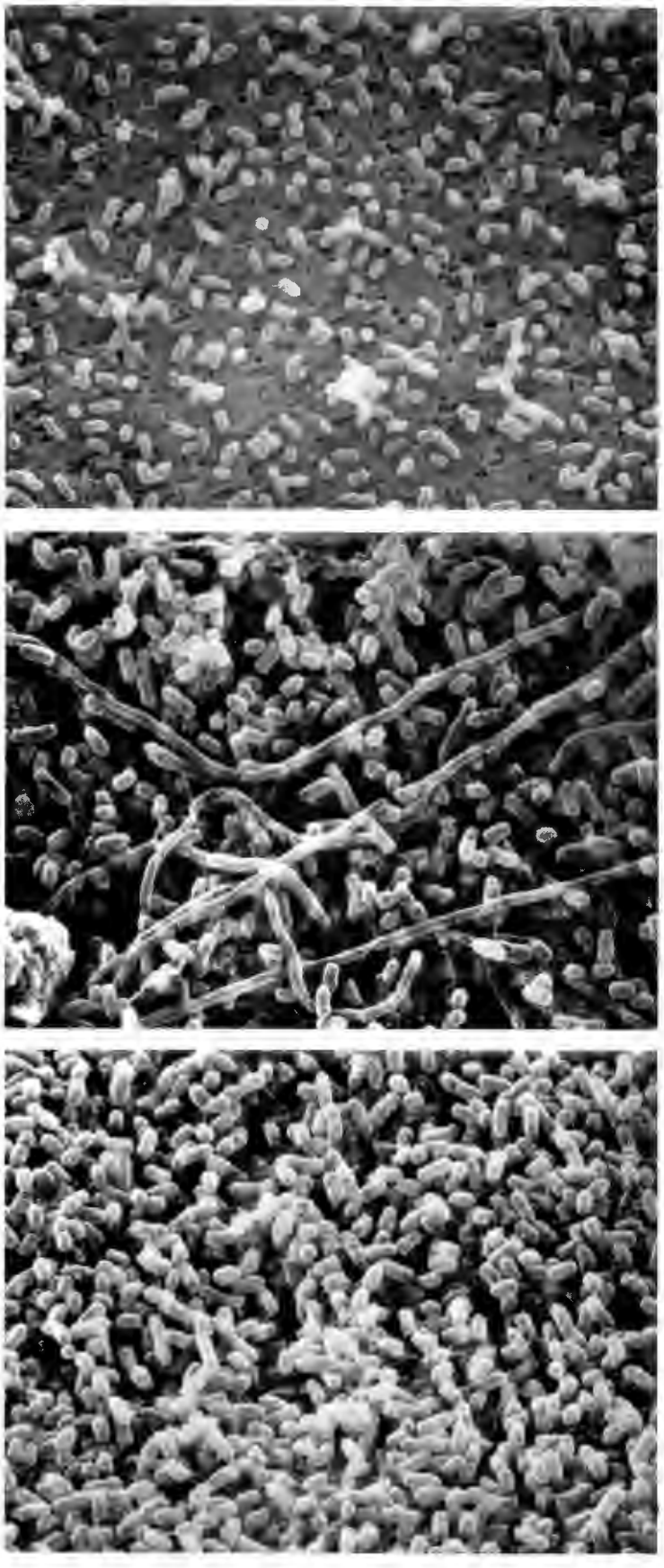


Plate 4.4. Scanning electron micrographs of stationary phase Achromobacter strain 14 cells from (a) a shaking culture, (b) a standing culture, and (c) a standing culture 16 h after addition of phage α3a.

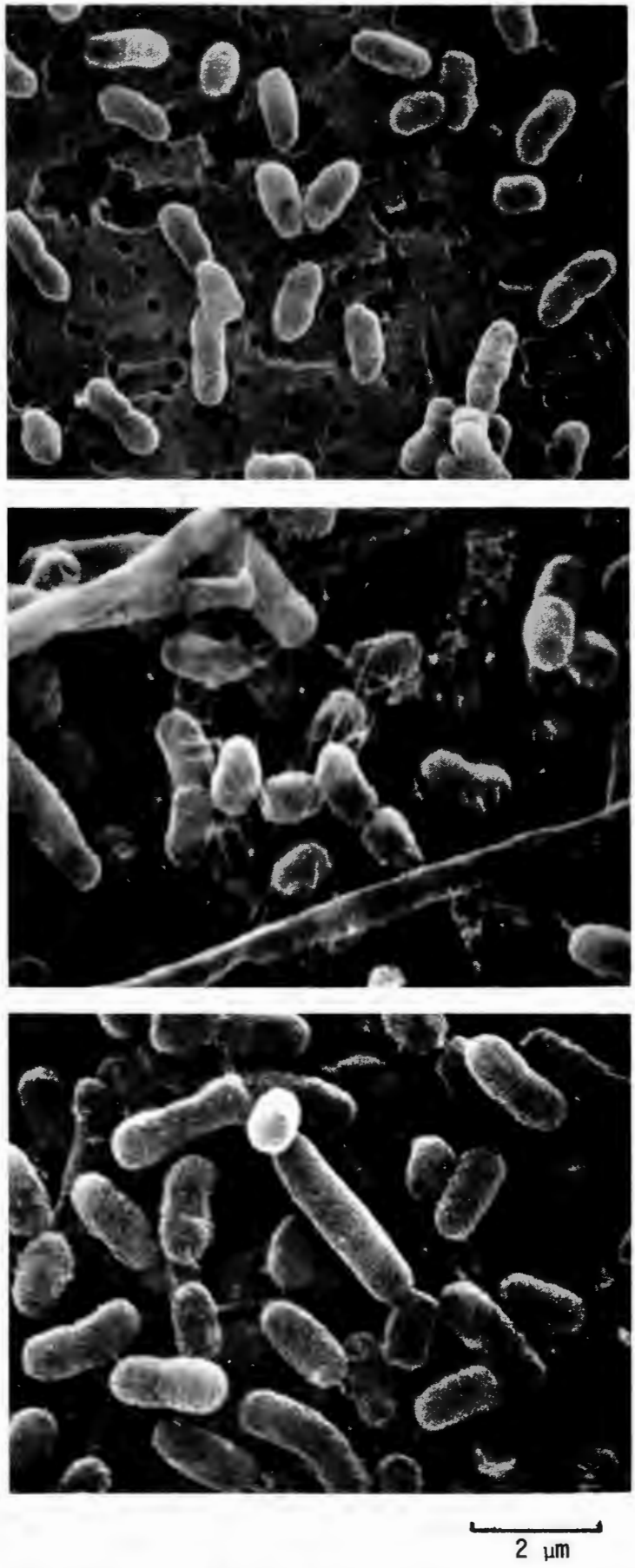


Plate 4.5. Scanning electron micrographs of stationary phase Achromobacter strain 14 cells from (a) a shaking culture, (b) a standing culture, and (c) a standing culture 16 h after addition of phage α3a.

Table 4.2 Characteristics of standing and shaking stationary phase Achromobacter strain 14 cultures.

	Shaking culture*	Standing culture$^{\phi}$
Viability of cells	+	++
Adsorption of phage α3a	+	+
RNA synthesis	+	+++
DNA synthesis	+-	-
Protein synthesis	+++++	+
Active transport	+	++++
Intracellular ATP	+	++
Resistance to UV irradiation	+	++
Heat resistance	+	++
Cell morphology	small non-motile	large motile

* 4-5-day-old cells which have been aerated continuously

$^{\phi}$ 4-5-day-old cells which have been standing for 24 h

and 'shaking cells' to cells whose aeration has been continued.

The first important characteristic to be considered is phage adsorption. Phage adsorbed equally well to aerated and standing stationary phase cells. Therefore, the inhibition of phage development in shaking cultures is not due to surface exclusion. This is supported by the fact that a drop of 74% of free phage occurred after 24 h in an aerated stationary phase Achromobacter strain 14 culture (see Table 2.4).

Interesting changes from shaking to standing cells involved macromolecular synthesis of nucleic acid and protein. There was no DNA synthesis in standing cells. However, in shaking cells there was a very small amount of incorporation of ^{3}H-adenine into DNA which was linear over 60 min. The incorporation was less than 1% of that obtained in exponential cells (correcting for OD). The significance of this is unknown at present. With respect to phage development in Achromobacter strain 14, it could be argued that since the shaking cells are synthesizing DNA they are growing slowly and since α3a development is inhibited in exponential cells, this would account for the inhibition in old shaking cells. This does not seem to be the case because even Achromobacter w.t. cells (which do not inhibit phage during exponential growth) do not support phage in stationary phase shaking cultures. One explanation for a very limited amount of DNA synthesis in shaking cultures may be that there is an increase in lysis of old cells and subsequent growth by a limited number of others. This would correlate with the fact that shaking cells lose viability more rapidly than standing cells over a period of 18 days. However, if there is regrowth in a small proportion of the shaking cells it does not compensate for the decline in viable cells.

From measurements of total RNA synthesis in shaking and standing stationary phase Achromobacter cells, it is apparent that removal from aeration stimulates incorporation of uracil into RNA (as reported by Woods, 1976). It is not known whether this stimulation represents proportionate increases in mRNA, rRNA and tRNA, whether one class is favoured or what role degradation of RNA plays in this apparent stimulation. It is likely that the increase observed in RNA synthesis caused by cessation of aeration is due to changes in regulation of stable RNA, since mRNA usually constitutes a minor fraction of total bacterial RNA (2 to 4.5% of total RNA in E. coli is mRNA; Forchhammer and Lindahl, 1971; Norris and Koch, 1972) which is independent of growth rate. Stable RNA, rRNA and tRNA constitute 73 to 80% and 15 to 24% of total RNA in E. coli, respectively (Norris and Koch, 1972).

It has also been shown that cellular energy levels increase in standing cultures. This was measured directly by assaying for cellular ATP and indirectly by measuring leucine uptake as an index of cellular energy levels. The level of ATP in shaking cells was only 50% of the level found in standing cells. Hansen et al. (1975) showed that breakdown of ppGpp in E. coli cells was facilitated when available energy was high. The level of ppGpp in the cell is dependent on the relative rates of ppGpp synthesis and degradation (Hansen et al., 1975). This means that the effect of ppGpp on transcriptional specificity (in particular stable RNA) is geared to both protein synthesis and energy metabolism. In stationary phase Achromobacter cultures removal from aeration results in an increase in RNA synthesis and an increase in the cellular ATP level. Since synthesis of rRNA in vivo is inversely correlated with ppGpp concentrations (Cashel, 1975), it is possible that in standing stationary phase Achromobacter cultures the

levels of ppGpp are decreased due to the higher energy levels. This may result in increased transcription of stable RNA genes. However, it must be taken into account that control of macromolecular synthesis and the "growth of a bacterium,even as simple as E. coli, is dependent on several thousands of reactions and the functions of hundreds of individual control mechanisms" (Nierlich, 1978). Furthermore, evidence suggests that control mechanisms in stationary phase cells may be distinct from those operating in growing cells (Koch and Deppe, 1971).

During growth, bacterial cells respond to changes in the environment by increasing or decreasing RNA and protein proportionately over the same period of time (Mandelstam, 1960). The finding that protein synthesis showed an opposite response to that of RNA when stationary phase Achromobacter cells were removed from aeration was somewhat unexpected. However, this finding may lend further support to the idea that regulation of macromolecular synthesis in stationary phase cells is very different to that during growth (Nierlich, 1978). Of all the physiological characteristics, protein synthesis was found to be the most profoundly affected by removal from aeration. In all experiments the protein synthesis levels observed in standing cells did not exceed 4% of the levels obtained in shaking cells. This undoubtedly represents a gross change in regulation at the level of protein synthesis.

Initially protein synthesis was measured after the cultures had been standing for 24 h and it was thought that rates of protein synthesis probably decreased gradually over this time. However, it was found that removal from shaking had an immediate inhibitory effect on ^{14}C-leucine incorporation. This rapid adaptation is a feature of the flexibility of stationary phase cells. Koch and Deppe (1971) showed

that the specific rate of protein synthesis increased by a factor of 7 in 2 min, after enrichment of a slowly growing E. coli culture - an adaptation opposite to the one described here.

It is important to remember that in this system, protein synthesis has been measured by the incorporation of exogenous ^{14}C-leucine. The results show that in standing cultures incorporation is reduced to very low levels. This does not necessarily mean that cellular protein synthesis has been reduced to negligible levels, since other factors such as amino acids pools and protein turnover (Nath and Koch, 1971) probably play a major role. Attempts have been made to measure protein turnover in standing and shaking stationary phase cells. Essentially the methods involve prelabelling of the cellular protein, washing out excess label, resuspension in an excess of the corresponding cold amino acid and determining the release of labelled amino acids. This is a measurement of the rate of protein degradation which is equalled by the protein synthesis rate (Mandelstam, 1968). Since protein synthesis in stationary phase Achromobacter cultures responded so rapidly to levels of aeration, it was desirable to find a method for measuring turnover which involved minimal manipulation of the cells. As yet, a method which adequately represents turnover under these conditions has not been developed. It seems highly likely that the changes in leucine incorporation measured do represent alteration in protein turnover.

The reduced incorporation of ^{14}C-leucine into protein in stationary phase standing cells was not due to the inability of the cells to transport leucine across the membrane. In fact, standing cells were found to have the capacity for far greater leucine uptake than shaking cells. Since the leucine uptake assay involved centrifugation, washing and final suspension in buffer, which undoubtedly aerated the cells, it

must be stressed that the levels of leucine uptake may not be representative of what is actually taking place under the standing conditions. However, they do indicate that both membrane potential and uptake systems exist in standing cells. It is possible that accelerated transport systems may compensate for possible low turnover levels and thus supply the cells with leucine for de novo protein synthesis.

Another criterion used to measure changes in the physiology of stationary phase cells, was their sensitivity to inactivation by UV radiation and heat. Standing cells were more resistant to inactivation by UV and heat than were shaking cells.

The response of a bacterial cell to a dose of UV irradiation is not simply a function of its inherent sensitivity but is also dependent on the repair processes to combat UV induced damage. It is well known that the repair of DNA damaged by far UV irradiation can be altered by changes in the physiological state of the cell (Tyrrell, Moss and Davies, 1972a; Peak, 1970). Bacterial cells possess at least three different repair mechanisms: the light dependent photoenzymatic repair and the two dark repair systems, excision and recombination repair. Tyrrell et al. (1972a), measured the repair capacity at different stages of growth in a number of E. coli strains. Excision repair and light dependent repair were less efficient in exponential cells. In a later study Tyrrell, Moss and Davies (1972b), correlated the decrease in light dependent repair to a decrease in the number of active photo-reactivating enzymes. There are two possibilites for the reduced sensitivity to UV inactivation in standing Achromobacter cells: That the sensitivity of the DNA itself is reduced or that there is an increase in the number of active repair enzymes, brought about by an increase in transcription of the repair genes. The small amount of

DNA synthesis observed in shaking cells compared to no DNA synthesis in standing cells may result in greater susceptibility to UV damage in shaking cells. Whether or not this DNA synthesis is sufficient to account for the difference in UV sensitivity is not known.

The killing of bacterial cells by heat is as a result of denaturation of protein (Davis et al., 1973). Standing stationary phase cells were more resistant to heat than shaking cells. If we consider the denaturation by heat of one particular enzyme there are three possible ways in which it could become more resistant to heat. Either it could become intrinsically more stable (the stability of proteins can alter with changes in the physiological state of the cell; Pine, 1972) or there could be more copies of protein present, or the rate of resynthesis of new copies may be increased. Since no measurements of the stability of individual enzymes have been made in stationary phase Achromobacter cells, it is impossible to say if one or any of the above possibilities is instrumental in altering the sensitivity of stationary phase cells to heat. In nature bacterial cells probably spend most of their time in the stationary phase state and the durability of cells in this state is an interesting aspect of survival. In addition, vigorous aeration is unlikely to occur in nature so the non-aerated stationary phase cultures are probably more representative of the natural state of the cells.

Achromobacter stationary phase cells also show gross changes in the morphology of the cells from shaking to standing conditions. Under standing conditions the cells appeared to increase in size, develop flagella and form long filaments of cells linked end to end. Since the concentrations of protein in standing and shaking cultures were identical, these morphological changes must show a reorganization of the available cellular components. These alterations in response to

the environment are likely to be brought about by the turnover of macromolecules (Mandelstam, 1968). The production of flagellin molecules, as demonstrated by the formation of flagella, is likely to be brought about by changes in transcriptional specificity such that the genes coding for flagellin are transcribed. Stationary phase cells are capable of adapting to the environment by changes at the transcriptional level (Mandelstam, 1957) and bacterial endospore formation is a morphological example of this type of differentiation (Pine, 1972).

Considering all the physiological aspects which have been studied, it appears that vigorous aeration of Achromobacter stationary phase cultures has a detrimental effect on the cell. Aeration adversely affects viability, renders the cells more sensitive to heat and UV, morphologically they look less 'healthy' than standing cells and have low energy levels. The high rate of protein synthesis in aerated cells is one aspect which does not seem to fit the general pattern. Since the ATP levels are low it seems that these elevated levels of protein synthesis may represent a 'sick' uncontrolled protein synthesis (similar to 'relaxed' mutants; Travers, 1976). It is highly likely that vigorous aeration of stationary phase cells (which is an unnatural state) impairs the protein synthesizing regulatory mechanisms such that synthesis continues unabated and wastefully utilises the cellular energy. Since degradation of protein in stationary phase cells probably continues at the same rate as synthesis there would be a futile cycling of amino acids. Standing cells however, rapidly become more resistant to the killing effects of heat and UV, and resemble exponential phase cells in morphology. This may indicate that although the protein synthesis declines, the proteins synthesized are regulated such that only appropriate proteins are made.

