GENETIC STUDIES ON COLLAGENOLYTIC ACHROMOBACTER STRAINS AND THEIR BACTERIOPHAGES

by

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CONTENTS

SUMMARY

A survey of collagenolytic aerobic bacteria from cured hides yielded three strains of Bacillus and eight of Achromobacter which degraded collagen at 0.4 M NaCl. Achromobacter sp. 2 was chosen for genetic studies due to its high collagenolytic activity and the lack of genetic information on Achromobacter. Four temperate bacteriophages specific for Achromobacter sp. 2 were isolated and their relationships studied. The phages caused lysogenic conversion resulting in the inability of lysogens to adsorb phage. Achromobacter sp. 2 was shown to be a cryptic lysogen as it was not immune to superinfection but had a very low rate of spontaneous induction which could be increased with mutagens. It is proposed that the cryptic lysogeny of this strain is maintained by a defective excision mechanism and the mode of prophage integration in the host chromosome. DNA extracted from phage α3a was used to transfect spheroplasts. The optimal conditions for the development of competence for transfection were determined. The presence of nuclease-attack on phage DNA under conditions of prolonged incubation of DNA and spheroplasts was proposed. A method for extracting Achromobacter DNA was devised which yielded purified, undegraded DNA, but it was not possible to transform Achromobacter sp. 2 with this DNA. The α phages were used to transduce a number of genetic markers into Achromobacter auxotrophs. The transductants had the ability to release the cryptic α3 prophage at a high rate while maintaining their sensitivity to homologous phage infection. It is proposed that this is due to complementation between the cryptic prophage and the residual phage functions in the transducing particles. The transductants segregated auxotrophs with a probability of 10^{-3} per cell per generation. It appears that an unusual system of generalised transduction is operating whereby the transducing particles contain both phage and bacterial DNA which is incorporated into the recipient genome by a single recombination event yielding unstable transductants.

In a study on induction of Escherichia coli (λ), carcinogenic nitrosamines were shown to be inducers of phage development. This provides a screening system for potentially harmful nitrosamines.

INTRODUCTION

Animal hides, used in the production of leather, are very susceptible to microbial attack. It has been shown (Woods, Atkinson, Cooper & Galloway, 1970) that there is no correlation between bacterial counts on a hide and the quality of the leather produced. There is a correlation, however, between bacteria which degrade collagen and leather decay (Woods, Rawlings, Cooper & Galloway, 1973). Collagen is the major protein constituent of leather and its degradation causes the upper layers of the leather to slough off. Tanning renders collagen resistant to collagenolytic attack by the highly specific collagenase enzymes, the only ones capable of degrading this protein. However, if collagenase attack occurs prior to tanning, inferior leather with loose grain will be produced no matter how well the tanning is done.

Bacterial degradation of collagen can occur during the delay between flaying and curing, between curing and desalination at the tannery or within the soak pit prior to tanning. Considerable decay has been shown to occur at all these stages (Woods, Welton, Thomson & Cooper, 1972) particularly prior to curing.

Bacterial collagenases have been isolated from Clostridium histolyticum (Gallop, Seifter & Meilman, 1957), Streptomyces madurae (Rippon, 1968) and Achromobacter iophagus (Welton & Woods, 1973). Tancous (1961) has identified the strain causing hide damage in a tannery in Cincinnati as a salt tolerant Clostridium capitovale and has isolated its collagenase. Other strains of bacteria with collagenolytic activity include C. perfringens (Neuman & Tytell, 1950), Bacteroides melaninogenicus (Gibbons & MacDonald, 1961), Staphylococcus aureus, C. tetani and other Bacteroides strains

(Waldvogel & Swartz, 1969), two strains of Pseudomonas (Adamcic & Clark, 1970), three Bacillus and eight Achromobacter strains (Thomson, Woods & Welton, 1972).

Collagen is a triple helix and the three polypeptide chains have a high glycine content. The sequence -gly-pro-X- (where X is often hydroxyproline) is a geometric requirement as glycine is sufficiently small to be accommodated within the helical structure (Steven, 1972). Collagenase cleaves the Y-gly bond producing peptides with NH_2-terminal glycine (Eisen, Bauer & Jeffrey, 1970).

The collagenolytic bacteria from cured hides are all halotolerant or halophilic. Extreme halophiles require at least 10-15% NaCl and the optimum is 20-25% NaCl (Larsen, 1962). They comprise the pigmented Halobacterium group and the Sarcina-Micrococcus group (Kushner, 1968). Moderate halophiles, requiring more than 3% NaCl, cover many groups of bacteria, are usually colourless and occur in large numbers in curing brines. Halotolerant strains do not require salt for growth but can multiply in concentrations of 10% or more.

Halophilism appears to be due to a multiple genetic system and no simple mutational event has been able to effect a change in their salt specificity (Larsen, 1962). Halophilic enzymes appear to be unstable due to a high degree of intramolecular electrostatic repulsion caused by negative charges as halophilic proteins have more aspartic and glutamic acid residues than non-halophiles (Reistad, 1970). The high internal cation concentration of halophiles helps to neutralize these charges to allow the protein to adopt an active configuration (Hochstein & Dalton, 1968). Removal of salt causes internal sulfhydryl groups to be exposed

(Hubbard & Miller, 1970). Cations are also required to stabilize the highly acidic halophile ribosomes (Larsen, 1967). Salts have an electrostatic as well as an osmotic role in maintaining the cell wall structure (Soo-Hoo & Brown, 1967). In slightly halophilic bacteria, Na^+ produces mechanical strength for the cell envelope and K^+ prevents cell lysis by balancing the internal osmotic pressure (Unemoto, Tsuruoka & Hayashi, 1973). There appears to be a specific requirement of Na^+ ions for the enzymes involved in cell wall synthesis (Larsen, 1962) and for extracellular proteinases (Norberg & Hofstein, 1969).

Halophiles appear to contain a satellite component of DNA in CsCl density gradient which may represent a clustering of genes necessary for the unique property of halophilism (Moore & McCarthy, 1969).

As preliminary investigations had indicated that leather decay was caused by collagenolytic activity by halophilic or halotolerant bacteria it was decided to study these collagenolytic strains. It was necessary to develop an assay for collagenase at different salt concentrations and then to screen hide bacteria for those producing the enzyme. With the ultimate aim of studying the genetic control of collagenase production genetic systems were developed. The bacterium used in this study was a strain of Achromobacter which had a high level of enzyme activity. The genetics of Achromobacter have not previously been investigated. Bacteriophages of Achromobacter were isolated and used in transfection and in the development of a transduction system. During the course of this study the phage-host relationships were also investigated.

ABBREVIATIONS AND NOMENCLATURE

AO	acridine orange
arg	arginine
5BU	5-bromouracil
c.	circa
cys	cystein
DAP	diaminopimelic acid
EDTA	ethylenediaminetetra-acetic acid
e.o.p.	efficiency of plating
his	histidine
MC	mitomycin C
met	methionine
MI	McIlvaine's buffer
m.o.i.	multiplicity of infection
NG	N-methyl-N'-nitro-N-nitrosoguanidine; nitrosoguanidine
O.D.	optical density
p.f.u.	plaque forming unit
pro	proline
SC	saline citrate buffer
SDS	sodium dodecyl sulphate
SNF	supernatant fluid
trp	tryptophan
u.v.	ultraviolet
w.t.	wild-type

Lysogeny is denoted by parenthesis: Achromobacter sp. 2 (α1)

THE INCIDENCE OF AEROBIC COLLAGENOLYTIC BACTERIA ON CURED HIDES

SUMMARY

The collagenolytic activity of 110 aerobic halophilic and halotolerant bacterial strains isolated from cured hides was determined. At 2.34% NaCl, 10% of the bacterial strains could denature purified collagen. Collagenolytic activity was not detected at 7% NaCl. Three of the collagenolytic strains were identified as Bacillus spp. and eight as Achromobacter spp. Nine of these eleven bacteria were also gelatinolytic. Australian hides which produce decay-free leather lacked collagenolytic bacteria.

INTRODUCTION

Since leather decay is attributed to bacterial degradation of collagen, the incidence of collagenolytic bacteria in cured hides is important. Although pure collagen is not denatured by common proteolytic enzymes, it may be denatured by specific collagenases or by physical and chemical means. Denatured collagen (gelatin) is susceptible to many proteolytic enzymes. Unfortunately, frequent use of unsuitable denatured substrates (e.g. hide powder), because of their availability and ease of assay, has resulted in erroneous reports of collagenolytic activity (Mandl, 1961). Eight bacterial strains have been shown to have collagenolytic activity under anaerobic conditions (Waldvogel & Swartz, 1969). The only report of collagenolytic activity in aerobic bacteria, as judged by denaturation of purified collagen, implicated two strains of Pseudomonas (Adamcic & Clark, 1970).

The present study was undertaken to determine the incidence of collagenolytic halophilic and halotolerant bacteria in hides. The salt requirements of gelatinolytic activity of the bacteria were also investigated and the strains were classified to the genus level.

The collagenolytic bacteria were isolated in order to carry out genetic studies with a view to determining the genetic control of collagenase production.

METHODS

Bacterial isolation. Plugs, 2 cm. in diameter, were taken from 10 randomly selected hides from eight different batches at a tannery (Woods, Atkinson, Cooper & Galloway, 1970). Two of the hide batches were from different Australian curers and the remainder from six different South African curers. Five plugs from each hide sample were cut into pieces and soaked in 50 ml. 10% NaCl at 4 °C for three days prior to plating on 10% NaCl agar to obtain isolated bacterial colonies.

Gelatinolytic activity. Bacterial strains were grown on 0.85 and 10% NaCl agar containing 0.25% gelatin. Utilization of gelatin was determined by flooding with acidified 15% $HgCl_2$ (Cowan & Steel, 1965).

Preparation of collagen. Neutral-salt-soluble collagen extracted from calf skin and purified by the method of Cooper & Davidson (1965) was used. The collagen was kept at 4 °C in the lyophilized form. A 0.2% solution of the collagen in 0.4 M (2.34%) NaCl, 0.01 M tris, 0.002 M $CaCl_2$, pH 7.6, was prepared by the method of Waldvogel & Swartz (1969).

Screening for collagenolytic activity. The method of Adamcic & Clark (1970) was followed, but modifications were made due to the high salt

concentrations used. To 3 ml. of 0.02% collagen solution in 2.34% NaCl, 0.01 M tris, 0.002 M $CaCl_2$ and 7% NaCl, 0.01 M tris, 0.002 M $CaCl_2$ at pH 7.6 was added 0.1 ml. of a bacterial suspension in buffer containing 10^9 bacteria/ml. Inoculated solutions were incubated aerobically and assayed for release of amino acids after 7 and 14 days using a ninhydrin colorimetric analysis (Rosen, 1957). Extinctions were read on a Unicam SP800 spectrophotometer at 570 nm. Controls at both 2.34 and 7% NaCl consisted of: (a) uninoculated collagen, (b) six bacterial strains, chosen at random, in buffer without collagen, (c) Clostridium histolyticum collagenase form II in the proportion enzyme:collagen, 1:10. Collagenolysis was determined by comparing the amount of amino acids released with that of the uninoculated controls and expressed as μmoles glycine/ml.

Identification of strains. Collagenolytic strains were classified according to Bergey's Manual of Determinative Bacteriology (Breed, Murray & Smith, 1957) using standard identification procedures (Cowan & Steel, 1966).

Salt tolerance. Bacterial strains were inoculated into nutrient broth containing 0.85%, 2.34%, 7%, 10%, 15% and 20% NaCl and incubated without aeration. Extinction at 660 nm. was measured over a period of 72 hr.

Pigmentation. Strains were streaked on salt agar and potato agar to give isolated colonies which were examined after 24 and 48 hr. incubation for pigment production.

Growth curve. As it is important to know the bacterial count corresponding to the age and optical density of a culture, cells were plated on salt agar to determine the count at different intervals during aerated growth.

RESULTS

Salt requirements of bacterial strains

Of 140 strains isolated, 51 were halotolerant (salt range of 0.85 to 20%) and 89 halophilic (salt range of 7 to 15% or 20%).

Collagenolytic activity

Eleven of 110 strains tested (76 South African, 34 Australian) at 2.34% NaCl showed collagenase activity under aerobic conditions. None of the 128 strains tested at 7% NaCl showed collagenolytic activity. Although the bacteria were isolated and subcultured for many generations on collagen-free media, only one strain, Achromobacter sp. 7, showed a long lag phase characteristic of the enzyme produced by one of the two Pseudomonas strains reported by Adamcic & Clark (1970). Significant enzymatic activity of the other strains was already detectable after seven days (Fig. 1-1). Collagenolytic activity was not observed when strains were tested in buffer without $CaCl_2$ (Gallop, Seifter & Meilman, 1957). The 11 collagenolytic strains were isolated from South African hides from five curers. The Australian hides, from two curers, did not contain collagenolytic bacteria. Thus the percentage of bacteria with collagenase from South African hides was 14.47% compared with 10% for all strains tested.

Gelatinolytic activity

Of the 11 collagenolytic strains, the nine halotolerant ones, growing from 0.85 to 20% NaCl had gelatinolytic activity while the two strains showing poor growth at 0.85% NaCl had no gelatinolytic activity. Thus degradation of gelatin by a bacterial strain is not necessarily an indication of collagenolytic activity.

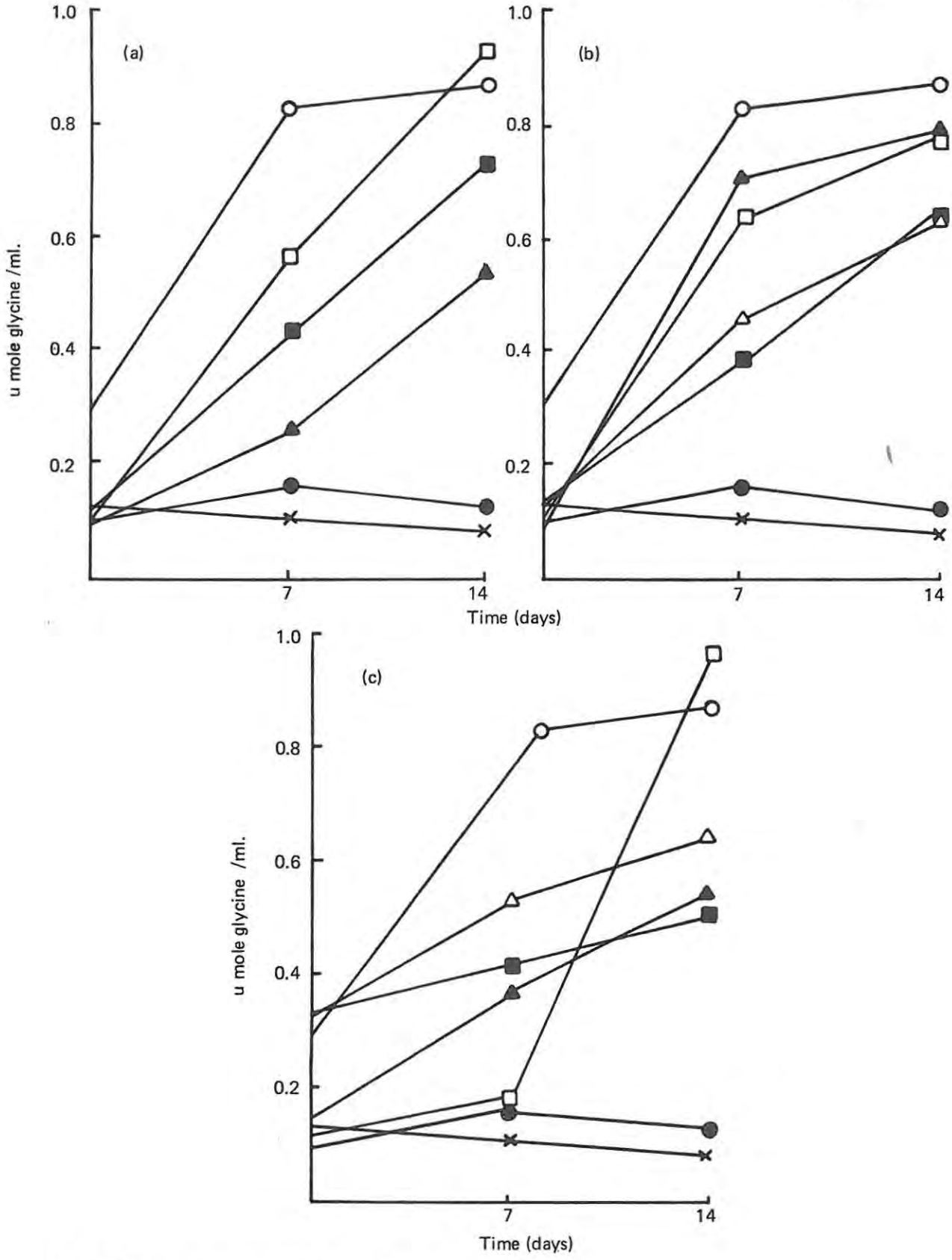


Fig. 1–1. Bacterial collagenolytic activity.