How could these observed changes in the physiology of stationary phase Achromobacter affect phage development? In evaluating this, it must be remembered that both Achromobacter w.t. and strain 14 inhibit phage development in stationary phase shaking cultures. Therefore the proposal of any model must apply to both strains.

With respect to phage growth in Achromobacter w.t.: Achromobacter sp.2 (w.t) contains a cryptic prophage (Thomson and Woods, 1974) which is not inducible and does not cause superinfection immunity. Superinfecting α3a can proceed with the vegetative growth cycle and produce phage progeny in exponential and stationary phase standing cultures. This means that in both these states all the genes required for phage development are transcribed. These obviously include the phage genes, are very likely to include a number of host genes (even virulent phage T4 is highly dependent on host functions; Takahashi, 1978) and may include some genes of the cryptic prophage. In addition, cellular conditions allow for phage morphogenesis and subsequent cell lysis with release of phage progeny. Thus, if there are any differences in transcriptional specificity in exponential phase and stationary phase standing cells they do not prevent phage growth.

Why is α3a development inhibited in w.t. stationary phase shaking cultures? There are numerous possibilities:

(i) As indicated by the physiological and morphological changes from shaking to standing, one possibility is that the transcriptional specificity in shaking cells is different to that in standing cells and genes required for phage development are not transcribed.

(ii) ATP levels in the cell may not be high enough for phage replication. DNA replication requires high levels of ATP (Moses and Richardson, 1970). There are indications that the establishment of lysogeny by temperate phages P22 and λ is influenced by cellular levels of cAMP (Hong, Smith and Ames, 1971; Grodzicker, Arditti and Eisen, 1972). Since the physiology of stationary phase Achromobacter is affected by aeration perhaps levels of cAMP alter, making lysogeny the preferred choice for α3a in stationary phase shaking cells.

(iii) There is one important aspect in phage infection of stationary phase cells which must be considered. Successful and productive infection of stationary phase cells (in which there is continuous synthesis and degradation of proteins) may rely on the ability of the phage to regulate cellular protein degradation. If degradation was not inhibited it is possible that pools of phage proteins would not accumulate. The rapid rate of protein degradation which is thought to occur in shaking cultures could contribute to inhibition of phage production. Stationary phase standing cells on the other hand show low levels of protein synthesis and probably have equally low levels of degradation. Productive phage development in these cells may be due to the ability of the phage to curtail this degradation or because the degradation is low enough not to affect phage protein accumulation.

The above three speculations may also apply to α3a infection of stationary phase Achromobacter strain 14 cells. As stated previously (1.1), the characteristics of strain 14 (a transductant) probably result from complementation between the resident cryptic prophage and residual functions in the transducing particle. It is quite likely that through this complementation α3a immunity products are formed. These may be produced abundantly during exponential growth and thus inhibit superinfecting α3a. In stationary phase, it is possible that due to alterations in the transcriptional patterns (which have been found for B. subtilis, see 6.1), immunity genes are not transcribed sufficiently and thus superinfecting phage is not repressed. It is likely that phage α3a is inhibited in shaking stationary phase Achromobacter strain 14 cultures for the same reasons as it is in w.t. cells and this inhibition is independent of interference by the defective prophage(s).

In conclusion, fundamental physiological differences exist between shaking and standing stationary phase Achromobacter cells. Some or all of these may influence the inhibition of phage production which is observed in shaking cultures.

CHAPTER V

DEFECTIVE LYSOGENY, PSEUDOLYSOGENY AND PHASE VARIATION IN ACHROMOBACTER STRAIN 14

5.1 INTRODUCTION

Genetic aberration in procaryotes may be caused by point mutations, deletions, duplications, inversions and transpositions. In addition, the fusion of genetic elements by additive recombination (without loss of genetic material) represents another class of aberration (Starlinger, 1977). The fusion of DNA molecules results from the ability of genetic elements, such as phages, plasmids, IS elements and transposons to insert at different sites in chromosomal or plasmid DNA (Schwesinger, 1977).

By inexact excision of phage DNA after induction (in the case of phage λ), or by multiple reinsertions at numerous sites on bacterial DNA molecules during the lytic cycle (in the case of phage Mu; Razzaki and Bukhari, 1975), phage particles can contain bacterial DNA at the end(s) of the genome.

The phenomenon of λ gal escape synthesis is a very good example of bacterial-phage DNA fusion in which the expression of the bacterial DNA falls partially under the phage control mechanisms. The primary λ integration site on the E. coli chromosome is between the gal and bio markers (Schwesinger, 1977). The gal operon is negatively controlled by the gal repressor and is induced (positive control) by galactose or fucose. RNA polymerase, cAMP and CAP protein are required for the

initiation of transcription (de Crombrugghe et al., 1973). Adhya et al. (1974) found that the induction of λ prophage resulted in the derepression of the gal operon, overriding the normal gal control mechanisms, i.e. constitutive cAMP independent gal enzyme synthesis. This was called λ gal escape synthesis. Escape synthesis required the activation of the λ sex promoter (PIℓ) and appeared to be mediated by the anti-rho action of the λ N gene product.

Early regulatory events in λ development, particularly regulation of the N gene, were studied using a system in which λ was fused to the tryptophan operon of E. coli. Non-defective, trp-transducing phages (λ trp) were derived from E. coli carrying a λ-φ80 hybrid prophage inserted adjacent to the trp operon and orientated in the same direction. Control of the expression of trp operon in transductants depended on whether the trp operator and promotor had been conserved. Expression of the trp genes in transductants with deleted trp promotors was as a result of read-through from phage genes. Thus, it was subject to repression by the λ immunity repressor (CI) and required the λ N product (Franklin, 1971).

Reznikoff et al. (1969) described a genetic system in which deletions were selected which fused the lac operon to the trp operon in E. coli. The operons were fused in such a way that the lac operator remained intact and transcription initiated at the trp promoter resulted in read-through into lac.

Achromobacter strain 14 was isolated as a trp^+ transductant (see 1.1). Trp^- recipients were transduced by α3a to trp^+ and since the transductants were unstable it was proposed that integration occurred by lysogeny rather than by a swopping event. However, no HFT lysates were found. A number of transductants tested showed varying sensitivity

to superinfecting phage suggesting that the transducing particles contained varying amounts of phage DNA (Woods and Thomson, 1975). Achromobacter strain 14 was initially resistant to superinfecting phage but after 4 clonings on minimal medium became 'semi-sensitive' (Woods, 1976). During this study the 'semi-sensitivity' has been found to be an unstable characteristic. Since the trp genes were presumably fused to phage genes in the transducing particle and may have remained associated in the transductant, it was thought that expression of the trp genes may affect expression of the phage genes or vice versa. This has been studied with respect to the stability of the semi-sensitive nature of strain 14 and the effect of trp expression on phage development.

The phenomenon of phase shift or phase variation has been reported in a number of systems. Zieg et al. (1977), Burt et al. (1978) and Neubauer and Calef (1970) described phase variation of flagella formation, colonial morphology and λ immunity, respectively.

Salmonella strains have the ability to switch from producing one flagella antigen (phase) to producing another. This phase variation occurs with a small probability which is two to three fold higher than the frequency of mutation. The structure of the flagella is specified by two genes, H1 and H2. The ability to switch from the expression of one gene to the other is linked to the H2 locus. When the H2 gene is turned on, H1 is turned off, but when H2 is inactive, H1 is expressed (Zieg et al., 1977; Silverman et al., 1979b). Zieg et al. (1977) and Silverman et al. (1979a) analysed the switch mechanism in a cloned DNA segment carrying the H2 gene and found it to be an invertible segment (c 800 base pairs) adjacent to the H2 gene. In one orientation, 'flip', H2 expression was turned on, whereas in the 'flop' orientation it was turned off. Evidence suggested that the H2 promoter is included in the inversion

region.

A similar mechanism has been postulated for the formation of infective and non-infective phage Mu particles (Bukhari and Ambrosia, 1978). The Mu genome contains heterogenous host sequences at both ends and a 3000 base sequence (the G segment) near the right end which can undergo inversion. Inversion occurs in the prophage state, is independent of the host recA functions and is thought to occur by the recombination between identical but inverted sequences of about 50 base pairs flanking the G segment. Approximately half the Mu particles resulting from induction contain the G segment in one orientation (flip) and are infective. The others contain the G segment in the 'flop' orientation and are non-infective. Howe, Schum and Taylor (1979) have shown that two essential genes, S and U (which appear to be involved in phage adsorption) are located in the invertible G segment.

Many polar mutations in bacteria have been found to be caused by insertions of short DNA sequences. These insertion sequences (IS) range from 800 to 1400 base pairs long. IS1 and IS4 exert polar effects when inserted in both orientations (I and II) but IS2 and IS3 exert polar effects only when inserted in orientation I. In orientation II, the IS2 element exerts a positive effect on gene expression (Shapiro, Adhya and Bukhari, 1977). Saedler *et al.* (1974) showed that, depending on its orientation, IS2 insertions in *E. coli* can serve to 'turn on' and 'turn off' genes. A number of mutations in λ have been found to be associated with the IS2 insertion element (Zissler *et al.*, 1977). One, the crg insertion, has a polar orientation and may have a direct influence on excision causing the formation of cryptic (cry) prophage (Fischer-Fantuzzi and Calef, 1964).

Another phase variation phenomenon, immunity phase shift, has been reported for defective λ prophages (Neubauef and Calef, 1970; Calef et al., 1971). The λ prophages contained two defects, one in the N gene and one in the early O or P genes concerned with DNA replication. Lysogens carrying the defective prophages could exist in two distinct regulatory phases, the im^+ phase and the im^- phase. In the im^+ phase, immunity against superinfecting homoimmune phage was present and early gene functions were repressed. The im^- phase was characterized by the absence of immunity, derepression of some early genes and channelling of superinfecting homoimmune phage towards the lytic cycle. The λ immunity phase shift was of a non-mutational nature since it was hereditary for the whole line of cells which were able to change back and forth between the phases.

It was proposed that the phases corresponded to the two possible alternatives that a temperate phage can display: lysogeny or lytic development. The im^- phase corresponding to the early stage of the prophage decision towards the lytic cycle. Due to the defective nature of the prophage it was unable to replicate and kill the host and was thus 'fixed' in this early stage for many generations. Thus, the phase shift was the result of two mutually antagonistic systems of negative control, viz. the CI repressor protein and its antagonist the antirepressor cro product (Oppenheim, Neubauer and Calef, 1970.)

Numerous phage-host relationships have been described which represent an intermediate state between true lysogeny and vegetative growth (Anderson and Cowles, 1958; Arditti and Coppo, 1965; Bott and Strauss, 1965; Romig and Brodetsky, 1961). These have become known as pseudolysogeny or the carrier state. In this state the phage genome exists in the cytoplasm without a mechanism to ensure

regular segregation to daughter cells at division. Phage are maintained in such cultures due to the presence of non-lysogenic sensitive bacteria which may be re-infected by phage liberated spontaneously by the lysogenic cells (Baess, 1971). This contrasts with true lysogeny where

> a lysogenic bacterium is a bacterium which perpetuates the capacity to form bacteriophages without intervention of exogenous bacteriophages,

(Lwoff and Gutmann, 1950).

Since the persistence of pseudolysogeny in bacterial populations depends on the presence of free phage, this state may be distinguished from stable lysogens by growth in the presence of phage-specific antiserum (Anderson and Cowles, 1958; Bott and Strauss, 1965). Inactivation of exogenous phage by antiserum or by growth in a medium which prevents phage adsorption (Kawakami and Landman, 1968) results in the enrichment of non-lysogenic segregants. The segregation of many cytoplasmic elements may be enhanced by treatment with acridine dyes and ethidium bromide and this has been used as a means of distinguishing between lysogens and pseudolysogens (Falkow and Baron, 1970; Arditti and Coppo, 1965). The cytoplasmic nature of the phage carrier state in B. subtilis and in a Salmonella typhosa hybrid was confirmed by density analysis of DNA from infected cells (Bott and Strauss, 1965; Falkow and Baron, 1970). These phage carrier states are distinct from the unusual prophage P1, which is not integrated but exists as a stable autonomous replicon in the cytoplasm (Zabrovitz, Segev and Cohen, 1977).

There appears to be great variability in the nature of the pseudolysogenic condition from strain to strain.

Phages in the pseudolysogenic state may be inducible (Bott and Strauss, 1965) or non-inducible (Arditti and Coppo, 1965) and despite their non-chromosomal nature have been found to be capable of transduction (Bott and Strauss, 1965; Thorne, 1961).

Kawakami and Landman (1968) attempted to define the nature of the carrier state of phage SP-10 in B. subtilis. They found that the stability of the carrier state varied with the growth phase of the bacteria. In addition, in exponential growth phase a large percentage of carrier cells became sensitive to virulent phage, although the same cells were insensitive during lag and stationary phase. This was apparently due to fluctuations in repressor levels. They proposed that the carrier state was maintained entirely by re-infection without replication of phage in the latent state.

Baron et al. (1970) and Falkow and Baron (1970) developed an E. coli - S. typhosa hybrid which could adsorb and be lysogenized by phage λ. The phage did not multiply nor kill the host. Many lysogens released phage initially but this ability segregated rapidly and permanently. To study the persistence of the viral genome a S. typhosa hybrid λdg transductant was used (λdg is a defective phage in which bacterial galactose genes replace part of the viral genome). Transductants were either stable with no release of phage or unstable. Of the unstable heterogenotes, 20% released phage and this characteristic was eliminated by treatment with antiserum. The ability to produce phage was not inducible, segregated independently of gal^+ and once lost did not reappear. The λdg DNA could be separated from the host chromosomal DNA confirming the plasmid nature of λ in S. typhosa. The characteristics of this host-phage relationship appear similar to the phage carrier state.

It was of interest to ascertain the nature of the host-phage

relationship in the Achromobacter transductant, strain 14 and a variant of this strain which released phage erratically and spontaneously.

5.2 METHODS

The ability of Achromobacter strain 14 to support phage α3a growth in stationary phase was tested in broth as described (2.2.11). Cultures were aerated for 3 days and allowed to stand for 24 h before phage addition. The phage-spray technique when used was carried out as described (2.2.12).

5.2.1 Effect of ageing and subculture on the stability of the phage-sensitive phenotype

An Achromobacter strain 14 clone which supported good phage growth in stationary phase (10^4 fold increase in 24 h) was subcultured onto a tryptone plate (plate 1) and stored at room temperature in a plastic bag (to prevent dessication). After 10 days the culture was sub-cultured again (plate 2) and a further subculture was made from this plate after 10 days (plate 3). After various lengths of time (4 to 36 days) the three cultures were innoculated into broth and tested for their ability to support α 3a in stationary phase.

5.2.2 The relationship between expression of the trp operon and stability of phage characteristics.

The effect of trp operon expression on the stability of phage sensitivity in Achromobacter strain 14 was examined by growing the

bacteria through several passages in minimal medium (MM) and MM + tryptophan (20 μg ml^{-1}) and subsequent testing for phage sensitivity. The presence of tryptophan represses the trp operon in E. coli (Franklin, 1971) and the assumption was made that a trp operon may exist in Achromobacter (F. Robb and W. Taylor, unpublished results).

Three sensitive Achromobacter strain 14 clones were innoculated into each of the above media and into tryptone broth (10 ml). The cultures were aerated for 24 h, diluted 10^{-2} in fresh medium and reincubated with aeration. This was repeated 8 times. Samples from each of the cultures were streaked onto tryptone plates to obtain isolated colonies. The sensitivity to phage α3a was tested by transfer of c. 20 colonies to tryptone plates. After incubation for 24 h, colonies were sprayed with α3a (c. 1 x 10^6 p.f.u. ml^{-1}) as described. The trp phenotype of the colonies was also tested. Colonies were transferred to MM plates and MM + tryptophan plates and after growth for 24 h a second transfer onto fresh plates was carried out (to eliminate carry-over of tryptophan from the tryptone plates).

5.2.3 The effect of trp operon expression on phage growth in stationary phase.

To determine this effect a suitable medium had to be developed. The medium found to be most suitable was an acid hydrolysed casein (AHC) medium containing tris (0.1M) and Mg SO_4 (0.1 mM). AHC does not contain tryptophan, therefore tryptophan can be added (20 μg ml^{-1}) to repress the trp operon.

Bacterial cultures were grown in tryptone, AHC and AHC + tryptophan (20 μg ml^{-1}) for 3 days with aeration and 1 day standing before measuring phage growth.

5.2.4 Characteristics of an Achromobacter strain 14 variant which spontaneously liberated phage.

During the course of this study one Achromobacter strain 14 clone (14^{*}) appeared to be able to support phage growth in standing and shaking stationary phase cultures. However, it was found that Achromobacter 14^{*} released phage spontaneously. This variant had arisen without superinfection with phage and has been partially characterized.

(i) Spontaneous phage release

This was tested by growing Achromobacter 14^{*} in tryptone broth with aeration. After a number of days (3-4) samples were removed and assayed for phage. Alternatively, colonies were spotted onto a sloppy agar plate which had been seeded with Achromobacter w.t. Colonies which released phage were surrounded by zones of lysis.

(ii) Phage adsorption and plaque formation

These were tested as described (2.2.6 and 2.2.4).

(iii) UV Induction

Overnight broth cultures were diluted 1:20 and incubated with aeration for 2 h. Cultures were pelleted by centrifugation and resuspended in PO_4 buffer. Cells were irradiated as described (4.2.7). One ml aliquots were removed at time intervals, 9 ml broth added and cultures were incubated for 90 min. After centrifugation the supernatents were sterilized with 10% v/v toluene and the phage titre assayed.