○—○ , Clostridium histolyticum collagenase + collagen;

×—× , uninoculated collagen; ●—● , bacteria + buffer.

(a) ■—■ , Bacillus sp. 1; ▲—▲ , Bacillus sp. 2;

□—□ , Bacillus sp. 3. (b) ■—■ , Achromobacter sp. 1;

▲—▲ , Achromobacter sp. 2; □—□ , Achromobacter sp. 3;

△—△ , Achromobacter sp. 4. (c) ■—■ , Achromobacter sp. 5;

▲—▲ , Achromobacter sp. 6; □—□ , Achromobacter sp. 7;

△—△ , Achromobacter sp. 8.

Identification of collagenolytic strains

Three of the collagenolytic strains were identified as Bacillus spp. and eight as Achromobacter spp. Their characteristics are shown in Tables 1-1 and 1-2.

Table 1-1. Characteristics of collagenolytic Bacillus strains

	Strain no.		
	1	2	3
Gram-variable rods	+	-	+
Gram-positive rods	-	+	-
Endospores	+	+	+
Capsules	-	-	-
Motility	+	+	+
Catalase and oxidase	+	+	+
Gelatinolytic activity	+	+	+
Starch hydrolysis	-	-	-
Production of acid, not gas from glucose, sucrose, mannose	+	+	+
Action on lactose	-	-	-
Nitrate reduction	+	+	+
Indole production	-	-	-
Obligate aerobe	-	+	+
Facultative anaerobe	+	-	-
Length (μm.)	1.8	1.8	1.6
Halotolerant with optimal NaCl concentration of 2.34%	+	+	+

Table 1-2. Characteristics of collagenolytic Achromobacter strains

	Strain no.				
	1	2, 3, 4	5, 6	7	8
Gram-negative rods	+	+	+	+	+
Endospores	-	-	-	-	-
Capsules	+	+	-	-	+
Motility	-	-	-	-	-
Catalase and oxidase	+	+	+	+	+
Gelatinolytic activity	+	+	+	-	-
Starch hydrolysis	-	-	-	-	-
Production of acid, not gas from glucose, sucrose, mannose	+	+	+	+	+
Action on lactose	-	-	-	-	-
Nitrate reduction	+	+	+	+	+
Litmus milk neutral	+	+	+	+	+
Indole production	+	-	-	-	+
Obligate aerobe	+	-	-	-	-
Facultative anaerobe	-	+	+	+	+
Length (μm.)	1.4	1.2	1.6	1.4	1.6
Halotolerant, with optimal NaCl concentration of 2.34%	+	+	+	-	-
Halotolerant, with poor growth at 0.85% and optimal at 7-15% NaCl	-	-	-	+	+

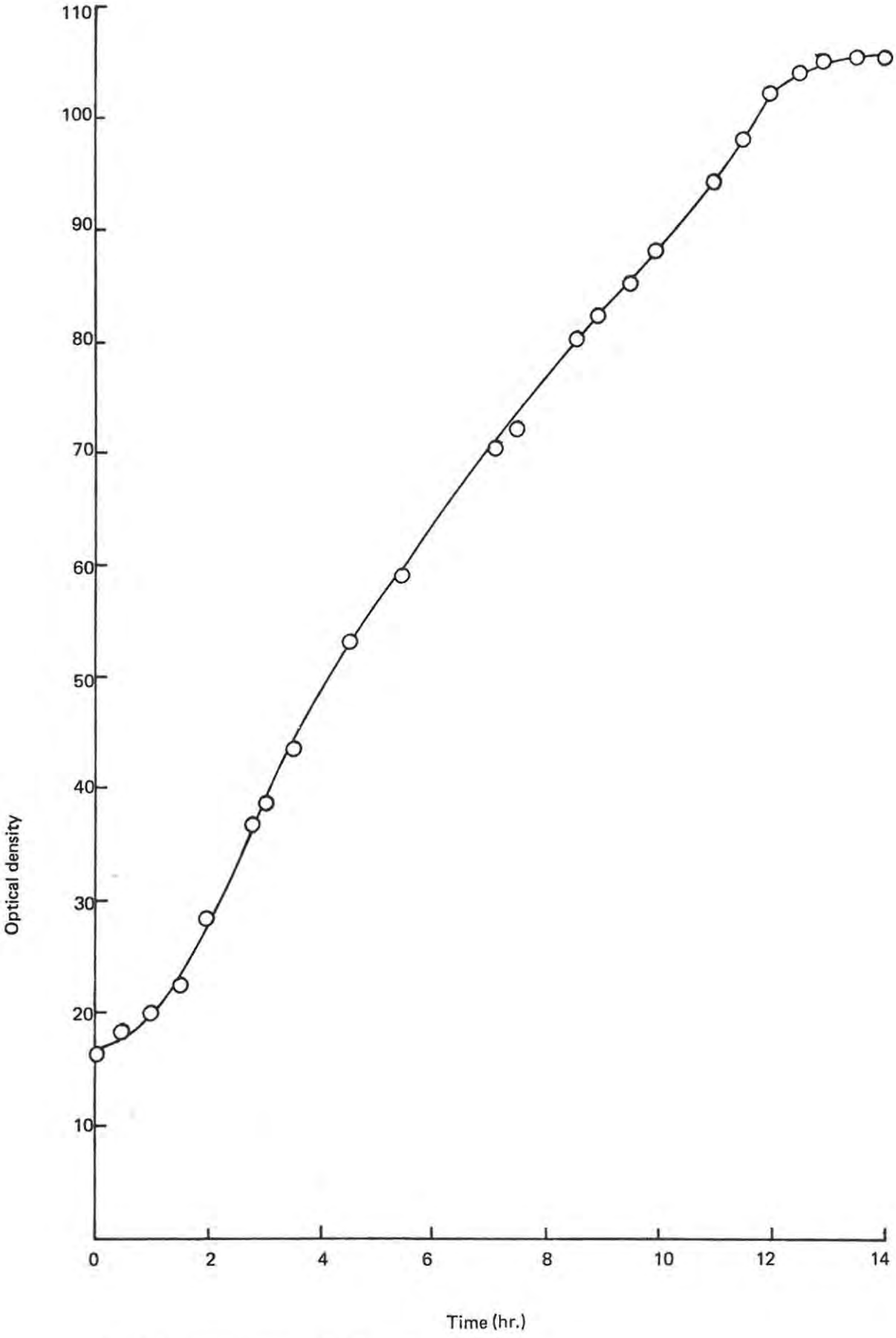


Fig. 1–2. Growth curve of Achromobacter sp. 2.