(iv) Stability of Achromobacter 14*

To test the stability of the spontaneous phage release the method of Choe (1969) was used. Overnight tryptone broth cultures were diluted 10^{-2} in broth containing α3a antiserum (k = 5) and grown to saturation. The serum cultures were again diluted 10^{-2} in serum-broth and the cycle repeated 4 times. The cultures were finally plated to obtain isolated colonies which were tested for phage production in broth cultures.

5.2.5 Characteristics of the spontaneously released phage (α3a*)

Areas of lysis surrounding Achromobacter 14* colonies on Achromobacter w.t. lawns were stabbed with a tooth pick, the phage suspended in tris-HCl buffer and treated with toluene. Dilutions of α3a* were plated on lawns of Achromobacter w.t., strain 14, 14* and 14* cultures which had been cured by antiserum treatment.

5.3 RESULTS

5.3.1 Loss of Achromobacter 14 phage sensitivity by ageing

Achromobacter strain 14 gradually lost the ability to support α3a growth in stationary phase broth cultures if maintained on one plate or subcultured. A sensitive culture (plate 1) was subcultured after 10 days (plate 2) and this was subcultured (plate 3) after a further 10 days. Plate 1 culture had lost the ability to support phage after 36 days, plate 2 after 21 days and plate 3 after 7 days (Table 5.1).

Table 5.1 Selection of phage resistance in Achromobacter strain 14 cultures as a result of prolonged storage.

Plate 1		Plate 2		Plate 3	
Age of culture (days)	Phage growth	Age of culture (days)	Phage growth	Age of culture (days)	Phage growth
4	+	4	+	3	+
7	+	16	+	7	-
14	+	21	-	8	-
36	-	30	-		

+ = increase in phage titre of 10^3 - 10^5 fold in 24 h.

- = less than 0.5 fold increase in phage titre in 24 h.

The loss of sensitivity to phage was not due to the inability to adsorb phage. Resistant cultures were tested for phage adsorption in exponential phase and they adsorbed 44 to 50% of phage added over a 30 min period.

5.3.2 Non permanent variation in sensitivity to phage

Cultures of strain 14 could alternate between resistance and sensitivity to phage α3a in an unpredictible manner during subculture on plates and in broth. For example, a culture grown from a lyophilised

stock may be sensitive to phage, but subculturing into fresh broth could result in resistance. After a few additional subcultures the sensitivity to phage α3a may be restored. It should be noted that once a culture had reached stationary phase and was sensitive to phage, it remained sensitive under those conditions. Throughout this study no strain 14 clones have been found to revert to supporting phage α3a development in exponential phase.

5.3.3 Relationship between expression of the trp operon and stability of phage characteristics.

Although preliminary experiments indicated that expression of the trp operon served to stabilise the sensitivity to phage of Achromobacter strain 14, further experiments indicated that this did not hold. There was no relationship between trp operon expression and the stability of the sensitivity to phage α3a (Table 5.2).

Table 5.2 Achromobacter strain 14 phage sensitivity after 8 passages in various media.

Medium	MM			MM + trp			Tryptone		
Original clones	4(2)	4(18)	4(19)	4(2)	4(18)	4(19)	4(2)	4(18)	4(19)
No. of α3a sensitive clones after 8 passages	21	19	15	22	21	19	19	19	22
No. of α3a resistant clones after 8 passages	2	0	3	1	1	1	1	2	0

The stability of phage sensitivity did not appear to be linked to growth on any one medium. No trp^- segregants were found, so that there was no correlation between phage resistance and trp segregation.

In another experiment, Achromobacter strain 14 cultures which had become resistant to phage α3a during storage were passaged through MM and MM + trp. The phage sensitivity tests showed that all colonies isolated after passage remained resistant. This is in contrast with the non-permanent resistance obtained by phase variation.

5.3.4 Comparison of phage development in tryptone, AHC and AHC + tryptophan cultures

Achromobacter strain 14

Ten colonies isolated from a lyophilised stock of Achromobacter strain 14 were tested for α3a development in stationary phase after growth in tryptone broth, AHC and AHC + tryptophan. Growth of α3a was comparable in all cultures indicating that expression of the trp operon did not affect phage development (Fig. 5.1).

However, Achromobacter strain 14 cultures which no longer supported good phage growth in tryptone broth (sensitivity had been lost by ageing of a plate culture and was permanent) were able to support phage growth if cultures were grown in AHC or AHC + trp (Fig. 5.2). Addition of tris or Mg SO_4 (which are constituents of AHC medium) to tryptone broth did not improve phage development in tryptone. Repeated subculturing of these cultures did not revert them to supporting good phage growth in tryptone broth.

Strain 14 cultures grown in AHC medium were unable to support phage α3a development in exponential phase or in shaking stationary cultures.

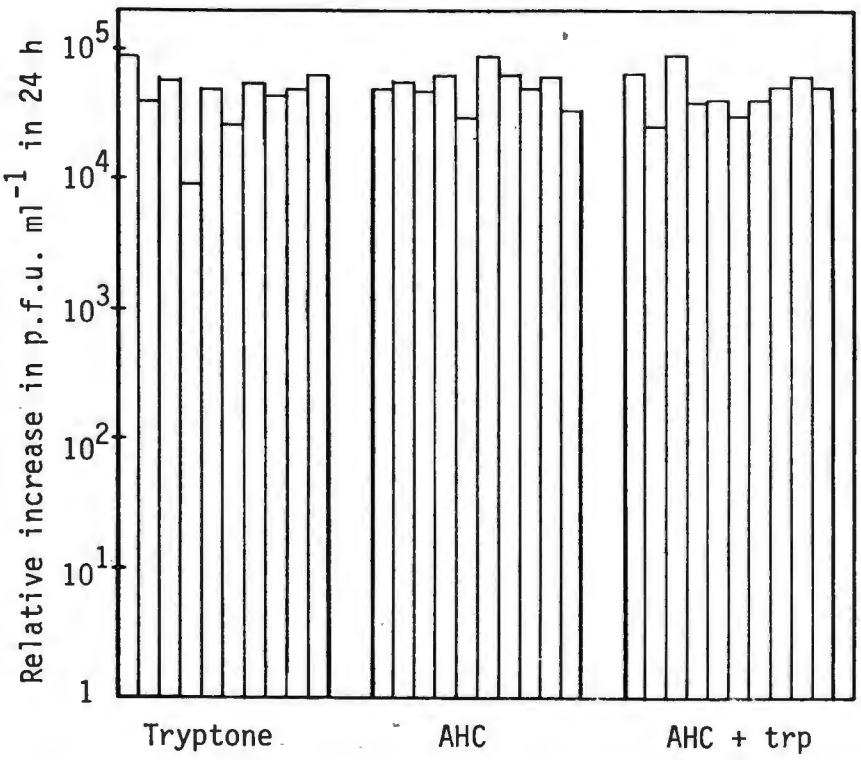


Fig 5.1 Comparison of phage α3a development in stationary phase Achromobacter strain 14 cultures, grown in tryptone, AHC and AHC + trp (20 μg ml^{-1}) Each bar represents a separate culture derived from a single clone.

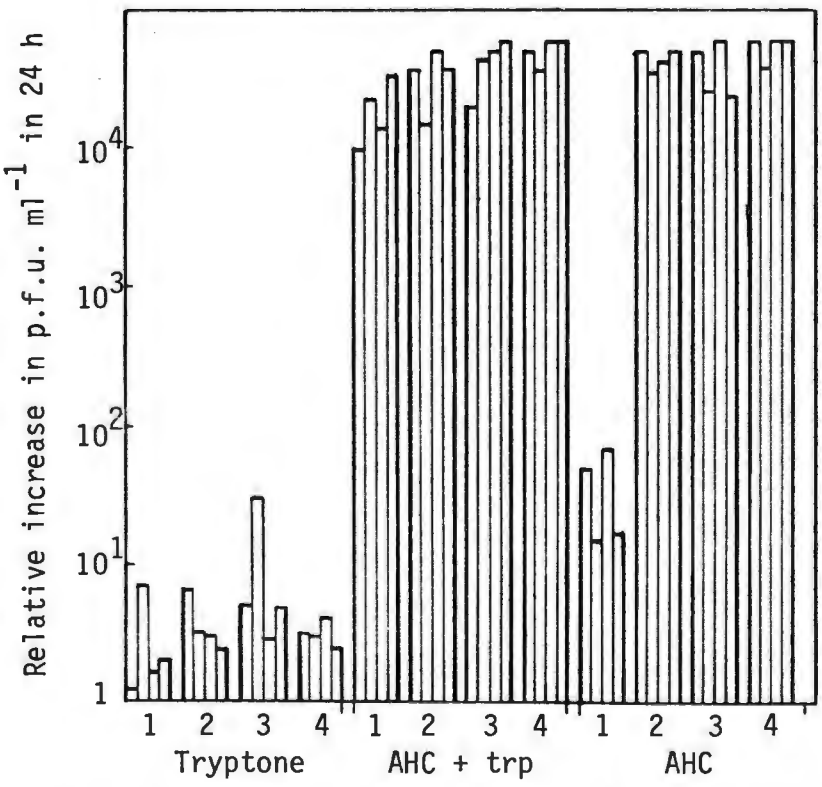


Fig. 5.2 Growth of phage α3a in Achromobacter strain 14 cultures. Resistance to α3a development is expressed in tryptone but not in AHC media. Individual clones, 1, 2, 3 and 4 were obtained from separate plate cultures and phage development measured in 4 identical cultures of each clone.

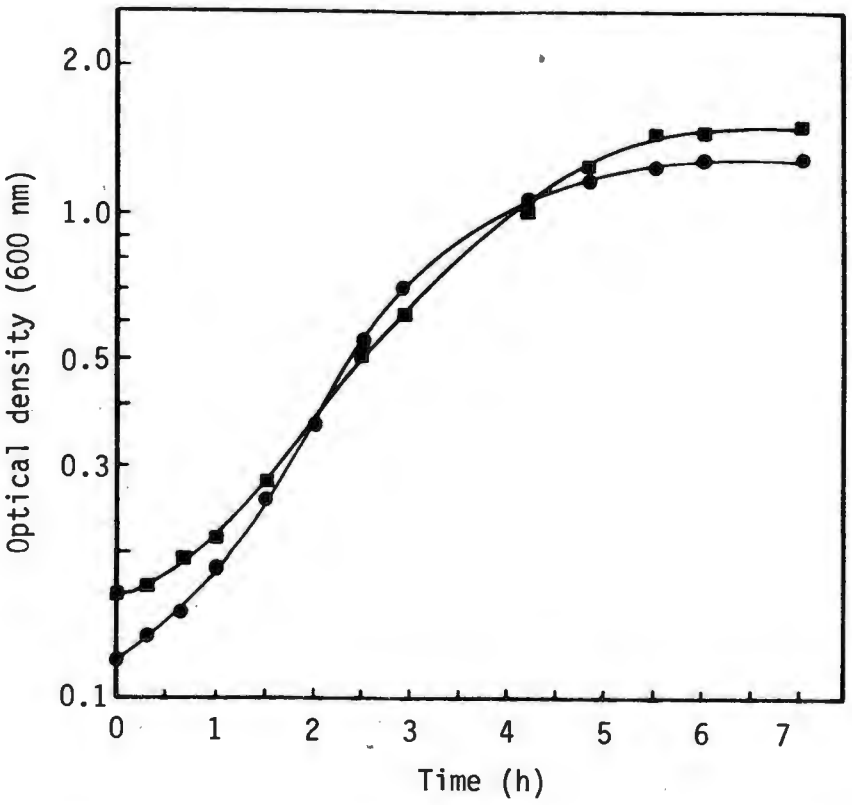


Fig. 5.3 Growth of Achromobacter strain 14 in tryptone (●) and AHC (■).

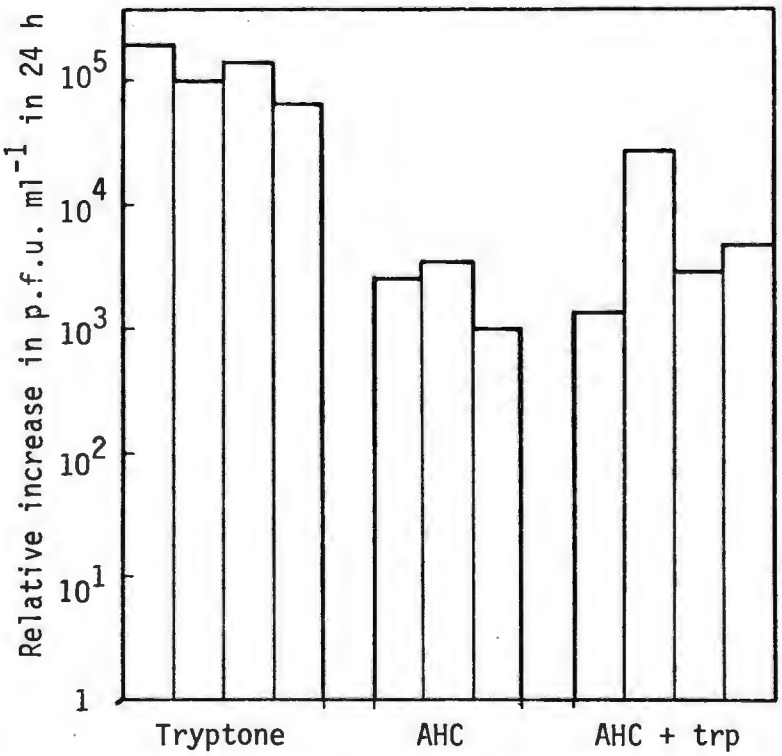


Fig. 5.4 Growth of phage α3a in Achromobacter w.t. stationary phase cultures grown in different media. Each bar represents a separate culture.

Bacterial growth measured at 30°C in AHC, AHC + trp and tryptone broth showed no major differences, although the final OD obtained in AHC and AHC + trp was higher (Fig. 5.3).

Achromobacter w.t.

Phage development in stationary phase Achromobacter w.t. cultures (measured over 24 h) was not as prolific in cultures which had been grown in AHC or AHC + trp as it was in tryptone broth cultures (Fig. 5.4). The average increase in tryptone broth was 10^5 fold and in AHC and AHC + trp, less than 10^4 fold in p.f.u. ml^{-1} over 24 h.

5.3.5 Characteristics of Achromobacter 14*

Spontaneous phage release from Achromobacter 14* broth cultures was erratic. Out of a total of 30 experiments phage were released spontaneously in 18. The titres of liberated phage measured in broth cultures after 3 - 4 days were 1×10^7 to 1×10^8 p.f.u. ml^{-1}.

Phage α3a did not adsorb to Achromobacter 14* (over 90 min) and did not form plaques on 14* lawns. The ability of Achromobacter 14* to release phage spontaneously was lost by passage through antiserum and phage did not reappear after successive subcultures in antiserum free medium. There was no UV induction of phage from Achromobacter 14*, w.t.,(as reported by Thomson and Woods, 1974) or from strain 14.

Achromobacter strain 14* colonies which had been cured by passage through antiserum remained resistant to α3a.

There was no plaque development on lawns of Achromobacter 14* even after 3 days incubation. These characteristics are summarised in Table 5.3.

Table 5.3 The nature of phage development in Achromobacter strains

Strain	Spontaneous release of phage	UV induction of phage	Growth of α3a		Growth of α3a*
			Exponential phase	Stationary phase	Exponential phase
w.t.	-	-	+	+	+
strain 14	-	-	-	+	+
trp⁻ strain$^{\phi}$ from which strain 14 was derived	-	-	+	+	
14*	+	-	-	-	-
14* cured	-		-	-	-

ϕ Woods and Thomson (1975) and Thomson, Ph.D. thesis

5.3.6 Characteristics of phage α3a*

Phage α3a* which had been released spontaneously from Achromobacter 14* were able to develop on exponentially growing lawns of Achromobacter strain 14 and w.t. Plaques were visible 7 h after plating and the e.o.p. was the same on Achromobacter strain 14 and w.t. lawns. Turbid plaques were formed on w.t. lawns and clear plaques on strain 14 lawns. Control plages showed that the strain 14 cultures did not support phage development of α3a in exponential phase.

Achromobacter 14* was totally resistant to α3a*. In addition, Achromobacter 14* colonies which had been cured with antiserum remained resistant to α3a and α3a*. Table 5.3 summarises these results.

Earlier in this study a phage mutant able to grow on exponential phase strain 14 was isolated. This mutant (α3aH') had an altered plaque morphology and produced circular halo type plaques on Achromobacter strain 14 and w.t. lawns (Robb et al., 1977). However, unlike α3a* growth of α3aH' in exponential strain 14 cultures was limited and variable.

5.4 DISCUSSION

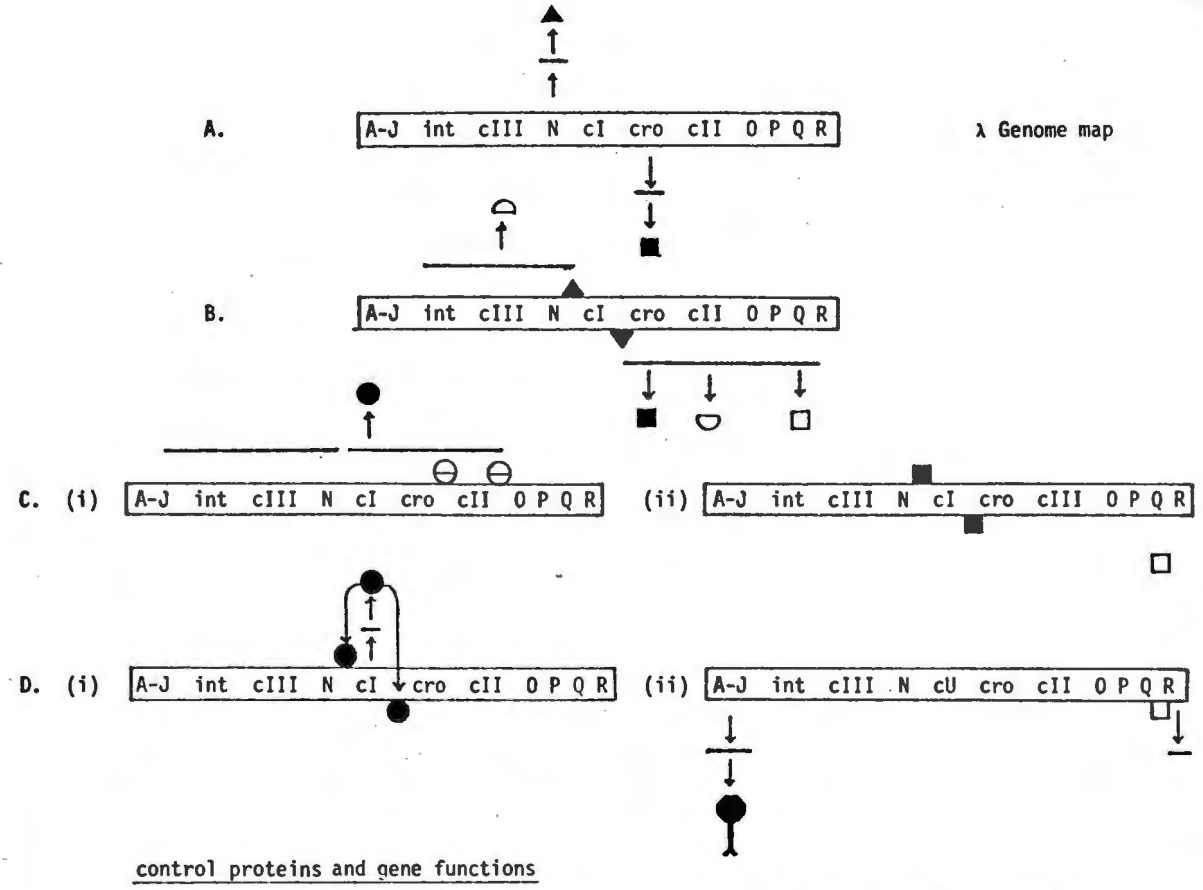
Achromobacter strain 14 appears to exhibit phase variation. Cultures alternate in an unpredictable manner between sensitivity and resistance to phage α3a in stationary phase. Some cultures have been found to be 'fixed' in the resistant phase (after prolonged ageing on plates) and no longer support phage development in tryptone broth. This does not appear to result from loss of genetic material since these same cultures are still able to support phage growth in AHC medium

which may indicate that the regulatory mechanisms alter in response to the environment.

This situation appears similar to immunity phase shift exhibited by defective λ prophages (Neubauer and Calef, 1970; Calef et al., 1971). These defective prophages could exist in either the im^+ phase and exhibit superinfection immunity, or in the im^- phase which was characterized by the absence of immunity. The phase shift was thought to correspond to the two alternative development pathways of lytic development or lysogeny (see 5.1). For clarity the developmental pathways of λ are presented diagramatically in Fig. 5.5. Cro gene product and the CI repressor protein bind to the same control regions but display antagonistic effects in determining the pathway followed. There are a number of factors which influence the choice between lysogeny and the lytic cycle but the mechanics of this choice and the interactions of the two controlling proteins are not clearly understood. It is thought that the critical decision in establishing one or other of the pathways may be quantitative in terms of concentrations of repressor and cro product and the antagonism may be as a result of competition for the DNA sites (Lewin, 1977). In addition, host functions are known to influence the establishment of lysogeny. E. coli mutants defective in cAMP or CAP protein have low levels of lysogeny whereas E. coli hfl mutants have high lysogeny levels (Belfort and Wulff, 1974). Fluctuations in transcription and translation in individual host cells may influence the pathway chosen (Lewin, 1977) by affecting expression of host and phage control proteins.

Such a system may be operative in Achromobacter strain 14 and could account for both the repression of phage in exponential phase and the phase variation. In phage sensitive Achromobacter strain 14 the im^+ state may exist during exponential growth but changes in cellular

Fig. 5.5 The control mechanisms of the lysogenic and lytic pathways of phage λ.



control proteins and gene functions

all expression	lysogeny	lytic development	head and tail	lysis	replication
N	cIII	cro	A - J	R	O & P
	cII	Q			
	cI				

A. Following infection the immediate early gene products N and cro are produced.

B. The N protein acts at the right and left control sites to allow transcription and translation of delayed early genes. These include the cII, cIII and int gene products, required for lysogeny and O, P and Q gene products required for lytic development. N and cro products are still produced.

C. Decision between lysogeny or lytic development.

(i) cIII and cII activate leftwards transcription of CI (repressor) and int (integrator) genes and inhibit rightward transcription of lytic genes.

(ii) cro protein inhibits transcription of delayed early genes (cII, cIII and Q). cII and cIII proteins decay rapidly and therefore cannot activate CI transcription. Q protein is stable ..

D. (i) Maintenance of lysogeny. CI repressor protein binds to early operators and represses nearly all λ transcription by preventing early gene transcription. CI protein also regulatesits continued synthesis.

(ii) Lytic development. The Q protein activates transcription of head, tail and lysis genes

Echols and Murialdo (1978); Lewin (1977)

conditions channel the defective prophage to the im^- state in stationary phase thus allowing superinfecting phage to develop. The phase variation would be a stationary phase phenomenon where strain 14 cultures were either channelled to the im^- state (sensitive to phage) or remained in the im^+ state (resistant to phage). The immunity state (im^+) in exponential phase appears constant and has been shown not to alter in response to the environment. However, the immunity state in stationary phase can be affected by growth in different media. For example, strain 14 cultures may be resistant to phage when grown in tryptone broth but sensitive in AHC medium. AHC medium appears to exert a specific positive effect on phage development in strain 14 cultures but not in w.t. cultures. This may be exerted on phage regulatory mechanisms not present in the w.t. strain. Kawakami and Landman (1968) found that the sensitivity to virulent phage of B. subtilis cells containing phage SP-10 in the carrier state varied with the growth phase. The variation appeared to be due to fluctuations in repressor levels.

Although there are a number of parallels between the λ immunity phase shift and that found for Achromobacter defective lysogens, this does not discount involvement of switch mechanism such as that found in Salmonella phase variation (Zieg et al., 1977).

There does not appear to be any relationship between the expression of the trp genes in strain 14 and the maintenance of the ability to support phage. Neither does trp gene expression affect phage production in stationary phase cultures. During the course of this study no trp^- segregants were isolated. However, Woods and Thomson (1975) reported that transduction by α3a was unstable and that the phage sensitivity of the transductants was not altered in trp^- segregants. This is difficult to explain but it seems to indicate a lack of association between the

trp genes and the phage genes in the transductant. A similar phenomenon was reported by Baron et al. (1970) where ability of λdg transductants to produce phage segregated independently of gal.

The fortuitous finding of a spontaneous variant of Achromobacter strain 14 which released phage and was immune to superinfection allows more insight into the nature of strain 14. The fact that this variant, 14^{*}, arose without superinfection by phage, lends further support to the idea that Achromobacter strain 14 is a 'partial' double lysogen (2.4). These results suggest that strain 14 cells do contain the full phage genome complement but they do not liberate phage and they allow superinfecting phage to adsorb (although with reduced efficiency 2.3.4). Variant 14^{*} on the other hand, releases phage spontaneously and is unable to adsorb phage. This implies that in strain 14 resident phage are defective in lytic development and there seems to be a defect in the expression of the immunity genes conferring lysogenic conversion. It appears that a mutational event has taken place in 14^{*} and overcome these defects.

Spontaneously liberated phage, $\alpha 3a^{*}$, are able to plate on Achromobacter strain 14 and w.t. with equal efficiency. They are also able to develop in exponentially growing cells of strain 14. This suggests that these phage are altered in such a way that the repression exhibited by exponential Achromobacter strain 14 cultures is overcome. If this repression is due to a phage repressor these findings may indicate that the phage repressor binding site is altered. Such mutations were found to exist in λvir which prevented the establishment of lysogeny and rendered the phage virulent (Sly, Rabideau and Kolber, 1971).

The nature of the lysogenic condition exhibited by Achromobacter 14^{*} appears to be similar to the pseudolysogenic or phage carrier state (Bott and Strauss, 1965; Kawakami and Landman, 1968). It is not a

stable characteristic, is cured permanently by treatment with antiserum and is not inducible. Double lysogens obtained by superinfecting the w.t. strain with α3a were stable and inducible (Woods and Thomson, 1975) so lysogeny in 14^{*} is clearly different. Curing of pseudolysogens by antiserum treatment usually results in enrichment of non-lysogenic segregants which are sensitive to superinfecting phage. However, after antiserum treatment of 14^{*} cells, all colonies isolated were totally resistant to phage infection by α3a or $\alpha 3a^{*}$ but were unable to release phage. A similar phenomenon was reported after curing a Bacteroides pseudolysogen (S. Burt, Ph.D. thesis) and seems to indicate residual phage functions in the cured lysogens.

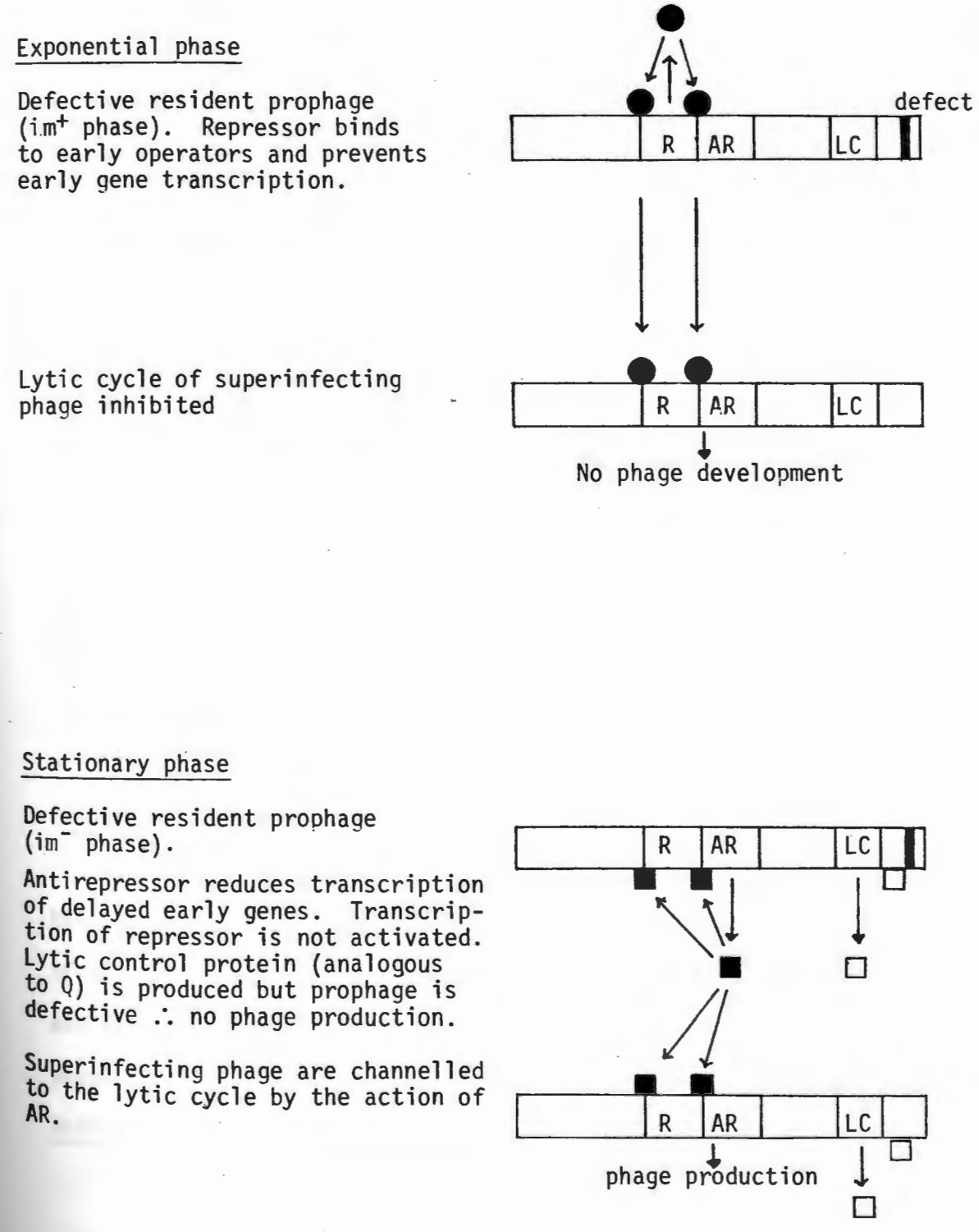
In terms of α3a phage development in strain 14 and 14^{*} cultures, a hypothetical model based on the λ control mechanisms has been constructed (Table 5.4 and Fig. 5.6). This model relies on fluctuations in repressor levels in strain 14, such that superinfecting phage are not able to develop in exponential phase bacteria due to high repressor levels but transcriptional or phage regulation alterations cause low repressor levels in stationary phase and allow development. It would be of interest to see whether Achromobacter 14^{*} would support phage development in stationary phase. This could be done using transfection methods to overcome the surface exclusion which exists in this strain.

It is of interest to note that α3a and $\alpha 3a^{*}$ infecting Achromobacter strain 14 are chanelled towards the lytic cycle as evidenced by clear plaque formation. The anti-repressor action of cro gene product was first recognized in the defective λ lysogens in the im^{-} state. It was found in these cells that a diffusible produce (cro), dominant in trans, could channel superinfecting phage towards the lytic cycle (first noticed by clear plaqe formation; Neubauer and Calef, 1970). A positive regulator of the lytic cycle seems to be implicated in

Table 5.4 *Hypothetical model for phage development in* Achromobacter *strain 14 and 14**

	Repressor production		Lysogenic conversion - surface exclusion		Phage α3a growth		Phage α3a* growth		Spontaneous phage release
	Log.	Stationary	Log.	Stationary	Log.	Stationary	Log.	Stationary	
strain 14	+	-	partial	partial	-	+	+	+	- resident phage may be defective in early genes, e.g. replication or excision
By a mutational event strain 14 is converted to:									
14*	+	-	+	+	-	-	-	-	+ resident phage no longer defective

Fig. 5.6 Model for phage development in Achromobacter strain 14.



Repressor R

Antirepressor AR

Lytic control protein LC (analogous to λ Q protein)

Achromobacter strain 14 cells which support phage development.

The hypothetical model would explain why phage α3a development is not initiated in exponential phase strain 14 cells but, once initiated in stationary phase cells, phage DNA and protein synthesis can continue if the infected cell enters exponential development (3.3.10). In λ, repressor protein CI prevents transcription of early genes from P_L and P_R by binding at O_L and O_R, thus inhibiting lytic development. However, once the control proteins (cro and Q) for lytic development have been produced in the absence of repressor, the lytic cycle is underway because the late genes (tail, head and lysis proteins) are not under repressor control but require Q for transcription (Echols and Murialdo, 1978). Thus, if the early stages of lytic development were able to take place in Achromobacter strain 14 stationary phase cells (due to absence of repressor) and produce the lytic control proteins, the lytic cycle could continue, even if exponential growth of the host was initiated and repressor protein was produced.

The discovery of an altered phage ($\alpha 3a^*$) which is able to propagate on exponential Achromobacter strain 14 cultures provides numerous possibilities for studying the developmental block of α3a in such cultures.

Due to the fact that this phage was a recent discovery such studies have not yet been started, but are likely to include restriction mapping of the two phages and analysis of phage macromolecular synthesis (mRNA and protein) after infection. This would determine the stage in the phage developmental cycle where α3a is blocked in exponential phase Achromobacter strain 14.

CHAPTER VI

PHAGE DEVELOPMENT IN ACHROMOBACTER RIFAMPICIN RESISTANT CELLS

6.1 INTRODUCTION

RNA polymerase (RPase) is structurally one of the most complex enzymes in the bacterial cell. It consists of four major types of subunit, α, σ, β and β' present in the molar ratio of 2:1:1:1. The enzyme can exist as the holoenzyme ($\alpha_2\beta\beta'\sigma$) or as the core enzyme ($\alpha_2\beta\beta'$) produced by the dissociation of the σ factor (Burgess et al., 1969; Zillig et al., 1970; Burgess, 1976). Other small polypeptides have been found to be associated with the holoenzyme in B. subtilis (Doi, 1977a) and in E. coli (Doi, 1977b). During purification approximately 60 to 70% of the enzyme is found as core enzyme. Sigma (σ) factor appears to dissociate fairly easily from the core enzyme during purification (Burgess et al., 1969) and it is possible that other factors are also loosely bound.

The core enzyme can initiate non-specific transcription but the σ factor is required for correct initiation at the promoter sites. Sigma factor binds to the β subunit, catalyses specific initiation and then dissociates from the core after completion of initiation (Losick, 1972; Zillig et al., 1972). The antibiotic rifampicin (rif) also binds to the β subunit and prevents the initiation step in RNA synthesis. The interaction of E. coli RPase with rif is reversible (Wehrli et al., 1976). Although similar in molecular weight, the β and β' subunits differ in function. The β' subunit is responsible for the binding of the RPase to the DNA (Zillig et al., 1970). The role of the α subunit is not as well defined but it is essential for

enzyme activity and may be involved in promoter recognition (Doi, 1977b; Goff and Webber, 1970; Travers, 1970).

To initiate transcription, RPase recognizes certain base sequences of the DNA (recognition site), binds to the DNA at the binding site and initiates transcription at the initiation site (Chamberlin, 1976). This whole region is called the promoter site. Base sequence analysis of phage and bacterial promoter regions which are recognized by the E. coli RPase, has revealed that although there are compositional similaries, there is little homology at the nucleotide level (Calos, 1978). The differing base sequences may determine the degree of affinity that the RPase has with the promoters and this may affect the level of transcription. Calos (1978) showed that a single base change in the promoter region of the lac repressor operon could convert a low level promoter into an 'up' promoter.