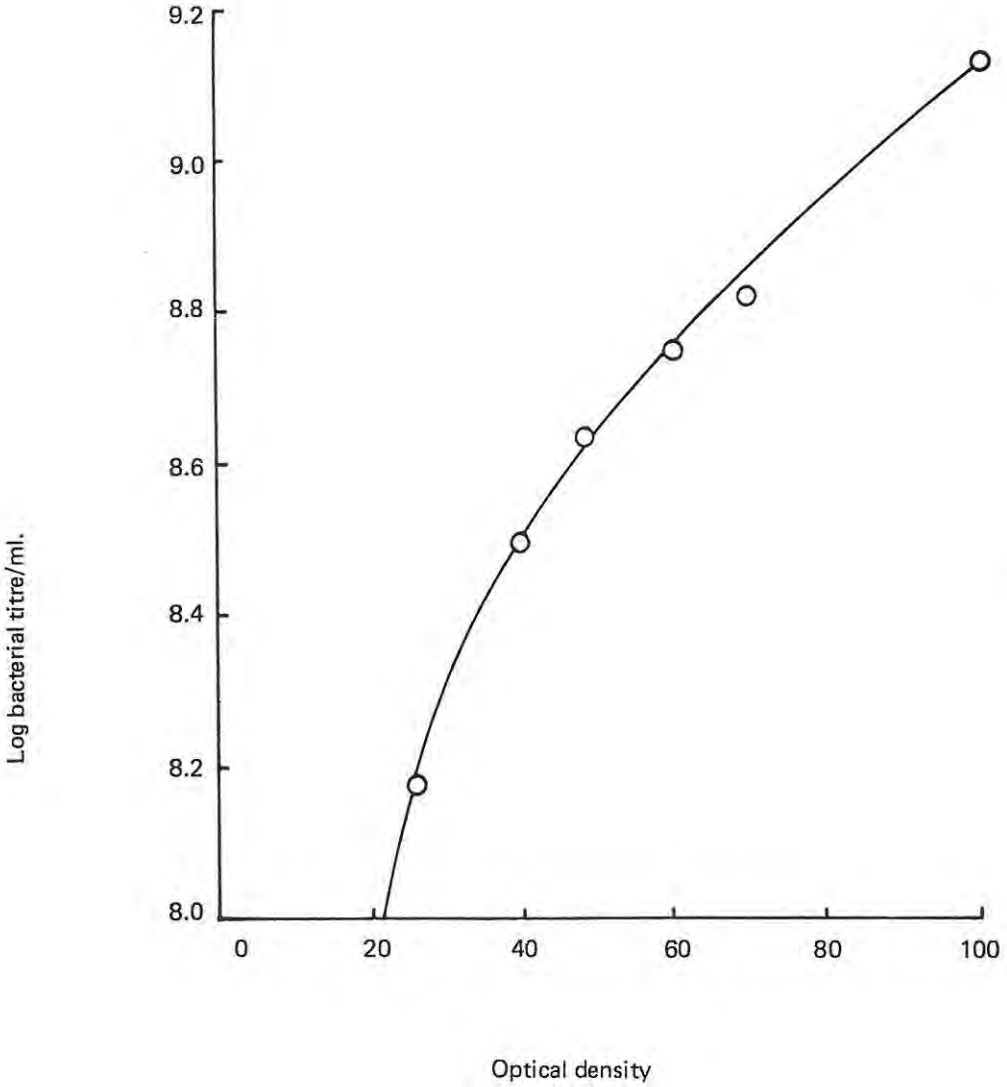


Fig. 1–3. Cell count of Achromobacter sp. 2 vs. optical density.

DISCUSSION

Aerobic collagenolytic activity was shown in 11 bacteria which appear to belong to eight different species. Conclusive demonstrations of true bacterial collagenases have been demonstrated only in four Clostridium spp., three Bacteroides spp., one strain of Staphylococcus aureus and two strains of Pseudomonas (Jennison, 1947; Neuman & Tytell, 1950; Gibbons & MacDonald, 1961; Waldvogel & Swartz, 1969; Adamcic & Clark, 1970).

The increased activity of the collagenase enzyme at 2.34% NaCl indicates that although the bacteria are tolerant of a wide range of salt concentrations, there is an optimal salt concentration for enzymatic activity. The lack of activity at 7% NaCl is likely to be due to an inability to produce the enzyme at that salt concentration or to the inactivation of the enzyme by salt. Inhibition of enzymes by salt concentrations greater than 3% has been shown in extremely halophilic bacteria which have an optimal salt concentration for growth of 20% (Louis & Fitt, 1971).

The observation that no Australian bacteria utilized collagen is important as it has been reported that Australian hides produce uniformly good leather with little hide decay (Hendry, Cooper & Woods, 1970). Recent comparative studies have confirmed the lack of collagenolytic bacteria on Australian hides (Rawlings & Woods, personal communication). This was despite the fact that the hides appeared to be in a bad state of decay. As expected from the absence of collagenase, these hides produced high quality leather. Thus visual examination of hides gives no indication of the true state of collagen decay. The ecological and

evolutionary aspects of collagenolytic bacteria from different environments is at present under investigation.

The lack of collagenolytic bacteria on Australian hides was also shown in a study of cured hides from one Australian and two South African curers (Woods, Welton & Thomson, 1972). The bacteria from South African cured hides possessed considerable collagenolytic activity under aerobic and anaerobic conditions at salt concentrations of 2.34% to 10%. The optimal salt concentration for collagenolysis varied but there was a trend indicating a decrease in collagenolysis with increasing salt concentration above 2.34% NaCl. Only one bacterial population showed collagenolysis at 10% NaCl. This emphasizes the importance of good curing as collagen denaturation is likely to occur in areas of hides containing less than 10% NaCl. Well-cured hides have an average salt concentration of 10-14% NaCl. The present results also indicated the inhibition of collagenase activity or production by 7% NaCl.

It has been shown (Woods, Welton, Thomson & Cooper, 1972) that raw hide bacteria possess considerable collagenolytic activity at 2.34% and 7% NaCl. Unlike the activity often found in bacteria from cured hides, that from raw hides showed no lag phase and the rate of collagen utilization was rapid and similar to that of pure enzyme. This rapid degradation of collagen was also found in the bacteria from soak pit water, but activity only occurred at 0.85% NaCl. Therefore, prior to the addition of hides, soak water was already contaminated with a bacterial population which could rapidly denature collagen at low salt concentrations.

In the above experiments collagenolytic activity has been shown under stringent conditions i.e. the substrate contained pure and undegraded collagen as the only protein nutrient. Under the less stringent conditions

found on the hide itself where non-collagenous proteins and other nutrients are present it is reasonable to assume that bacterial growth and thus collagenolytic activity will be even greater.

The rapid initial rate of collagenolytic activity of bacteria from raw hides is of practical importance in delayed curing which is known to increase the incidence of decay. The inhibitory effect of high salt concentrations emphasizes the importance of rapid and good curing.

Subsequent to this work a collagenolytic strain of Achromobacter iophagus has been isolated from cured hides (Welton & Woods, 1973). The activity of this strain was greater than that of any reported here and collagenolytic activity was shown from 0.85% to 15% NaCl. It was therefore thought to be responsible for the significant decay in hides poorly cured or subjected to an extended period of delayed curing (Cooper, Galloway & Woods, 1972).

As Achromobacter sp. 2 showed a high level of collagenase activity, this strain was chosen for genetic studies. This choice was also influenced by the fact that the genetics of Achromobacter have not previously been studied. As the first step in this study, bacteriophages for Achromobacter were investigated.

THE ISOLATION AND CHARACTERIZATION OF BACTERIOPHAGES OF ACHROMOBACTER

SUMMARY

Phages specific for three strains of Achromobacter were isolated. Phage α1 came from a hide soak solution, α2 and α3b were induced by ultraviolet from strains 9 and 2 respectively and α3a was isolated from a spontaneous plaque on lawns of Achromobacter sp. 2. Host range, morphology, G+C (%), serology, adsorption kinetics and ultraviolet and thermal inactivation were used to determine the relatedness of the phages.

INTRODUCTION

The name bacteriophage (eater of bacteria) was given by d'Herelle (1926) to a bacteriolytic substance isolated from faeces. Today phages are recognized as a group of bacteria-specific viruses, although d'Herelle considered that all phages belonged to a single variable species Protobios bacteriophagus. An unfortunate swing in the opposite direction is now becoming tempered by the realization that the number of different phage types is not equivalent to the number of phages isolated. Relationships between different phages are now becoming increasingly evident (e.g. Grabnar & Hartman, 1968).

One of d'Herelle's most remarkable discoveries was the lytic cycle of phage development whereby phages can multiply within their bacterial hosts before being released by cell lysis. It is this serial transmission between bacteria that enables the visualization of plaques on a bacterial lawn upon which so much of phage research depends.

The early years of phage research were mainly devoted to attempts to use phages therapeutically. These proved largely futile and work with phage was somewhat confused until the three states of phage existence were determined. These are the prophage (Lwoff & Gutmann, 1950) in which the phage exists in a heritable symbiotic relationship with its host known as lysogeny, the vegetative or reproductive form within the cell and the mature state of the metabolically inert phage outside its host. Not all phages can exist in the prophage state which has led to the distinction between those that can (temperate) and those that cannot (virulent). However, the distinction between the two is not always clear-cut. The fundamental criteria of lysogeny are prophage inheritance by all cells of a clone and immunity to superinfection by homologous phage (Lwoff, 1953). The discovery of subtly different kinds of phage-host relationships e.g. cryptic lysogeny (Fischer-Fantuzzi & Calef, 1964) and pseudolysogeny (Lwoff, 1953) has rendered the distinction even more tenuous. Moreover, external conditions can influence the temperate phage as to its choice between entering the lytic cycle or lysogenizing its host (Echols, 1972).

It is essential when studying a group of organisms involving different types to develop some system of classification. Numerous systems of this nature have been proposed for phages (see Adams, 1959) but the allocation to the categories of genus and species in the Linnaean binomial nomenclature is not, at least at present, a feasible idea. One of the most effective classification systems was devised by Delbrück (1946). He classified seven coliphages into four serological groups which correlated with plaque morphology, electron microscopic morphology, single-step growth characteristics and rate of adsorption to host cells. These criteria together with host range have consistently been used to characterize phages and appear to be the most effective means of classification.