Following initial binding of RPase to the DNA, there is a conformational change in the DNA of the promoter region. Once this transition has taken place, an RNA chain can be initiated very rapidly. The transition often occurs over a narrow temperature range, the midpoint of which (t) defines the transition temperature. Travers (1976) has proposed that each promoter has a characteristic t for opening by RPase holoenzyme. Accessory regulatory factors which bind either to the DNA or to the RPase can alter the affinity of the RPase for the promoter by changing t, i.e. an increase in t, results in decreased affinity for the promoter. He proposed that RPase is an allosteric enzyme, whose activity and specificity are controlled by interactions with regulatory factors and these may play an important role in gene selection.

A number of accessory regulatory factors have been found to exert

positive or negative control on transcriptional levels. The cAMP-CAP protein complex has a positive effect on transcription of the lac operon. The complex is able to bind to the lac promoter region and facilitate binding of the RPase by causing destabilization of the DNA (Majors, 1975). It is believed that this is a major control mechanism for transcription of catabolite repressed genes (Pastan and Adhya, 1976).

A number of other DNA binding proteins in E. coli have been shown to affect the specificity of transcription, e.g. the D, HU and HI factors (Doi, 1977b) and the TFI factor (Geiduschek et al., 1977). Inhibition of initiation by these factors (e.g. TFI) is not by steric blockage as is the inhibition by repressor molecules (e.g. λ repressor; Maurer, Maniatis and Ptashne, 1974).

The guanosine nucleotide (ppGpp) specifically inhibits the formation of rRNA-RPase complexes. In vitro the presence of ppGpp increases t for opening rRNA promoters but has little effect on other promoters, such as those of phage ϕ80. Experimental evidence suggests that ppGpp alters the properties of the RPase itself, possibly causing a structural alteration (Travers, 1976).

Other factors associated with the translational machinery affect the specific binding of E. coli holoenzyme to rRNA promoters. N-formyl methionyl tRNA binds to the RPase and reduces rRNA transcription (Pongs and Ulbrich, 1976) whereas elongation factor TuTs decreases t for rRNA synthesis (Travers, 1976).

During phage infection there are a number of ways by which selective gene transcription can occur.

New phage specific polymerases are synthesised several minutes after infection of E. coli by T7 and T3 phages. They consist of

single polypeptides of molecular weight 110,000 and 107,000 for T7 and T3 respectively. They do not recognize E. coli promoters but show strong preference for their respective DNAs. Phage transcription does not require the host RPase (Bautz, 1976). A new RPase enzyme has also been identified after infection of B. subtilis with phage PBS2 (Clark, Losick and Pero, 1974). This enzyme has poor activity on DNA templates other than PBS2 DNA.

In contrast, phage SP01 requires the B. subtilis RPase core for transcription of its genome (Geiduschek and Sklar, 1969) but modifies it to change its promoter selection. After infection, phage specified polypeptides associate with the host core and specifically transcribe 'middle' and 'late' genes (Fox and Pero, 1974; Fox, Losick and Pero, 1976; Swanton, Smith and Shub, 1975). These altered RPase forms lack host σ factor and no longer recognize host promoter sites. One of the modified RPase forms (enzyme B) has been shown to bind selectively to SP01 DNA fragments containing middle gene promoters (Talkington and Pero, 1978).

Phage λ does not alter the initiation specificity of the host RPase but changes its ability to terminate transcription. The λ N gene product interacts with the host RPase and has an anti-termination effect such that transcription proceeds beyond phage termination sites during the lytic cycle (Georgopoulos, 1971; Sternberg, 1976).

Transcriptional control has been shown to be central to the regulation of bacterial morphogenesis. Bacterial sporulation represents a relatively simple form of cellular morphogenetic differentiation. A temporal sequence of events leads to the development of a spore which is distinct from the vegetative cell in physiological functions, physical and biochemical properties and in morphology (Hanson et al., 1970).

There is sequential selective expression of genes during the sporulation process. This has been shown by the appearance of sporulation specific

proteins and mRNA species. Sporulation specific proteins in B. subtilis are either essential for sporulation such as serine protease or non-essential, such as metalloprotease (Dancer and Mandelstam, 1975 a and b).

A number of studies have indicated that during sporulation of B. subtilis the rate of RNA synthesis changes dramatically at different times (Hussey et al., 1971; Leighton, 1973; Sumida-Yasumoto and Doi, 1977) and this may be the result of the turning on and off of certain sporulation genes. DNA-RNA hybridization studies with mRNA from sporulating cells and spores have shown that there is a sequential appearance of classes of mRNA which are not present in vegetative cells. They also showed that vegetative mRNA species are transcribed simultaneously with sporulation specific mRNA species (Hanson et al., 1970; Jeng and Doi, 1974; Sumida-Yasumoto and Doi, 1974). A small percentage of vegetative genes are turned off but even at the late stages of sporulation, 60% of the mRNA corresponds to vegetative mRNA (Doi, 1977a). This indicates that at least two types of transcriptional apparatus must exist simultaneously to transcribe the vegetative and sporulation specific genes. Studies have shown that the β subunit (which is involved in initiation and therefore in promoter selection) of the RPase core is functional throughout sporulation (Doi, 1977b).

The study of RPase mutants resistant to the polymerase specific antibiotics, rifampicin and streptolydigan, strongly suggest that RPase plays an important role in the control of sporulation. Rifampicin and streptolydigan resistant mutants of B. subtilis either produce abnormal spores or do not sporulate (Doi, Brown, Rodgers and Hsu, 1970; Sonenshein and Losick, 1970; Sonenshein, Cami, Brevet and Cote, 1974).

A range of temperature sensitive rif resistant mutants, which are only temperature sensitive during sporulation, has been isolated (Leighton, 1973; Sumida-Yasumoto and Doi, 1977). The mutants grow

at the same rate as the wild-type at the non-permissive temperature but sporulation is blocked at different stages. These results and subsequent studies (Linn et al., 1975; Fukuda and Doi, 1977) indicate that the vegetative core enzyme is used during sporulation and associates with sporulation specific regulatory polypeptides to change the template specificity. Incorrect conformation of the mutant RPase core at the non-permissive temperature inhibits this interaction.

So far no gross changes in the basic structure of the B. subtilis RPase core have been observed at any stage of sporulation. Earlier results (Losick, Shorenstein and Sonenshein, 1970) showed a structural alteration in the β subunit of sporulation RPase but this was later shown to result from proteolytic cleavage during purification (Linn et al., 1973; Orrego et al., 1973). However, there is the possibility that minor modifications of the core subunits such as phosphorylation or adenylylation do occur. Such modifications have not been detected using the SDS-polyacrylamide gel electrophoresis methods which have been used to analyse the RPase core (Doi, 1977a). In B. thuringiensis structural modifications of the core have been reported to occur during sporulation (Klier and Lecadet, 1974).

One of the earliest observations in the analysis of the sporulation RPase was that it has a lower affinity for σ factor (Losick et al., 1970; Losick, Shorenstein and Sonenshein, 1970; Brevet, 1974; Greenleaf et al., 1973; Segall et al., 1974). Supporting the idea that other regulatory polypeptides interact with the RPase core to change its specificity, a number of sporulation specific polypeptides which bind to the RPase core have been isolated.

At stage II to III in sporulating cells two forms of RPase have

been identified (Linn et al., 1975; Fukuda and Doi, 1977). One form (enzyme I) appears to be identical to vegetative holoenzyme ($\alpha_2\beta\beta'\sigma$). The other form (enzyme II) has the composition $\alpha_2\beta\beta'\delta^1$, where δ^1 is a sporulation specific peptide with a molecular weight of 27,000. At stage V in sporulation, a third enzyme form (enzyme III) has been identified (Fukuda and Doi, 1977), with the composition $\alpha_2\beta\beta'\delta^2$. δ^2 is a second sporulation specific factor with the molecular weight of about 20,000. Enzymes II and III differ from Enzyme I by their greater affinity to DNA cellulose columns, by their higher specific activities on several DNA templates, and by the tighter association of δ^1 and δ^2 to the RPase core, relative to the association that σ has with enzyme I. One rif resistant ts mutant (Ts 1), blocked in sporulation, is unable to make the δ-containing forms of RPase at the restrictive temperature (Sumida-Yasumoto and Doi, 1977).

Evidence suggests that enzyme II has a different promoter recognition to enzyme I (Doi, 1977b). The antibiotic netropsin, which binds to A-T rich regions of DNA, inhibits enzyme II much more than enzyme I when B. subtilis DNA is used as the template. This indicates that δ^1 and σ may recognize a different spectrum of promoter sequences, and also that sporulation specific promoters may be qualitatively different to vegetative promoters.

The presence of the vegetative polymerase in sporulating cells is consistent with the continued synthesis of vegetative mRNA during sporulation.

It has been shown that phage-host interaction in sporulating cells may be useful in investigating aspects of bacterial differentiation (Yehle and Doi, 1967; Sonenshein and Roscoe, 1969; Osburne and Sonenshein, 1976). Depending on which phage is used, phage development

is inhibited at a specific time during the sporulation cycle (see 3.1). The precise reasons for this inhibition are not understood and a number of ideas have emerged.

Yehle and Doi (1967) proposed that differential expression of the phage genome in sporulating cells is due to the relative abilities of the phages to suppress the host genome.

Another possibility that has been suggested (Losick and Sonenshein, 1969; Sonenshein and Roscoe, 1969; Sonenshein et al., 1974), is that host RPase changes in structure and specificity during sporulation, and thereby interferes with viral gene expression. Since the transcription of some phages requires the interaction of phage coded polypeptides with host RPase, it is possible that changes in the host core prevent these interactions.

Other studies on the growth characteristics of a number of phages in sporulating cells have indicated that the complexity of the phage and the dependence on host cell functions may affect the ability of the phage to replicate (Harding and Ito, 1976; Ito, Kawamura and Yanofsky, 1976; Kawamura and Ito, 1974; Kawamura and Ito, 1977c). Host factors required for the multiplication of various phages may be lost or altered at different times during sporulation.

The interactions of phage α3a with the various Achromobacter strains are particularly interesting. Phase specific phage growth may indicate that there are changes in template specificity in Achromobacter, which interfere with viral expression. In addition, in strains which support α3a growth in stationary phase, phage development is restricted to cells in a particular state (standing cultures). Analysis of this restriction may provide some insight into stationary phase metabolic control mechanisms and their effect on phage development.

The following approach has been used to assess the role of

transcriptional control in differential phage development in Achromobacter. Achromobacter Lp (referred to as w.t. by Woods, 1976 and Robb et al., 1977) only supports phage α3a development in exponential phase. Rif resistant Achromobacter Lp mutants were obtained and examined for altered phage development. The activity of the altered RPase was tested in vitro in the presence and absence of rif.

6.2 METHODS

6.2.1 Isolation of Rifampicin-resistant Achromobacter mutants

Achromobacter strains which have been collectively referred to as wild-type show differences in their abilities to support phage in exponential and stationary phase. Woods (1976) and Robb et al. (1977) reported that Achromobacter w.t. was unable to support phage α3a in stationary phase. In this dissertation Achromobacter w.t. refers to a strain which is able to support phage development in both growth phases. To avoid confusion, the Achromobacter strain unable to support phage in stationary phase will be referred to as Achromobacter Lp (Log phase phage development).

Rif resistant mutants of Achromobacter Lp were isolated by streaking the bacteria onto tryptone agar plates containing a rif gradient. Mutant colonies which were able to grow in the presence of rif were cloned and maintained on tryptone plates containing rif (50 $\mu g\ ml^{-1}$).

6.2.2 RNA Synthesis

RNA synthesis was determined by incorporation of ^{3}H-uracil (2 $\mu g\ ml^{-1}$, 0.4 $\mu Ci\ ml^{-1}$) into trichloroacetic acid (TCA) - precipitable

material as described (4.2.2). Rif was added to exponential phase cultures to give a final concentration of 25 μg ml^{-1}.

6.2.3 Extraction and assay of RNA polymerase

Exponential phase cultures in tryptone broth were harvested by centrifugation, washed twice and resuspended in 5 ml buffer C containing 0.5 M tris-HCl, 0.1 mM EDTA, 0.1 mM dithiothreitol and 10% (v/v) glycerol (Clark, Losick and Pero, 1974). This volume contained 2 - 4 g of cells. The cells were disrupted by sonication at 4°C (4 x 30 sec bursts) in buffer C containing 0.5 mM phenylmethylsulphonyl fluoride (PMSF, protease inhibitor) and centrifuged at 24 000 g for 15 min. The supernatant (crude extract) was assayed for RNA polymerase activity. Enzyme activity was lost rapidly during storage at 0°C.

RNA polymerase assay

The assay for RPase measured conversion of ^{3}H from the labelled nucleoside triphosphate into an acid-insoluble form. The method of Chamberlin and Berg (1962) was used with modifications.

The reaction mixture (250 μl) contained 40.0 mM tris-HCl (pH 7.9), 4.0 mM $MgCl_2$, 1.0 mM $MnCl_2$, 0.15 M KCl, 12.0 mM mercaptoethanol, 0.4 mM ATP, CTP, GTP, 0.4 mM ^{3}H-UTP (0.4 μCi ml^{-1}), 250 μg ml^{-1} salmon sperm DNA (Sigma) as template, 50 μl of the crude extract and 0, 2.5, 20 and 40 μg ml^{-1} rif. The GTP was dissolved on the day of use. The mixtures were incubated for 10 min at 30°C and 0.25 ml cold 10% (w/v) TCA was added to stop the reaction. After standing at 4°C for 30 min the samples were filtered and washed with 2 x 5 ml 5% (w/v) TCA and 5 ml 1% (v/v) acetic acid on Whatman GF-C filters. The filters were dried and counted as described (4.2.2). Enzyme units were

determined as nmol/min/mg protein. Protein was determined by the method of Lowry *et al.* (1951).

6.2.4 The effect of rifampicin on exponential and stationary phase *Achromobacter*.

There were indications that stationary phase cells were irreversibly inhibited by *rif*, whereas exponential phase cells were able to recover if the antibiotic was removed. To examine this, stationary phase (3 day old) and exponential phase cultures of strain 14 were diluted (*c.* 2×10^5 cells ml^{-1}) into broth containing 0.3175 $\mu g\ ml^{-1}$ *rif* (minimum inhibitory concentration; m.i.c.). These cultures were allowed to stand at 30^oC and at various times, samples were diluted 10^{-1} and 10^{-2} and plated on tryptone plates.

6.3 RESULTS

6.3.1 Isolation of rifampicin resistant mutants

Achromobacter w.t., strain 14 and Lp were sensitive to *rif* (1 $\mu g\ ml^{-1}$ to 40 $\mu g\ ml^{-1}$).

Rif resistant colonies of *Achromobacter* Lp were obtained. One mutant, rif-I, was selected and had the same growth rate in liquid tryptone medium with or without 2.5 $\mu g\ ml^{-1}$ *rif* after growing in rifampicin free medium.

6.3.2 The effect of rifampicin on RNA synthesis

RNA synthesis in *Achromobacter* Lp was inhibited by the addition of 25 $\mu g\ ml^{-1}$ *rif* whereas RNA synthesis of *Achromobacter* rif-I was not

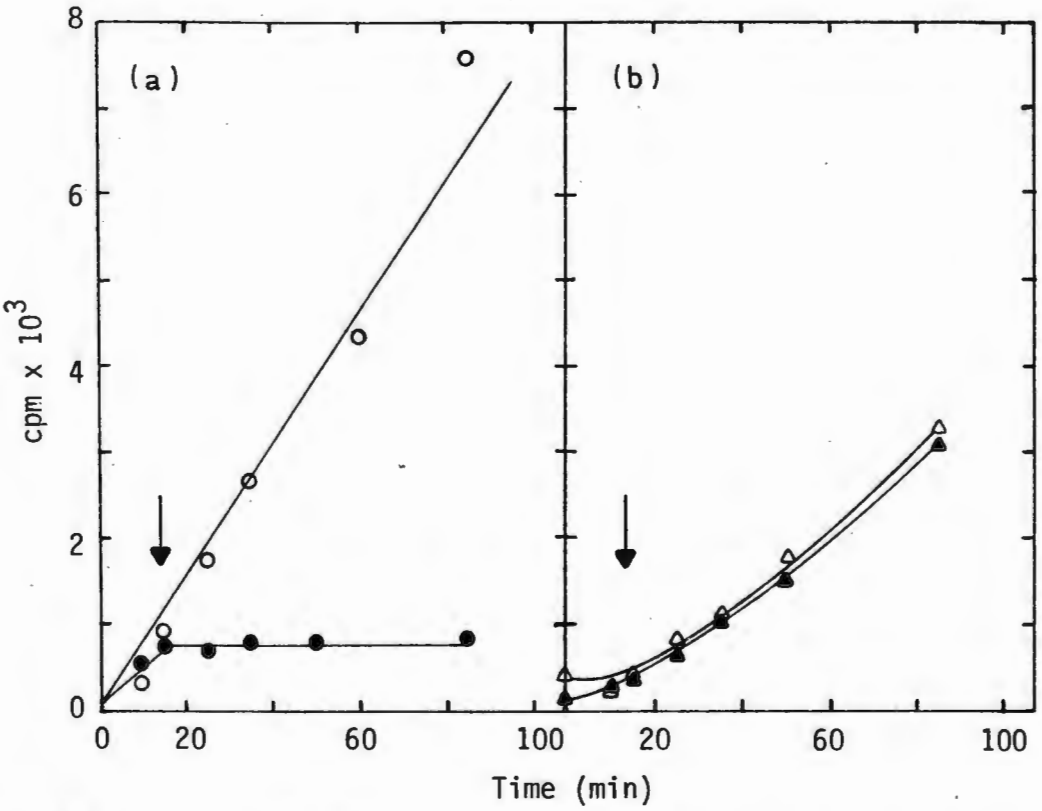


Fig. 6.1 Effect of rifampicin on RNA synthesis in exponential phase Achromobacter Lp and rif-I cultures. Incorporation of ^{3}H-uracil into TCA-precipitable material.
(a) Achromobacter Lp cells with (●—●) and without (o—o) rifampicin. (b) Rif-I mutant cells with (▲—▲) and without (△—△) rifampicin. ↓ Time of addition of 25 μg ml^{-1} rifampicin.

affected (Fig 6.1). Addition of rif under these conditions caused cessation of growth in Achromobacter Lp cultures, but the growth rate of Achromobacter rif-I was not affected (Table 6.1).

Table 6.1 Bacterial growth inhibition by rifampicin (25 μg ml^{-1}).

Time (min)	Additions	Achromobacter Lp		Achromobacter rif-I	
		- rif	+ rif	- rif	- rif
* 0	rif	0.125 ϕ	0.15	0.10	0.10
10		0.14	0.16	0.11	0.12
15					
25		0.16	0.20	0.13	0.14
35		0.18	0.20	0.14	0.16
50		0.22	0.20	0.16	0.175
70		0.27	0.20	0.19	0.20
85		0.34	0.21	0.23	0.24

* these times correspond to those in Fig 6.1.

ϕ optical density at 600 nm.

6.3.3 RNA polymerase activity

The specific activities of crude extracts of RPase from Achromobacter Lp and rif-I strains were investigated in order to show that the rif-resistance was due to an alteration in the RPase. The results indicate

that RPase from the rif-I mutant strain was resistant to 40 μg ml^{-1} rif, whereas Achromobacter Lp RPase activity was inhibited by rif (Table 6.2).

Table 6.2 Specific activities of crude extracts of RNA polymerase from Achromobacter Lp and rif-I strains.

Bacterial strain	Rifampicin (μg ml^{-1})	Specific activity *	% Activity
Lp	0	0.697	100
	20	0.0078	1
	40	0.040	6
rif-I	0	0.224	100
	2.5	0.196	88
	20	0.196	88
	40	0.226	100

* Specific activity expressed as units per mg protein.

6.3.4 Altered phage growth in Achromobacter rif-I mutant

Phage development in broth cultures

The growth of phage α3a was measured (see 2.2.11) in stationary phase standing and shaking cultures of Achromobacter Lp, rif-I and

strain 14 (Fig 6.2). The rif-I mutant was able to support α3a growth in stationary phase (standing cultures), with an increase of 8×10^2 fold in p.f.u. ml^{-1} over 48 h. An increase of 6×10^4 fold was observed in strain 14 cultures over the same time.

No increases in phage titre were observed in any of the shaking cultures or in standing cultures of Achromobacter Lp.

Exponentially growing cultures of Achromobacter rif-I and Lp supported phage growth (measured as described in 2.2.9). Increases of 5×10^2 to 10^4 fold in p.f.u. ml^{-1} were observed in aerated cultures after 4 h. No phage growth was observed in exponential Achromobacter strain 14 cultures.

Plaque formation

The plaque morphology of phage α3a on Achromobacter rif-I lawns was altered. Phage α3a formed circular semi-turbid plaques on Achromobacter Lp but formed halo-type plaques on rif-I lawns. The e.o.p. on the rif-I strain was 1.0 (relative to plating on Achromobacter Lp) and approximately 50% of the plaques were visible after overnight incubation, i.e. developing on the growing background lawn. These plaques were initially small, clear and irregular, but developed into circular halo-type plaques after a further 24 h incubation. The remaining 50% of the plaques developed after 36 - 48 h incubation. The plaque morphology of these stationary phase plaques was initially of a semi-turbid type, but they also developed into the halo type.

The number and size of plaques which appeared on Achromobacter Lp lawns did not increase (as observed with Achromobacter w.t., 2.3.6) with further incubation. This indicates that phage development did

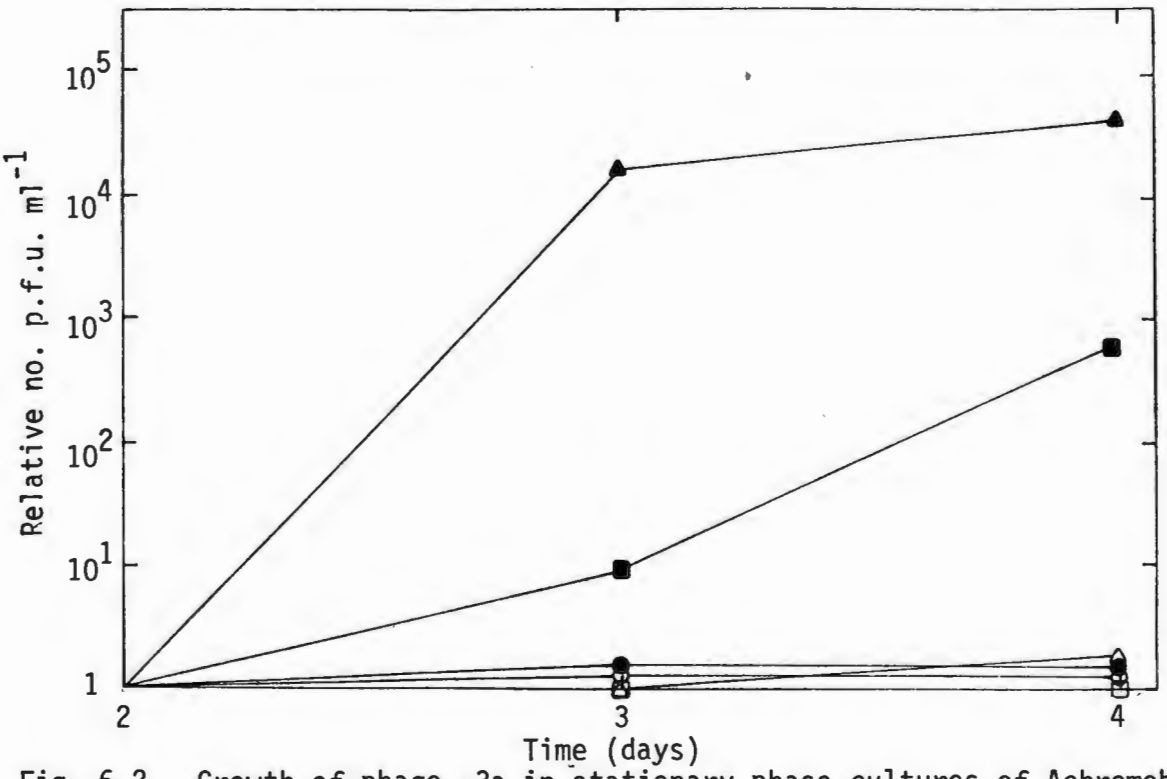


Fig. 6.2 Growth of phage α3a in stationary phase cultures of Achromobacter Lp 14 and rif-I strains. Stationary phase (2-day-old) cultures were infected with α3a (1 x 10^4 p.f.u. ml^{-1}) and re-incubated with and without shaking: Re-incubated with shaking: o—o, Lp; △—△, strain 14; and □—□, rif-I. Re-incubated without shaking: ●—●, Lp; ▲—▲, strain 14; and ■—■, rif-I.

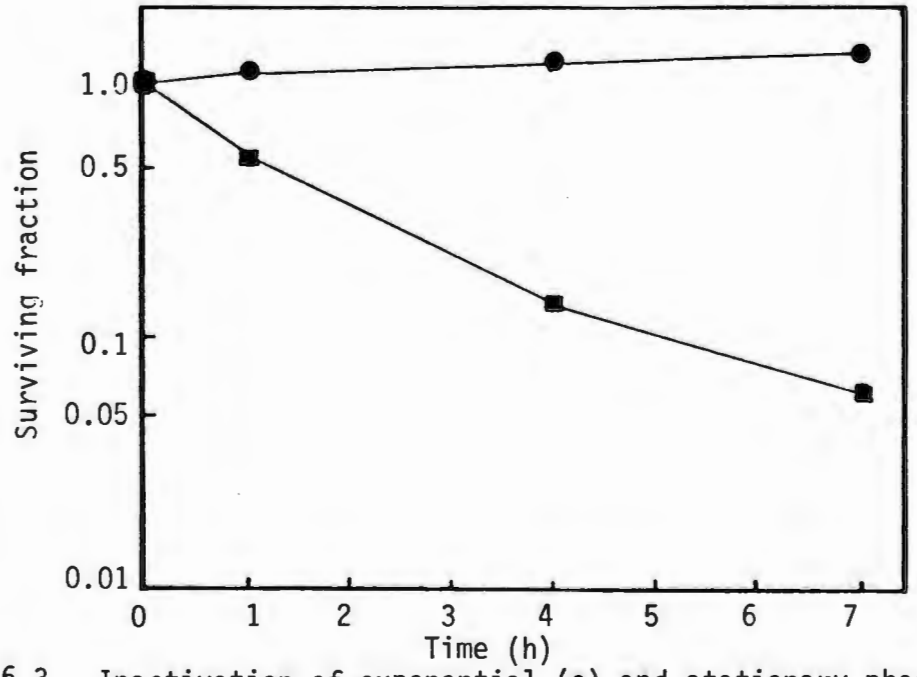


Fig. 6.3 Inactivation of exponential (●) and stationary phase (■) Achromobacter strain 14 cells by rifampicin. Refer to 6.2.4.

not continue when the host cells entered stationary phase.

6.3.5 Irreversible inhibition of stationary phase cells by rifampicin

Continued incubation of Achromobacter strain 14 stationary phase cells in the presence of rif (at the m.i.c.), resulted in the killing of 94% of the bacterial population over 7 h (Fig 6.3). Removal of the antibiotic by dilution and plating did not result in recovery.

In contrast, the exponential phase cells were not killed by incubation with rif. In the presence of rif, they showed limited growth, but were able to recover from the inhibition and produce colonies on plating. The limited growth observed was comparable with that obtained when exponential cultures were diluted into broth containing 15, 20 and 40 $\mu g\ ml^{-1}$ rif.

6.4 DISCUSSION

A rif resistant mutation in an Achromobacter Lp strain alters the development of phage α3a in these cells. The mutant strain Achromobacter rif-I is altered in plaque morphology and is able to support phage growth in stationary phase, as well as in exponential growth phase. The parent strain is only able to support phage development in exponential phase.

The rif resistance is not due to the exclusion of rif from the cell, but is the result of an alteration in the RPase, as shown by the resistance of the enzyme to the antibiotic in vitro. In vivo and in vitro, RNA synthesis by Achromobacter rif-I RPase is unaffected by rif ($25\ \mu g\ ml^{-1}$), whereas Achromobacter Lp RNA synthesis is inhibited.

In B. subtilis and E. coli the β subunit of the RPase core contains the rif binding site (Sumida-Yasumoto and Doi, 1977; Zillig et al., 1970). Since the β subunit is involved in initiation of transcription and interaction with σ factor and other regulatory polypeptides (see 6.1), changes in the β subunit (such as resistance to rif) may alter its capacity for initiation and/or regulation. Such a change may or may not affect transcriptional specificity.

It is likely that there is biochemical differentiation in terms of regulation of transcription from exponential growth phase to stationary phase in Achromobacter cells, such as that found in sporulating B. subtilis (Sumida-Yasumoto and Doi, 1977). The fact that a mutation in the RPase of Achromobacter rif-I enables these cells to support phage growth in stationary phase but does not appear to affect phage production in exponential growth phase, indicates qualitative changes in the transcriptional machinery. These could affect phage development directly or indirectly. For example, the stationary phase RPase of Achromobacter Lp may not be able to transcribe α3a DNA or associate with putative α3a regulatory polypeptides. Alternatively, host components required for phage development may not be transcribed (see 6.1). The rif resistant mutation overcomes this inhibition.

Further support for qualitative changes in the RPase from exponential phase growth to stationary phase is shown by the differing responses of Achromobacter to rif. Rif inhibition of exponential phase cells is reversible, but stationary phase cells appear to be irreversibly inhibited and are killed by the antibiotic. Sumida-Yasumoto and Doi (1977) found that rif had a different effect on RPase activity depending on the growth stage of the cell and the results

indicated that the RPase in vegetative and sporulating cells had different affinities for rif. They suggested that this was further support for the hypothesis that the RPase from vegetative and sporulating cells differed in structure or conformation.

The Achromobacter w.t. and strain 14 are normally able to support phage development in stationary phase. Phage α3a is, however, inhibited in exponential phase cultures of strain 14. As suggested in 5.4 this could result from immunity phase shift which may be affected by promoter selection of phage or host components.

Successful phage development in old cells is an unusual phenomenon, and its study in terms of transcriptional regulation may provide valuable information concerning host-phage interactions. Since no other phage has been reported to develop in very old cells this suggests that the Achromobacter host cells and phage α3a have unusual properties.

CHAPTER VII

PRELIMINARY STUDIES ON INTRACELLULAR PHAGE DEVELOPMENT

7.1 INTRODUCTION

A number of situations have been described in which a bacteriophage is able to infect a potential host cell but is unable to complete the lytic cycle. These have been termed 'abortive infections' (Adams, 1959). The best understood abortive infections are those which are brought about by the selective degradation of the phage genome by restriction endonucleases (Boyer, 1971). Other prophage-mediated abortive infections which do not appear to involve phage DNA degradation have been reported in B. subtilis lysogenic for phage SP02 (Rettenmier and Hemphill, 1973) and in E. coli lysogenic for phage P2 (Brégégère, 1978). In the latter case, interference of λ infection results in a peculiar type of host killing accompanied by a shut-down of host protein synthesis and degradation of bacterial tRNA (Brégégère, 1976).

To determine the nature of the inhibition of phage α3a in exponential Achromobacter strain 14, it is important to establish the sequence of events during infection of the w.t. strain in which phage development is not blocked. Such studies would involve analysis of phage specific DNA, RNA and protein synthesis.

The techniques used to study the macromolecular development of phage after infection depend largely on the nature of the phage, the host and the effect that the phage has on host cell metabolism. For example, some phage genomes contain unusual nucleotide bases which are not found

in its host. E. coli phages T4, T2 and T6 contain hydroxymethylcytosine (HMC) in place of cytosine (Kornberg et al., 1959) and B. subtilis phages SP82, φe and SP01 contain hydroxymethyluracil (HMU) in place of thymine (Rettenmier and Hemphill, 1973). In these cases phage specific DNA synthesis may be detected by differential labelling and can be distinguished from host DNA synthesis.

Analysis of phage specific DNA in B. subtilis has been simplified by the finding that replication of B. subtilis DNA is specifically inhibited by 6-(p-hydroxyphenylazo)-uracil (HPUra) (Brown, 1972). Under conditions where host synthesis is completely inhibited, phage replication proceeds normally (Schachtele et al., 1973; Kawamura and Ito, 1974).

To identify phage proteins by isotope labelling it is important to inhibit the synthesis of host proteins. Phages such as T4 (Snustad and Bursch, 1977), φe and SP01 (Kawamura and Ito, 1974) suppress host functions and if host protein synthesis is shut down sufficiently phage specific proteins can be identified. A number of phages such as λ (Murialdo and Siminovitch, 1971), φX174 (Gelfand and Hayashi, 1969) and φ29 (Carrascosa et al., 1973) do not interfere with host functions significantly and it is difficult to differentiate between host and phage induced proteins. However, this can be accomplished by UV irradiation of the host cells before infection. UV damages the host DNA and decreases host protein synthesis dramatically. Ptashne (1967) used such a system for the isolation of the λ repressor protein. Irradiation of host cells and analysis of protein by polyacrylamide gel electrophoresis has been used to characterize phage induced proteins of phages T7 (Studier and Maizel, 1969), φ29 (Carrascosa et al., 1973), φX174 (Gelfand and Hayashi, 1969; Godson, 1971) and λ (Murialdo and Siminovitch, 1971 and 1972; Schwartz, 1970). Kawamura and Ito (1977 a and b) analysed the

synthesis of phage specific mRNA in UV irradiated ϕ29 infected B. subtilis. The electrophoretic patterns of RNA were comparable with those of ϕ29 specific RNA isolated from unirradiated, infected cells (Kawamura and Ito, 1977 b). Using a combination of heteroduplex mapping of phage T7 deletions with genetic and electrophoretic analysis of T7 RNA and protein in UV irradiated cells, Simon and Studier (1973) were able to construct a detailed physical map of a large segment of T7 DNA.

Transfection, which is the infection of a host cell by virus nucleic acid devoid of its coat, has been used to solve many important problems in the field of procaryotic molecular biology (for an excellent cataloguing of the applications of transfection assays, see Benzinger, 1978).

One application of the transfection assay has been in determining the activity of λ DNA after induction in the presence of a P2 prophage (Brégégère, 1978). Phage λ is unable to grow in P2 lysogens, and Brégégère (1978) showed that the phage λ DNA extracted from cells undergoing P2-λ interference was still active in a transfection assay. This indicated that the inhibition of λ replication was not due to an irreversible alteration of the λ DNA substrate.

This chapter describes preliminary studies which may ultimately be used to determine the nature of phage α3a inhibition in exponential phase Achromobacter strain 14 cells.

7.2 METHODS

7.2.1 DNA, RNA and protein synthesis in phage α3a infected cells

DNA, RNA and protein synthesis was compared in infected and uninfected exponential cultures of Achromobacter w.t. and strain 14. The experiments were carried out to determine:

(i) whether phage development resulted in any difference in total DNA, RNA or protein synthesis in Achromobacter w.t. cultures and

(ii) whether abortive phage infection of strain 14 resulted in any observable difference in macromolecular synthesis, either as a result of phage synthesis or by suppression of host synthesis.