The use of serology to define the phylogenetic relationships between phages is based on antigenic similarities which are largely under genetic control (Fodor & Adams, 1955). Although phage head and tail antigens have been recognized, the neutralizing antibody appears to react exclusively with those of the tail (Lanni & Lanni, 1953). The J cistron is the only structural gene involved in the synthesis of serum-blocking antigen in λ (Buchwald & Siminovitch, 1969).

Plaque morphology is another criterion which can be used as a rapid preliminary characterization. However, as external factors such as the age of the host culture can affect this it is a classification procedure which must be used with caution. Certain phage characteristics can be deduced from plaque morphology. Turbid plaques are usually formed by phages capable of lysogenization although the cells causing the turbid appearance may be merely resistant to phage (Thomson & Woods, 1973). A diversity of plaque size is usually observed with phages having a slow rate of adsorption to their hosts (Sagik, 1954). Wide haloes surrounding plaques are due to the production of lysin.

Bacterial cell walls harbour specific phage receptor sites in the lipoprotein or lipopolysaccharide, removal of which renders the cells resistant to phage attack (Watson & Paigen, 1972). As different bacteria can have receptor sites for more than one phage, the host range of a phage can be useful in classification.

Morphological criteria have been widely used in phage classification and Bradley (1967) has proposed the division of phages into six basic morphological types. With the development of the negative staining method for phage electron microscopy (Brenner & Horne, 1959), morphology has become a major taxonomic tool and appears to be a more useful criterion for phages than for bacteria.

The latent period of phages is remarkably constant under standard environmental conditions but care must be taken in its use as very small changes in temperature or nutrition can cause considerable variation (Adams, 1959).

The base composition of the DNA of organisms is highly characteristic and can be obtained from the thermal denaturation temperature (Tm) (Marmur & Doty, 1962). When DNA is heated in solution a sharp increase in its extinction coefficient occurs at the temperature when the native, double-stranded structure denatures. The temperature corresponding to the mid-point of the absorbance rise is linearly related to the average DNA base composition, a higher G-C content conferring a higher thermal stability.

Thermal inactivation, largely dependent on phage DNA (Parkinson & Huskey, 1971) and ultraviolet inactivation, under genetic control (Streisinger, 1956), can also be used in characterization. The main lethal event during heat inactivation is the release of DNA (Yamagishi, Eguchi, Matsuo & Ozeki, 1973). Coetzee (1958) showed that each of 14 Proteus phages had a characteristic rate of inactivation at 60 °C, pH 7.4. These phages fell into 7 serological groups each showing a different range of inactivation rates.

True lysogenic bacteria are immune to superinfection by homologous phage which enables the distinction between certain phages (Kaiser & Jacob, 1957) although exceptions do occur (Buck, Anacker, Newman & Eisenstark, 1963).

There are other more sophisticated techniques that may be used to classify phages. Mixed infection by unrelated phages will result in mutual exclusion but by related ones can yield recombinants (Adams, 1959).

The similarity of cohesive ends of DNA is another criterion which can be used but it only applies to certain groups of phages (Baldwin, Barrand, Fritsch, Goldthwait & Jacob, 1966).

All these identifying characteristics are subject to mutation and indeed the study of phage genetics owes much to work carried out on mutant host range (Luria, 1945) and plaque morphology (Hershey, 1946). However, used in conjunction with each other, these characteristics together prove a useful tool in establishing phage relationships.

Bacteriophages of Achromobacter have not previously been isolated. A search for such phages was therefore initiated, with a view to developing transfection and transduction systems. The isolated phages were characterized according to the criteria, mentioned above, of morphology, serology, nucleic acid content, ultraviolet and thermal inactivation, single-step growth characteristics and adsorption kinetics.

METHODS

Bacteria. Achromobacter spp. 1-8 (Thomson et al., 1972), A. iophagus (Welton & Woods, 1973) and a further 11 Achromobacter strains were used. All were halotolerant, collagenolytic bacteria isolated from hides.

Bacteriophage isolation. Cured hide samples (c. 2 g.) were cut into pieces and soaked in 50 ml. 10% NaCl at 4 °C for 3 days. The liquid was decanted, centrifuged at 2,000 g for 20 min., the supernatant sterilized with 10% (v/v) chloroform and tested for phage by the modified double-agar-layer method (Adams, 1959) of Hershey, Kalmanson & Bronfenbrenner (1943).

Thirty soak solutions were tested. In order to isolate phage, a plaque was stabbed with a needle which was then washed in 1 ml. phage buffer containing 0.1 ml. chloroform. This suspension was tested for serial transfer on fresh lawns of bacteria and used to prepare phage lysates.

To screen for spontaneous phage liberation, double-agar-layer plates were seeded with Achromobacter sp. 2 and incubated for 18 hr. Plaques were stabbed as above.

In order to induce lysogenic phages overnight broth cultures of the 20 Achromobacter strains were diluted 1:10 in nutrient broth and incubated for 5 hrs. Cultures were centrifuged at 2,000 g for 15 min. and resuspended in phage buffer. The source of ultraviolet irradiation was a Hanovia lamp (2537 Å) used at a height of 24 cm. and switched on 20 min. before use. The lamp was calibrated with phage T2 (Fig. 2-10) and found to deliver 7.2 ergs. $mm.^{-2}$ $sec.^{-1}$ as 99% inactivation of T2 is equivalent to 133 ergs. $mm.^{-2}$ (Tessman, 1956).

Cell samples, less than 2 mm. deep to avoid shielding were irradiated in glass Petri dishes with continuous agitation to ensure uniform irradiation. All irradiations were carried out in the dark while post-irradiation treatment was in subdued light. Plates were incubated in the dark to avoid photo-reactivation (Kelner, 1949). One ml. aliquots were removed after 0.5, 1, 5 and 10 min. irradiation, pooled and an equal volume phage broth added. Cultures were incubated for 18 hr., centrifuged at 2,000 g for 10 min. and the supernatant sterilized with 10% (v/v) chloroform before testing for phage.

Preparation of high titres of phage. Overnight cultures were diluted 1:10 in fresh nutrient broth and incubated with aeration for 5 hr. Equal volumes of bacteria and phage (c. 10^6 p.f.u./ml.) were mixed and allowed to stand at room temperature for 10 min. Aliquots (0.2 ml.) were added to tubes containing 2.5 ml. molten toplayer salt agar and poured onto the surface of thick, freshly-poured salt agar plates. After overnight incubation 2.5 ml. 0.4 M NaCl was added to the surface of each plate and the soft agar layer scraped off. The mixture was allowed to stand for 2 hr. at room temperature to facilitate phage entry into the liquid phase. Agar and bacterial debris were removed by centrifugation at 2,000 g for 20 min., the lysate sterilized with 0.1 vol. chloroform and stored at 4°C to minimize phage inactivation. This method yielded titres of 10^{10} to 10^{11} p.f.u./ml.

Assay of phage lysates. The method of Adams (1959) was used. Phage lysates were diluted appropriately in 0.4 M NaCl. The indicator strain was prepared as above for high titre preparations and the salt agar plates were well dried before use.

Concentration and purification of lysates. Lysates (c. 10^{11} p.f.u./ml.) were centrifuged at alternate cycles of 10,000 g for 10 min. and 35,000 g for 90 min. Phage pellets were allowed to resuspend overnight at 4°C.

For the preparation of antiphage serum, phage was further purified by zone electrophoresis (Polson and Russel, 1967). A sucrose density gradient was prepared in a zone electrophoresis apparatus which consisted of a vertical U tube with an electrode vessel attached near the top of each limb. The gradient was prepared by attaching an Erlenmeyer flask of 40% sucrose in borate buffer to another of buffer alone which was

attached to a capillary tube at the base of the U tube. Sucrose was allowed to flow into the buffer and the mixture entered the U tube to form the gradient. The phage sample was suspended in 2 ml. borate sugar + 0.2 ml. borate buffer pH 8.6 to which was added a small quantity of phenol red as a reference. The preparation was introduced into the apparatus by placing it in a syringe fitted to the bottom of the capillary tube and forcing the sample gently into the column. Reversible silver-silver chloride electrodes were placed in the electrode vessels and a voltage gradient of 3-4 V/cm. applied to the column. With the left-hand electrode positive, an upward migration resulted. With air cooling a current of 20-22 mA could be used without convection occurring. Electrophoresis was continued for 12 to 18 hr. before 1 cm. samples were removed via the capillary tube and assayed for plaque formers.

Electron microscopy. One drop of concentrated, purified phage suspended in 0.1 M ammonium acetate (pH 7.2) was mixed with a drop of 2% potassium phosphotungstate (pH 7.4) according to Brenner & Horne (1959). The phage suspension was mounted on a carbon-coated copper grid, the excess withdrawn by touching with filter paper and the preparation air dried. Electron micrographs were taken with a Hitachi HU 11B (Hitachi Ltd., Tokyo) electron microscope.