A method of dual labelling with ^{3}H and ^{14}C was developed to measure the synthesis of DNA, RNA and protein in a single culture.

Exponential tryptone broth cultures were adjusted to the same OD (0.3) using prewarmed broth. The cultures were divided into 2 x 10 ml aliquots in 150 ml flasks. Phage was added (m.o.i. 10) to one flask and 5 min later ^{3}H-adenine (1 μCi ml^{-1}), adenine (2 μg ml^{-1}), ^{14}C-L-leucine (0.5 μCi ml^{-1}) and L-leucine (10 μg ml^{-1}) were added to both flasks. Cultures were aerated for 120 min and at each sampling time, 2 x 0.5 ml aliquots were withdrawn and added to an equal volume of 1N NaOH (to determine DNA counts) and to 10% TCA containing 1 mg ml^{-1} adenine and leucine on ice (to determine total nucleic acid and protein counts). Hydrolysis of the RNA in the NaOH treated sample was carried out as described (4.2.3). Treatment of

the TCA sample and filtering of both samples was as described in 4.2.2.

In double isotope analysis it is desirable to obtain the minimum amount of spill-over between the carbon and tritium channels during counting without eliminating too great a percentage of counts. The method used for the adjustment of the carbon and tritium channels was that of Neame and Homewood (1974). The lower and upper discriminator settings used for the tritium channel were 0 and 2.3 respectively and for the carbon channel, 2.8 and 10 respectively. The spill-over from the tritium to the carbon channel was between 0.01% and 0.02% and 16% from the carbon to the tritium channel. The acceptable spill-over levels depend on the number of tritium and carbon counts relative to one another.

7.2.2 Phage α3a development in UV irradiated host cells

Phage development in UV irradiated Achromobacter w.t. cells was examined with the aim of finding a system to detect phage macromolecular synthesis specifically.

Dosage effect of UV on DNA and protein synthesis in uninfected and α3a infected cells.

Achromobacter w.t. was grown to a cell density of 1.2×10^8 cells ml^{-1} in AHC medium at 30°C, centrifuged (3 000g for 10 min) and resuspended in chilled PO_4 buffer (0.1M). Samples (8 ml) were irradiated on ice as described (4.2.7). At various time intervals 1.2 ml samples were removed and placed in microcentrifuge tubes in the dark in ice. The bacteria were pelleted and resuspended in 1.2 ml chilled AHC medium.

One ml samples were placed in small tubes containing ^{3}H-adenine ($1\mu Ci\ ml^{-1}$), adenine ($2\ \mu g\ ml^{-1}$).

^{14}C-L-leucine (0.5 μCi ml^{-1}) and L-leucine (10 μg ml^{-1}). The samples were divided into 0.5 ml aliquots and to one, phage α3a was added (m.o.i. 10). After 5 min on ice the cultures were warmed to 30°C and incubated with aeration for 200 min. After this time, half the sample was treated with NaOH (for incorporation into DNA) and half with TCA (for incorporation into protein) (7.2.1).

Filtering and counting were carried out as described (4.2.2 and 7.2.1).

Phage production after irradiation

Cells were irradiated as described above. Phage α3a was added (3×10^4 p.f.u. ml^{-1}) to 1 ml cell samples, and the phage yield was measured after 200 min of aeration at 30°C.

7.2.3 Transfection with phage α3a DNA

High titre phage lysates were prepared and purified as described (2.2.2 and 2.2.3).

Phage DNA extraction

(i) Concentrated phage lysates were resuspended in 0.01M-Tris buffer (pH 7.6). Sodium dodecyl sulphate was added to a final concentration of 1.0% and incubated at room temperature for 30 min or until opalescence disappeared. The nucleic acid was extracted using the phenol method of Sjöström, Lindberg and Philipson (1972).

(ii) The method of Thomson and Woods (1973) was used.

Transfection methods

Three transfection methods were employed:

(i) the penicillin sphaeroplast method of Thomson and Woods (1973);

(ii) the calcium shock method of Mandel and Higa (1970), and

(iii) the method of Kushner (1978).

7.3 RESULTS

7.3.1 DNA, RNA and protein synthesis in phage infected and uninfected exponential phase cultures

DNA Synthesis

There was a measurable increase in the incorporation of ^{3}H-adenine into DNA in infected w.t. cultures compared to that in uninfected cultures (Fig 7.1a). The incorporation in the two cultures was similar during the first 45 min but from 45 to 120 min DNA synthesis in the uninfected culture appeared to plateau while the level doubled in the infected culture during the same time. The latent period for phage α3a under these conditions was 100 min and it can therefore be concluded that the increased DNA synthesis was due to phage replication.

In exponential cultures of strain 14 the addition of phage did not result in increased DNA synthesis (Fig 7.1b). The total DNA synthesis was consistently lower in infected cultures than in uninfected cultures (similar results in 4 experiments). It was thought that although phage α3a did not appear to develop in these cultures, it may be capable of suppressing host DNA synthesis. To determine whether the rate of incorporation in these cultures was significantly different, samples from unlabelled infected and uninfected cultures were removed

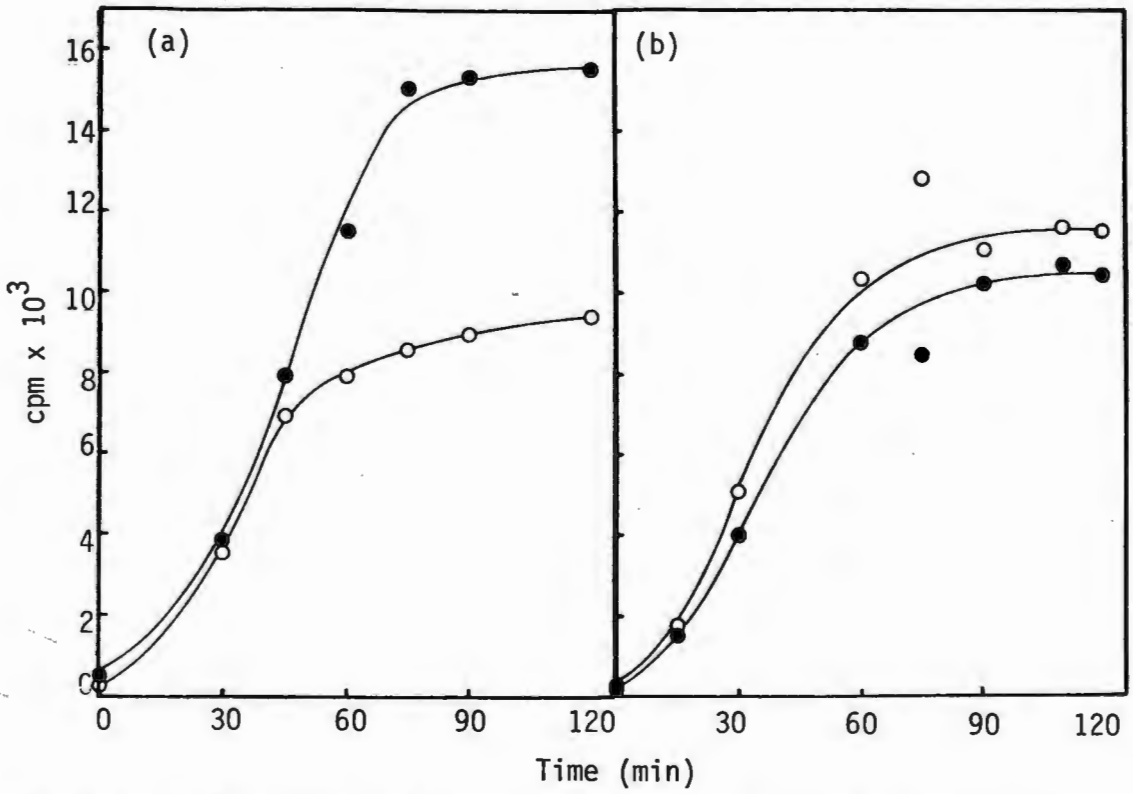


Fig. 7.1 DNA synthesis in exponential cultures of Achromobacter w.t. (a) and strain 14 (b) which had been infected with phage α3a (m.o.i. 10) (●) or were uninfected (o). Incorporation of ^{3}H-adenine into NaOH hydrolysed TCA-precipitable material was measured.

(at times 30, 60 and 120 min) and incorporation of ^{3}H-adenine measured over 15 min. The rates did not appear to differ appreciably (Table 7.1).

Table 7.1 Rate of DNA synthesis in infected and uninfected exponential phase strain 14 cultures.

Time (min) after phage addition	DNA synthesis (counts/OD unit/min)	
	+ phage α3a	- phage α3a
30	593	715
60	868	725
120	363	433

Infected and uninfected exponential phase strain 14 cultures also had identical growth curves which seems to indicate that phage α3a does not suppress host functions.

RNA and protein synthesis

The synthesis of RNA was calculated by subtracting the counts obtained for DNA synthesis (NaOH treated samples) from the counts obtained for ^{3}H-adenine incorporation into total nucleic acid (from the TCA treated sample).

RNA synthesis in Achromobacter w.t. was slightly decreased in infected cells and slightly increased in infected cells of strain 14 (Fig 7.2a and b, respectively).

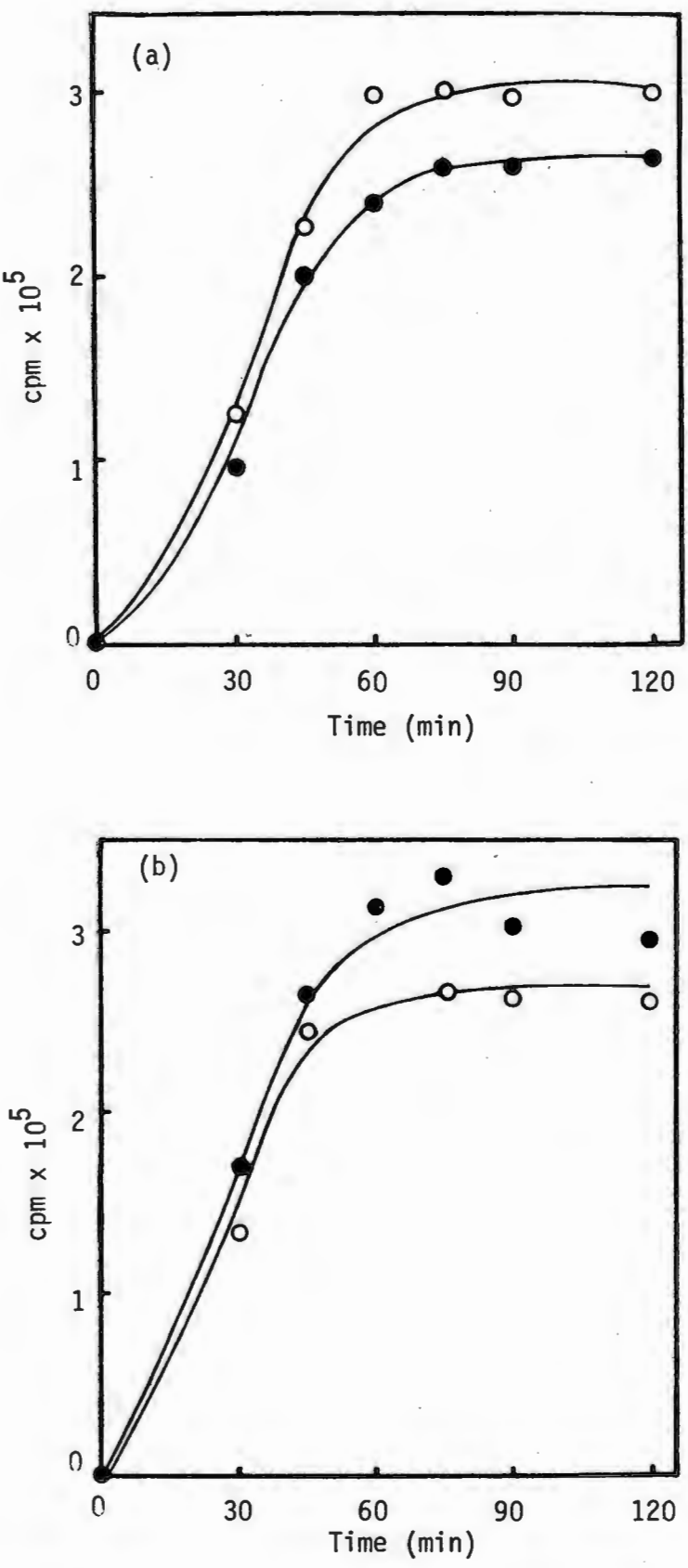


Fig. 7.2 RNA synthesis in exponential cultures of Achromobacter w.t. (a) and strain 14 (b) which had been infected with phage α3a (m.o.i. 10) (●) or were uninfected (o). Incorporation of ^{3}H-adenine into RNA calculated by subtracting counts for incorporation into DNA from total counts in TCA precipitate.

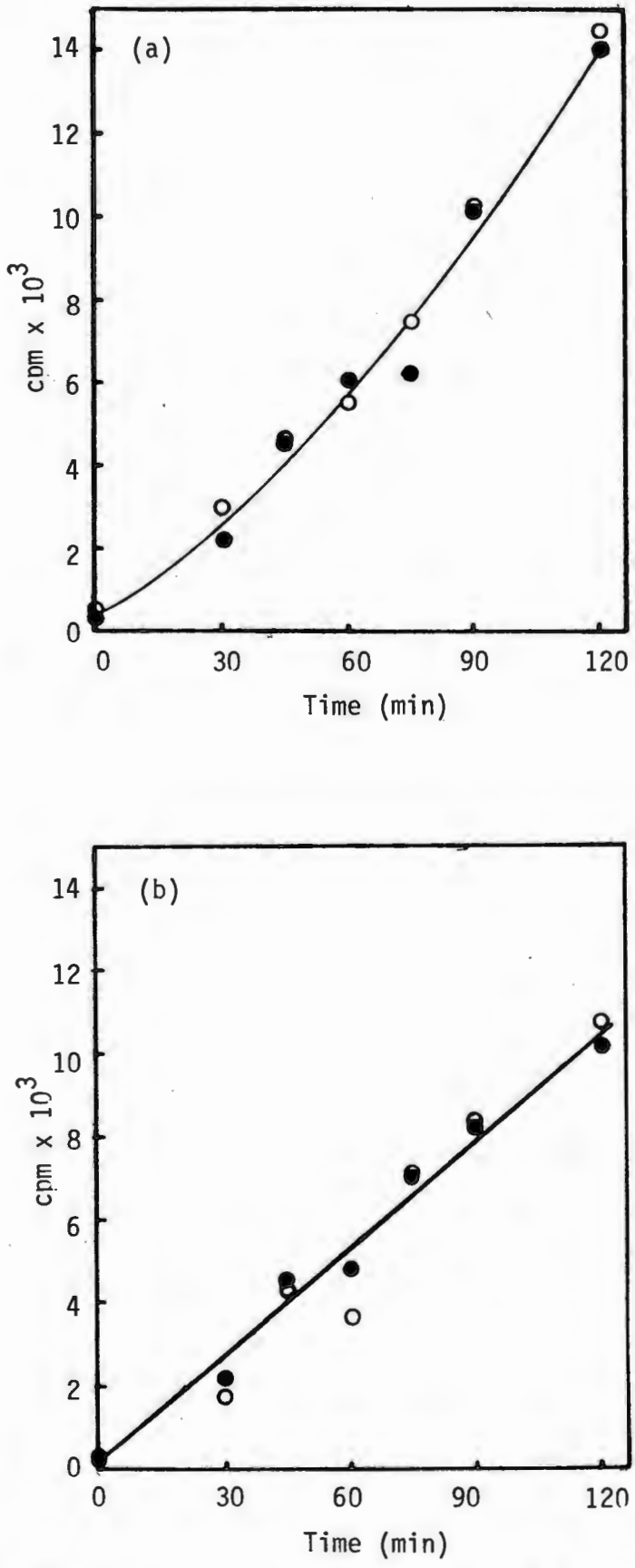


Fig. 7.3 Protein synthesis in exponential Achromobacter w.t. (a) and strain 14 (b) cultures which had been infected with phage α3a (m.o.i. 10) (●) or were uninfected (o). The incorporation of ^{14}C-leucine into TCA-precipitable material was determined.

There was no observable difference in protein synthesis in Achromobacter w.t. or strain 14 cultures due to phage development (Fig 7.3a and b, respectively).

7.3.2 Phage development in UV irradiated Achromobacter w.t. cells

UV dose effect on protein synthesis

It is important to examine protein synthesis at a range of UV doses in order to find the optimal dose where host synthesis is preferentially inhibited, but infection with phage causes a stimulation of amino acid incorporation. This was done by irradiating Achromobacter w.t. cell suspensions with different doses of UV and measuring amino acid incorporation with and without α3a infection. Incorporation was measured at a late time after infection (200 min) (Murialdo and Siminovitch, 1971; Gelfand and Hayashi, 1969).

Incorporation of ^{14}C-leucine decreased with increasing UV dose both in infected and uninfected Achromobacter w.t. cells, but there was no stimulation of incorporation by phage α3a infection (Fig 7.4) even though the UV doses used were comparatively low. After a dose of only 112 $J\,m^{-2}$, incorporation in uninfected cells of Achromobacter was reduced to 3.1% of that in unirradiated cells, but no stimulation resulted from infection.