Nucleic acid staining. Nucleic acid type was determined by the method of Bradley (1966). Successive drops of purified phage were placed on a clean microscope slide and dried. Samples were fixed in Carnoy's fluid at room temperature for 5 min., washed briefly in absolute ethanol and dried in warm air. Phage was stained in 0.01% acridine orange (AO) in modified McIlvaine's (MI) buffer pH 3.8 for 5 min. Slides were rinsed twice in MI buffer and soaked in 0.15 M Na_2HPO_4 for 15 min. Excess liquid

was removed and the colour of the spot observed under ultraviolet irradiation (2537 Å). The slide was dipped in molybdic acid solution and the colour change observed for 15 to 90 sec. A second AO-stained slide was placed in a dish of 0.1 M tartaric acid and colour change monitored for 2 to 5 min.

The results were confirmed by treatment with RNAse and DNAse. A Carnoy-fixed slide was soaked in MI buffer for 5 min., placed in 0.1% RNAse in MI buffer and incubated at 37 °C for 2 hr. before being stained with AO as above. A second fixed slide was soaked in phosphate acetate buffer (pH 5.5) for 5-10 min., incubated for 2 hr. at 37 °C in 0.02% DNAse in phosphate acetate buffer. The slide was soaked in MI buffer for 5-10 min. and stained with AO. Colours obtained for different nucleic acid types are given in Table 2-1.

Phage DNA extraction. A modification of the phenol extraction method of Mandell & Hershey (1960) was used. An equal volume of water-saturated phenol was added to purified phage (1×10^{11} p.f.u./ml.) in standard saline-citrate buffer (SC) (pH 7.0). The tube was rotated on an angled turntable at 45 rev./min. for 15 min. The mixture was chilled at 4 °C, centrifuged at 2,000 g for 5 min. and the phenol layer removed with a Pasteur pipette. The phenol extraction was repeated three times and the final product dialysed at 4 °C for 24 hr. against three changes of the saline-citrate buffer. The concentration of DNA was determined by ultraviolet absorption at 260 nm. according to the relationship:

$$\mu g.\ DNA/ml. = \frac{Abs._{254\ m\mu} + 0.0048}{0.021}$$

The presence and concentration of DNA was confirmed by the modified Dische diphenylamine reaction of Burton (1956). One ml. of nucleic acid solution was mixed with 2 ml. diphenylamine reagent and placed in a boiling water bath for 10 min. The mixture was chilled in ice and the optical density at 600 nm. was measured and compared with values obtained for standard solutions of DNA.

Determination of the base compositions of phage DNA. These were determined from the thermal denaturation temperatures (Tm) (Marmur & Doty, 1962). Phage DNA was dissolved in standard saline citrate (Marmur, 1961) in a 3 ml. quartz cuvette with a 1 cm. light path to give an absorbance of approximately 0.7 at 254 nm. The blank was SC alone. The samples were placed in a Unicam spectrophotometer thermal chamber and the temperature raised by $\frac{1}{4}$°C per min. The absorbance at each temperature was divided by the value at 25°C and the ratio (relative absorbance) plotted against temperature. The temperature corresponding to half the sharp increase in absorbance when the double-stranded DNA denatures is designated the thermal denaturation temperature. The % (G+C) was calculated from the formula: Tm = % (G+C) . 0.41 + 69.3 (Marmur & Doty, 1962). Absorption spectra were recorded before and after heating.

Serology. Phage purified by zone electrophoresis was used to inject 1 ml. aliquots intravenously into rabbits at weekly intervals. Blood was collected 28 days after the initial injection, allowed to clot, refrigerated overnight and centrifuged at 2,000 g for 10 min. The serum was decanted and its neutralizing power determined (Hershey, Kalmanson & Bronfenbrenner, 1943).

Serum was diluted in physiological saline (0.85%) and 1 ml. aliquots added to 0.1 ml. phage (c. 10^7 p.f.u./ml.). Samples were withdrawn at

intervals, diluted 1:100 to arrest the antigen-antibody reaction and assayed for free phage. Inactivation was plotted as a function of time and the neutralizing power was expressed as k, the velocity constant of phage inactivation according to the formula: $k = 2.3 \frac{D}{t} \log \frac{P_o}{P}$. In this equation D is the serum dilution factor, P_o is the initial phage titre and P is the titre at time t.

Single-step growth curves. Overnight broth cultures of Achromobacter sp. 2 were diluted 1:10 in phage broth and incubated at 37 °C with aeration for 90 min. (c. 5×10^7 cells/ml.). To 0.9 ml. cells phage was added at m.o.i. 1. After 5 min. adsorption at 37 °C the reaction was stopped by the addition of antiphage serum (k = 5) for 5 min. The mixture was diluted to 10^{-4} and 10^{-6}, incubated at 37 °C with aeration and aliquots assayed for infective centres and free phage at intervals. Aliquots from the 10^{-2} tube were diluted 1:100 in 0.4 M NaCl containing 10% (v/v) chloroform to measure intracellular phage.

Phage adsorption. Overnight broth cultures of Achromobacter sp. 2 were diluted 1:10 in phage broth and incubated with aeration to give 2×10^8 cells/ml. To 9 ml. bacteria, 1 ml. phage (1×10^9 p.f.u./ml.) was added and the mixture incubated. At intervals (0 to 10 min.) samples were diluted 1:100 in chilled 0.4 M NaCl, centrifuged, the supernatant sterilized with chloroform (10% v/v) and assayed for free phage. The rate constant K was calculated from the formula:

$$K = \frac{2.3}{Bt} \log \frac{P_o}{P} \text{ ml. min.}^{-1}$$

where B = concentration of cells/ml., P_o = initial phage concentration and P = phage concentration at t min.

Ultraviolet inactivation. Phage (5×10^7 p.f.u./ml.) was irradiated in phage buffer. Aliquots were assayed at different time intervals for plaque-forming ability. To measure the inactivation rate of vegatative phage, overnight broth cultures of Achromobacter sp. 2 were diluted 1:10 in phage broth and incubated with aeration to a concentration of 1×10^8 cells/ml. The cells were centrifuged and resuspended in phage buffer. To 1 ml. of cells was added 0.1 ml. phage (1×10^9 p.f.u./ml.). After adsorption for 10 min. antiserum (k = 5) was added to neutralise unadsorbed phage. The mixture was diluted 1:10 in cold phage buffer, irradiated in a Petri dish on a layer of crushed ice and plated for infective centres before the end of the latent period. All assays were carried out in subdued light to eliminate photo-reactivation.

Thermal inactivation. Phage suspended in phage buffer (pH 7.8) at a titre of 1×10^9 was placed in a waterbath at 60 °C. Aliquots of phage were assayed at different time intervals.

RESULTS

Bacteriophage isolation

Bacteriophages for Achromobacter are in our experience rare and only 4 were isolated. Phage α1 came from a hide soak solution and α2 was induced by ultraviolet irradiation of Achromobacter sp. 9. Phage α3a was isolated from a single spontaneous plaque on a total of 250 lawns of Achromobacter sp. 2 and α3b was induced by ultraviolet irradiation of the same strain. The ability of the α phages to form plaques on all the Achromobacter strains was determined. Each of the phages plated only on Achromobacter spp. 2, 3 and 4. The narrow host range makes these phages ideal for use as a taxonomic tool. The phages form turbid plaques with distinct cloudy centres (Fig. 2-1).

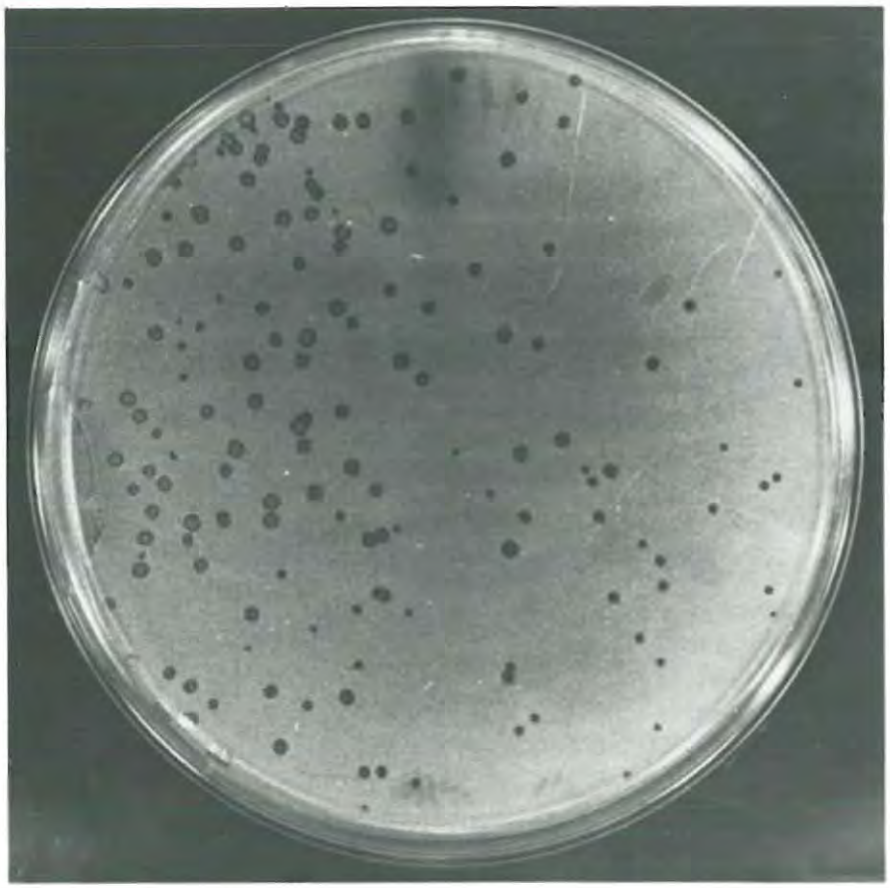


Fig. 2–1. The characteristic turbid plaques of the α phages.

The requirement for aeration for 5 hr. of indicator strains before plating phage was very stringent. When cultures were aerated for different periods the plaques appeared as pinpricks. High titres of phage (c. 10^{11} p.f.u./ml.) were unstable when stored at 4 °C in the presence of chloroform, showing a loss of infectivity of two logs after one week. Stability was maximal

under these conditions between pH 7 and pH 8.

Phage purification by zone electrophoresis proved highly efficient with the majority of plaque-forming units migrating in a narrow band (Fig. 2-2).

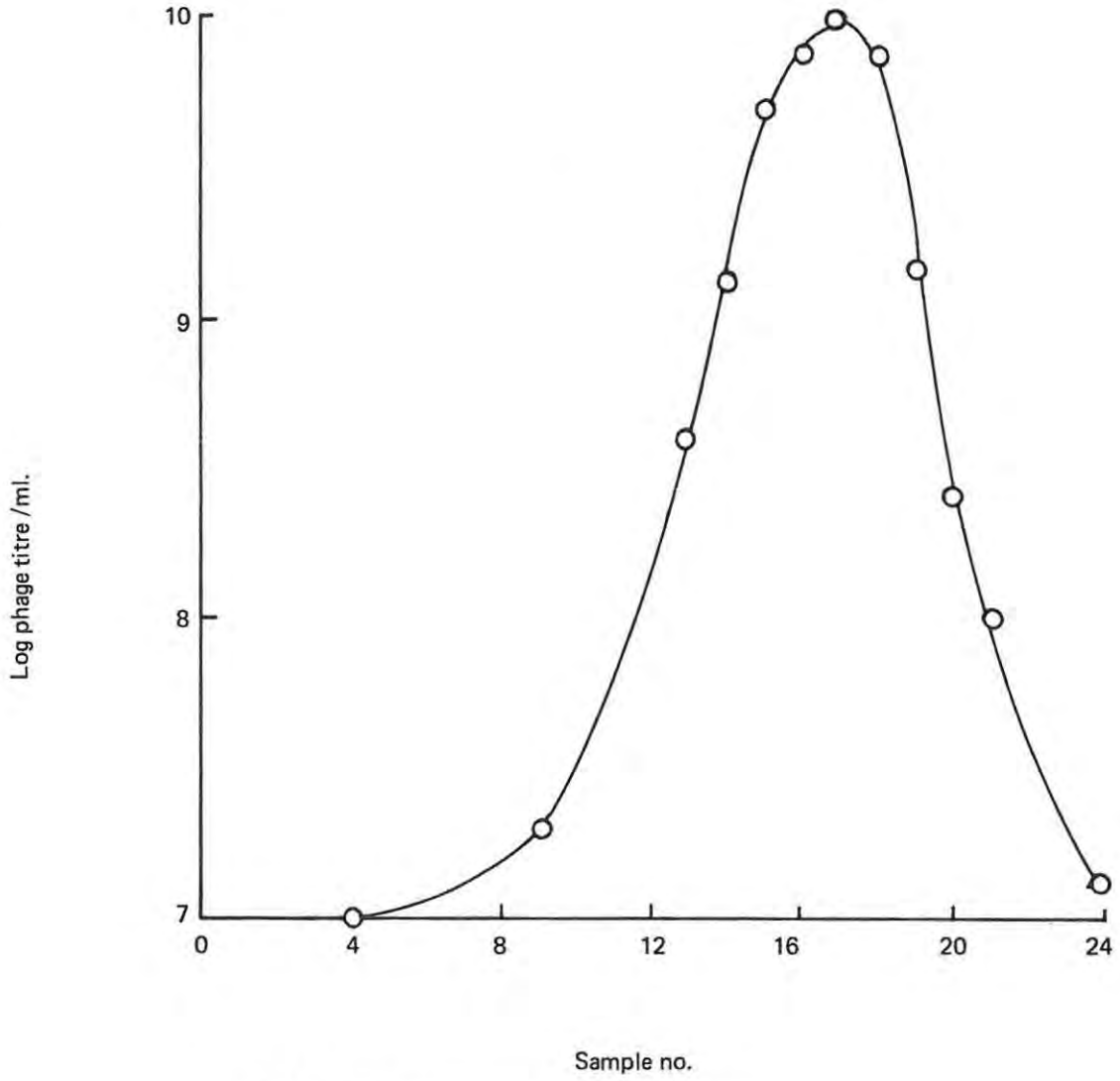


Fig. 2–2. Phage purification by zone electrophoresis.