UV dose effect on DNA synthesis

Host DNA synthesis was inhibited in UV irradiated cells and infection with α3a resulted in stimulation of incorporation of ^{3}H-adenine. Incorporation (measured after 200 min) decreased in infected and uninfected cells as the UV dose was increased (Fig 7.5). In uninfected cells incorporation was reduced to 39 and 3.7% of

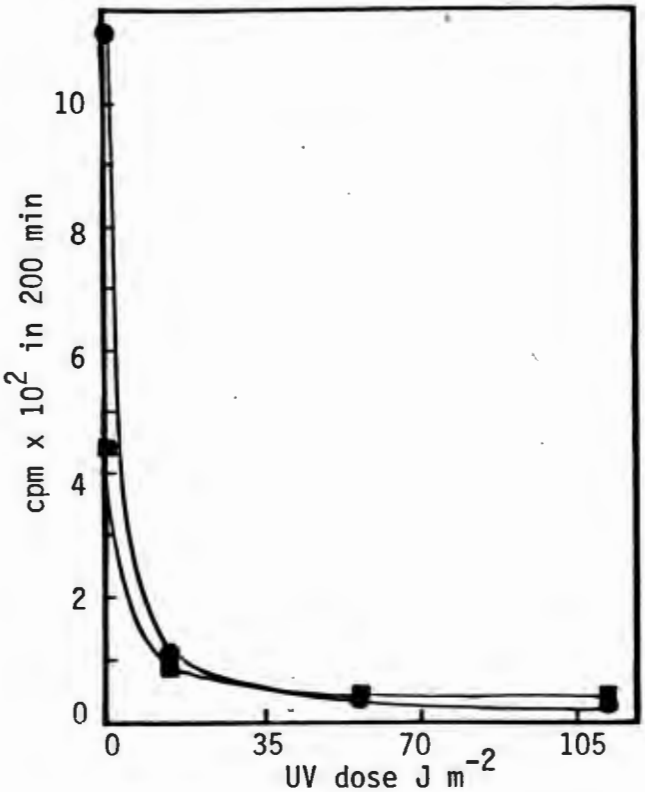


Fig. 7.4 Protein synthesis in UV irradiated phage infected (■) and uninfected (●) Achromobacter w.t. cells. Exponential phase cells were irradiated for various times. ^{14}C-leucine and phage (m.o.i. 10, in the case of infected cells) were added and incorporation into TCA-precipitable material measured after 200 min.

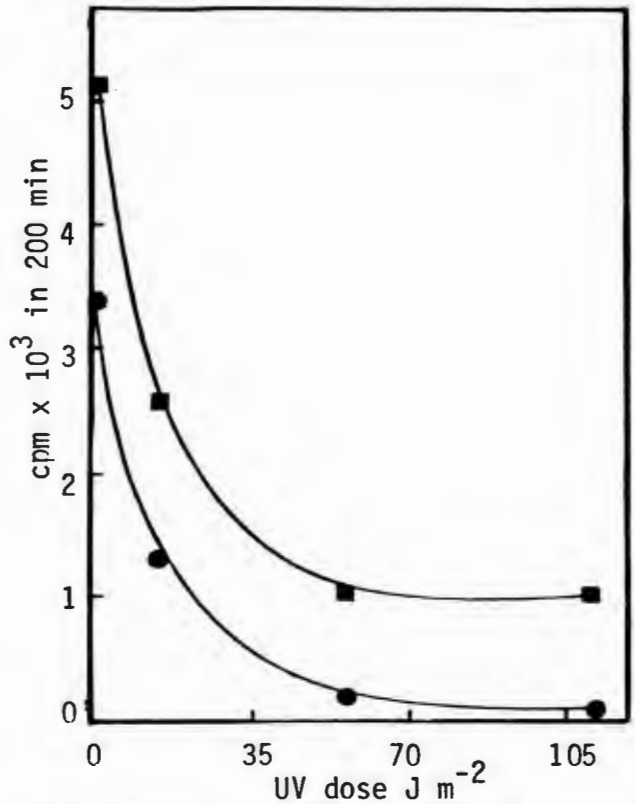


Fig. 7.5 DNA synthesis in UV irradiated phage infected (■) and uninfected (●) Achromobacter w.t. cells. Samples were treated as described for Fig. 7.4 but incorporation of ^{3}H-adenine into NaOH hydrolysed TCA-precipitable materials was measured.

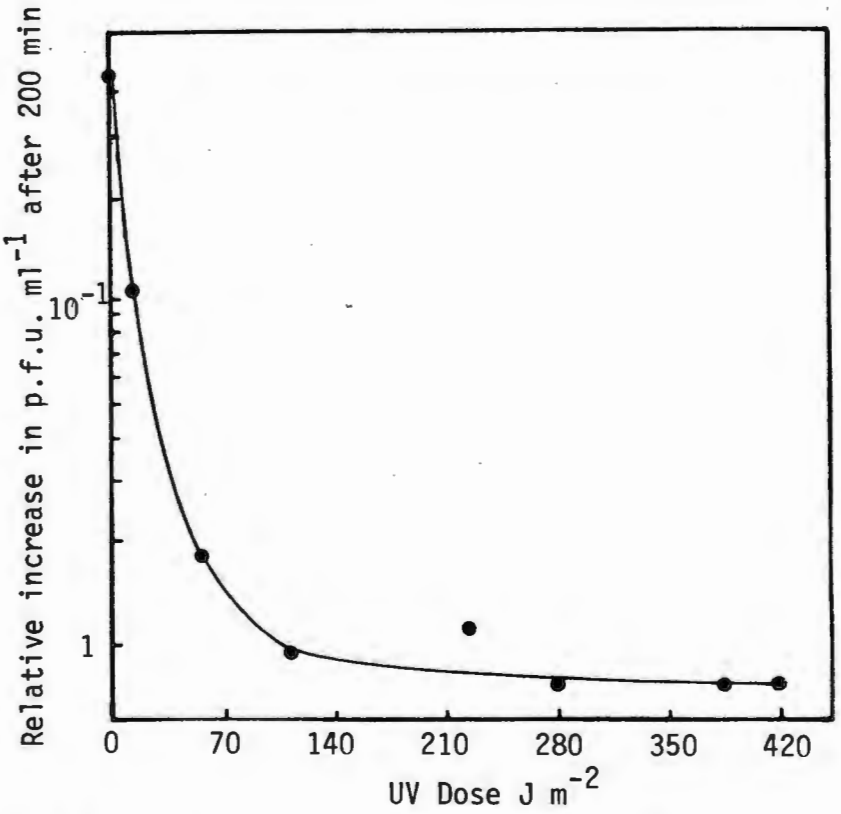


Fig. 7.6 Phage production in UV irradiated Achromobacter w.t. cells. Cell samples were pre-irradiated for various times, phage α3a was added (c. 3 x 10^4 p.f.u. ml^{-1}) and the phage titre assayed after 200 min of incubation with aeration.

that of unirradiated cells for doses of 14 and 112 J m^{-2}, respectively. Stimulation of incorporation by infection increased as the UV dose increased, reaching 1.9 fold at 14 J m^{-2} and 8.5 fold at 112 J m^{-2}. The stimulation of unirradiated cells by infection was 1.5 fold (consistent with results in 7.3.1). UV doses exceeding 112 J m^{-2} resulted in reduced stimulation by infection (results not shown).

Phage production in UV irradiated cells

Phage α3a production was severely inhibited in UV irradiated Achromobacter w.t. cells, and showed extreme sensitivity to low UV doses. In unirradiated cells there was an increase of 4.3×10^1 fold in p.f.u. ml^{-1}, but in cells which had been irradiated with 112 J m^{-2} there was no detectable increase (Fig 7.6).

The latent period for phage production in AHC medium was c. 100 min (similar to that in tryptone broth; Thomson and Woods, 1974) and the average burst size was 230 as determined by a single step growth experiment (Adams, 1959).

7.3.3 Transfection

No transfection of Achromobacter w.t. with phage α3a DNA was obtained using any of the methods described.

7.4 DISCUSSION

Using dual labelling techniques a method was developed in which DNA, RNA and protein synthesis could be measured in a single culture.

Measurement of DNA synthesis in infected and uninfected exponential cultures of Achromobacter w.t. showed that without attempting to inhibit

host DNA synthesis preferentially, phage infection stimulated incorporation of ^{3}H-adenine into DNA. However, exponential cultures of strain 14 did not show any stimulation of DNA synthesis by α3a infection. Either phage α3a DNA is not replicated in exponential strain 14 cells, or the levels are too low to detect without preferential inhibition of host DNA synthesis.

Phage infection of exponential strain 14 did not appear to have any detrimental effect on host metabolism (as found with λ-P2 interference; Brégégère, 1976) as indicated by normal growth rates of infected cells.

UV irradiation of Achromobacter w.t. cells was effective in the inhibition of DNA and protein synthesis. Irradiation prior to infection with phage α3a had an interesting effect on phage production, protein and DNA synthesis.

DNA synthesis was stimulated by infection of UV irradiated cells, suggesting that phage α3a is able to replicate in irradiated cells. However, the incorporation of amino acids into protein in irradiated cells was sensitive to low UV doses and was not stimulated by infection. A dose of 112 J m^{-2} reduced incorporation in uninfected cells to 3.1% of that in unirradiated cells and there was no stimulation by infection. In comparison, Murialdo and Siminovitch (1971) found that after irradiation of 1200 J m^{-2}, incorporation of amino acids in uninfected cells was reduced to 0.2% of that in unirradiated cells, but this level was stimulated 10-fold by infection with λ.

Phage production was also severely inhibited by low UV doses of the host cells prior to infection with no increase in p.f.u. ml^{-1} at 112 J m^{-2}.

It appears that although phage DNA synthesis can proceed in

irradiated cells, the integrity of the host chromosome is required for phage protein synthesis and consequently for phage production. Irradiation of E. coli prior to infection with λ inhibits synthesis of some late λ proteins (Schwartz, 1970), but despite this there is an observable stimulation of amino acid incorporation (Murialdo and Siminovitch, 1971) which did not occur in Achromobacter infected cells.

It is possible that phage α3a development, in particular protein synthesis, is dependent on an interaction with the host chromosome. The host may produce a product essential for phage protein synthesis and production may be destroyed by irradiation. A number of E. coli genes are essential for bacteriophage development and a variety of bacterial mutants have been reported which inhibit phage λ or T4. Host mutants such as gro N (Georgopoulos, 1971), induce a specific block in viral transcription. Other mutants inhibit viral replication (gro P, Georgopoulous and Herskowitz, 1971) and another class (gro E and tab C) block viral morphogenesis (Georgopoulos and Hohn, 1978; Hendrix and Tsui, 1978; Takahashi, 1978).

On the other hand, the infecting α3a may interact with the cryptic prophage which is present in Achromobacter w.t. and thought to be integrated in the chromosome. Phage α3a may require cryptic phage functions and the interaction may be of the P4 - P2 transactivation type. Temperate phage P4 does not carry a complete set of genes for particle formation and is able to activate all the head, tail and lysis genes of a P2 prophage (Six, 1975; Souza et al., 1977; Barclay and Dove, 1978a and b).

Attempts to develop a transfection assay to determine the activity of phage DNA in infected exponential Achromobacter strain 14 have been unsuccessful, although Woods and Thomson (1973) were able to transfect Achromobacter sphaeroplasts with α3a DNA.

Alternative ways of examining the infected phage DNA may be to analyse the DNA by centrifugation on neutral and alkaline sucrose gradients or by agarose gel electrophoresis techniques.

CONCLUSION

The aim of this study was to characterize phage α3a growth in stationary phase Achromobacter cells with particular reference to α3a development in Achromobacter strain 14. In Achromobacter strain 14 phage development is blocked in exponential phase but is not restricted in stationary phase. Although the opposite situation, involving unrestricted phage development in exponential phase and inhibition in stationary phase is common, this is the first detailed report of restricted phage development being alleviated once the host cells reach stationary phase.

Phage growth characteristics in stationary phase strain 14 differ from those in exponential Achromobacter w.t. cells. The latent period is longer (6 - 9 h compared with 100 min) and the burst sizes larger (709 compared with 153). In both Achromobacter strain 14 and w.t. stationary phase cultures, phage development displays a peculiar requirement for low levels of aeration. This suggests that phage development requires that the host cell be in a particular physiological state. Reduction in aeration has been shown to mediate numerous physiological and morphological changes in the host cell. Since regulatory mechanisms operating in stationary phase gram-negative bacteria are obscure, the Achromobacter-phage α3a interaction could provide an ideal system for studying differentiation in stationary phase. Such studies may reveal new control mechanisms or novel modes of action of known control mechanisms.

Changes in the host transcriptional apparatus have been shown to affect differential phage development in Achromobacter Lp, a strain which only supports phage in exponential phase. By altering the RNA

polymerase this strain was able to support phage in stationary phase. Future work in this area should include the isolation of more rifampicin resistant mutants and the determination of their phage characteristics as well as the biochemical characterization of RNA polymerases extracted at different times during bacterial growth and phage infection.

In terms of the differential phage growth in Achromobacter strain 14, there are indications that resident phage control mechanisms which exert their effect on superinfecting phage, are influenced by the growth phase of the bacterium. Further studies on phage $\alpha 3a^{x}$ which is able to overcome the developmental block in exponential phase strain 14 will prove useful in determining how these control mechanisms operate.

The bacterium Achromobacter and its phages are relatively uncharacterized in terms of what is known about organisms such as E. coli, B. subtilis and bacteriophage lambda. In an attempt to explain and understand the unique system of bacteriophage growth described in this study, I have followed the approaches dictated by decades of researching these well characterized microorganisms.

Undoubtedly much can be gained from this type of approach, but it is well to remember that one cannot draw too many parallels between known systems and new systems. Novel modes of regulation may exist which may dictate the use of new methods of analysis.

APPENDIX

MEDIA AND SOLUTIONS

All H_2O used was glass distilled.

Acid hydrolysed casein medium (AHC)

Vitamin Free Casamino Acids	20g
NaCl	23.4g
Glucose	1.5g
Tris Base	12.114g
$MgSO_4.7H_2O$ (stock solution 0.1M)	1ml
H_2O	1ℓ

Adjust to pH 7.0 using HCl.

AHC + tryptophan

Stock solution of tryptophan made up in distilled water, sterilized by filtration and added to AHC medium at final concentration of 20 μg ml^{-1}.

AHC bottom agar

AHC medium	1ℓ
Oxoid agar no. 3	15g

AHC sloppy agar

AHC medium containing	
3g glucose instead of 1.5g	1ℓ
Oxoid agar no. 3	6g

Antibiotics

Stock solutions made up as follows and stored at 4°C:

	Dissolved in	Final concentration
chloramphenicol	95% ethanol	1 mg ml^{-1}
nalidixic acid	0.1N NaOH	1 mg ml^{-1}
rifampicin	95% ethanol	1 mg ml^{-1}

Minimal medium

Salts solution:	$K_2H\ PO_4$	10.5g
	$KH_2\ PO_4$	4.5g
	Sodium-citrate $2H_2O$	0.47g
	$(NH_4)_2\ SO_4$	1g
	H_2O	100ml
Glucose:	NaCl	23.4g
	H_2O	1ℓ
Water agar:	NaCl solution	1ℓ
	Oxoid Ion agar (No 2)	12g
To make up:	NaCl sol. or water agar	70ml
	Salts solution	8ml
	Glucose	1ml
	$MgSO_4$ (stock sol. (0.1M)	316μℓ (0.4mM)

Phosphate buffer (0.1M)

Solution A (1M $KH_2\ PO_4$):	$KH_2\ PO_4$	13.6g
	H_2O	100ml

Solution B (1M $K_2H\ PO_4$):	$K_2H\ PO_4$	17.4g
	H_2O	100ml

Mix 70ml sol. A with 100 ml sol. B (approximately) until the pH is 6.9 (10x buffer).

To make up:	H_2O	900ml
	NaCl	23.4g
	10x buffer	100ml
	$MgSO_4$ (stock sol. 0.1M)	1ml
		(0.1mM)

Transport buffer (double strength)

H_2O	76ml
NaCl	2.34g
10x buffer	20ml
Glucose (20% w/v sol.)	4ml
$MgSO_4$ (0.1M stock sol.)	0.2ml

Scinitillation coctail

10x solution:	Primary Fluor diphenyl ozazole (PPO)	26.7g
	Toluene	1000ml

Store in the dark. Dilute 10x with toluene for use.

Tris-HCl buffer

Tris Base	1.12114g
$MgSO_4.7H_2O$	2.464g
NaCl	23.4g
H_2O	1ℓ

Adjust to pH 8 with HCl

Tris-maleic buffer (TM)

Consists of minimal medium in which the phosphate salts are replaced by tris and maleic acid, each at a final concentration of 0.05M. Adjust to pH 6.

Tryptone broth

Tryptone	13g
NaCl	23.4g
Glucose	1.5g
H_2O	1ℓ

Tryptone bottom agar

Tryptone broth	1ℓ
Oxoid agar no. 3	15g

Sloppy agar

Tryptone	10g
NaCl	23.4g
Glucose	3g
Oxoid agar no. 3	6g
H_2O	1ℓ

All sterilization was by autoclaving unless stated otherwise.

ABBREVIATIONS AND NOMENCLATURE

arg	arginine
c.	circa
cml.	chloramphenicol
cys	cystein
e.o.p.	efficiency of plating
ileu	isoleucine
leu	leucine
LIV	leucine, isoleucine and valine
met	methionine
m.i.c.	minimum inhibitory concentration
MC	mitomycin C
MM	minimal medium
m.o.i.	multiplicity of infection
nal	nalidixic acid
NTG	N-methyl-N'-nitro-nitrosoguanidine
OD	Optical density
p.f.u.	plaque forming unit
PMSF	phenylmethylsulphonyl fluoride
pro	proline
rif	rifampicin
RPase	RNA polymerase
SC	standard container
TCA	trichloroacetic acid
TFL	5'5'5' trifluoroleucine
TM	tris-maleic
trp	tryptophan
ts	temperature sensitive
UV	ultraviolet
w.t.	wild-type

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