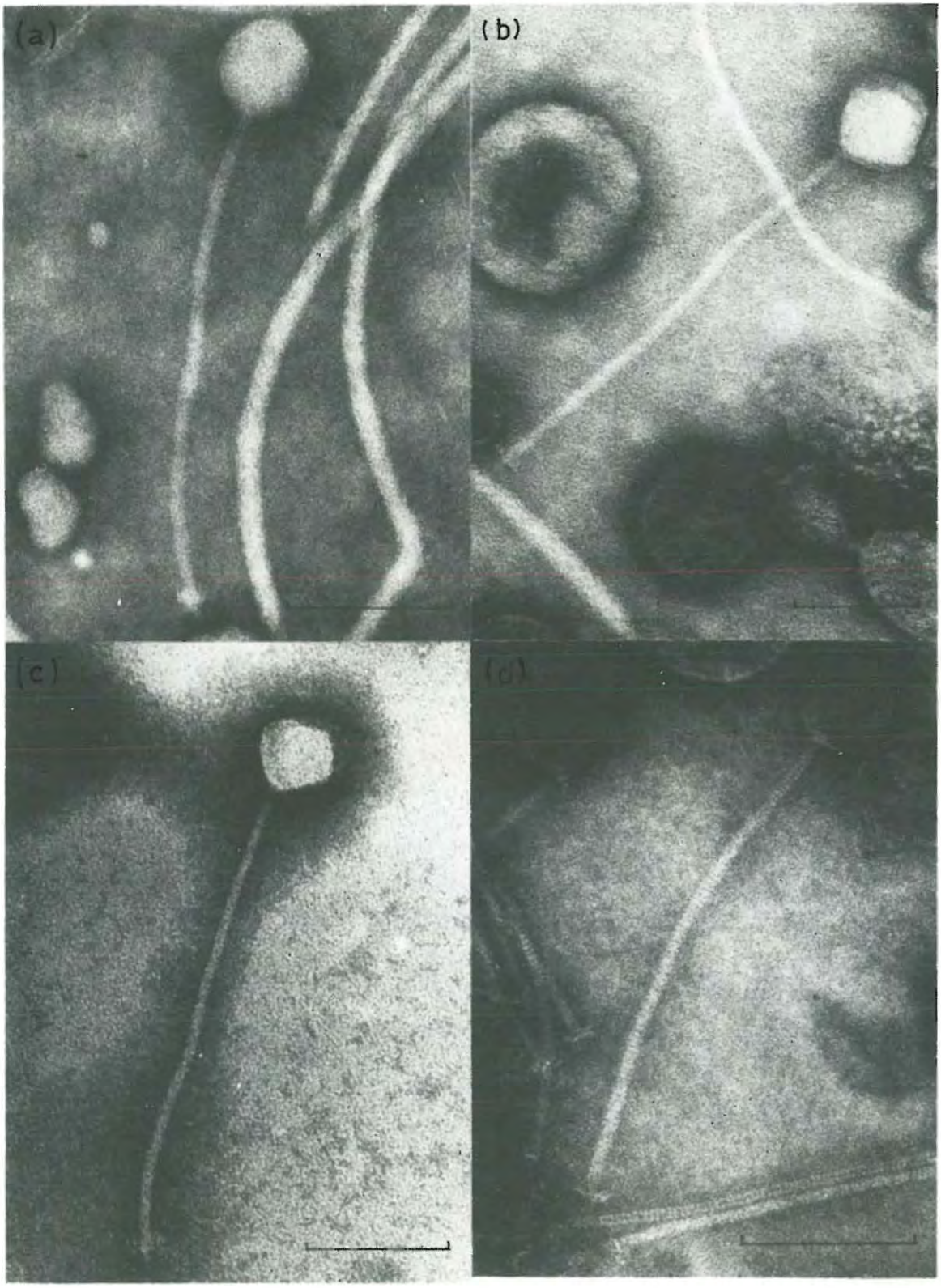


Fig. 2–3. Electron micrographs of phages. (a) α 1; (b) α 2; (c) α 3a; (d) α 3b. The bar represents 100 nm.

Morphology

Electron micrographs of the phages (Fig. 2-3) show that they all had hexagonal heads with no visible neck. The tail length of the phages varied slightly: α1, 3009 Å; α2, 3867 Å; α3a, 3450 Å and α3b, 3178 Å. The proximal half of the tail showed cross striations and short tail-fibres originated near the tip. The extreme length of the tails possibly accounts for the instability of these phages. All preparations observed under the electron microscope consisted largely of tail fragments with or without heads attached. Due to the loss of DNA phage heads were often collapsed losing their hexagonal structure. Numbers of phages attached to a lysed bacterial cell were frequently observed (Fig. 2-4).

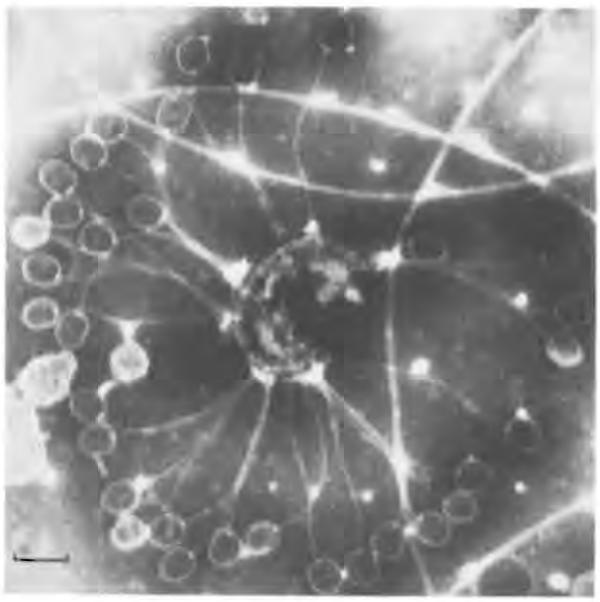


Fig. 2–4. Electron micrograph of α 3a phages attached to cell debris of Achromobacter sp. 2. The bar represents 100 nm.

Nucleic acid determination

The nature of the phage nucleic acid was determined by staining with acridine orange (Bradley, 1966) and shown to be 2-DNA by the green fluorescence under ultraviolet light. This fluorescence remained after treatment with molybdic acid, tartaric acid and ribonuclease but disappeared after treatment with deoxyribonuclease. Standard colours obtained by acridine orange staining of different nucleic acids is shown (Table 2-1). The results were confirmed by the Dische diphenylamine reaction.

Table 2-1. Colours of stained nucleic acid preparations*

Nucleic acid	Treatment				
	Na_2HPO_4	Molybdic acid	Tartaric acid	RNAse	DNAse
2-DNA	Green	Green	Green	-**	+**
1-DNA	Red	Pale green	Pale green	-	+
2-RNA	Green	Green (fades)	Pale red	-	-
1-RNA	Red	Pale red	Red	+	-

* After Bradley (1966)

** - resistant
+ sensitive

Samples of DNA showed maximum absorption at 258 nm. and a trough at 230 nm. (Fig. 2-5). The ratios of A_{258}/A_{280} and A_{258}/A_{230} were 2.2 and 1.95 respectively, indicating the purity of the DNA.

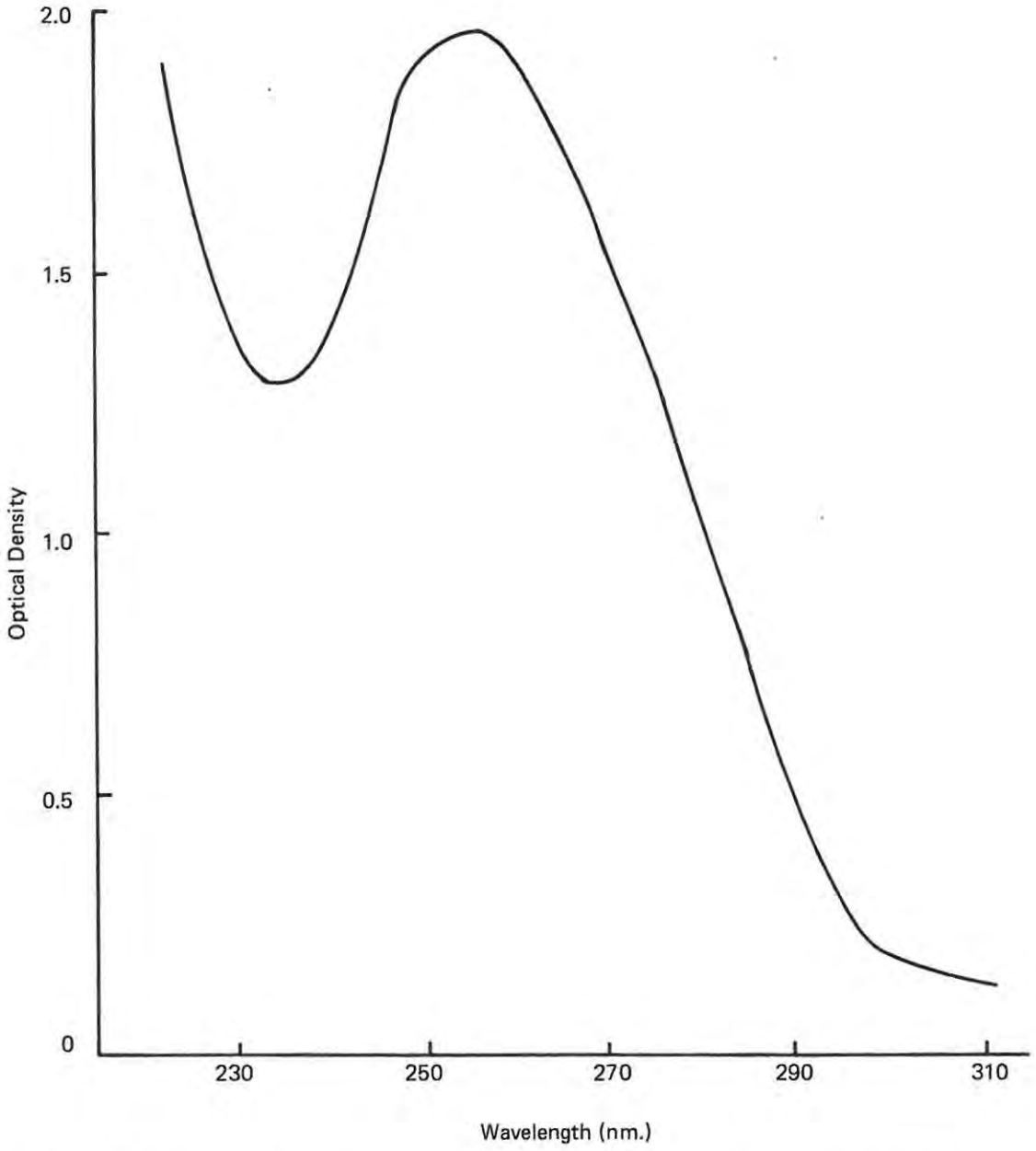


Fig. 2–5. Ultraviolet absorption spectrum of phage DNA. Maximal absorption was at 258 nm. and a trough occurred at 230 nm.

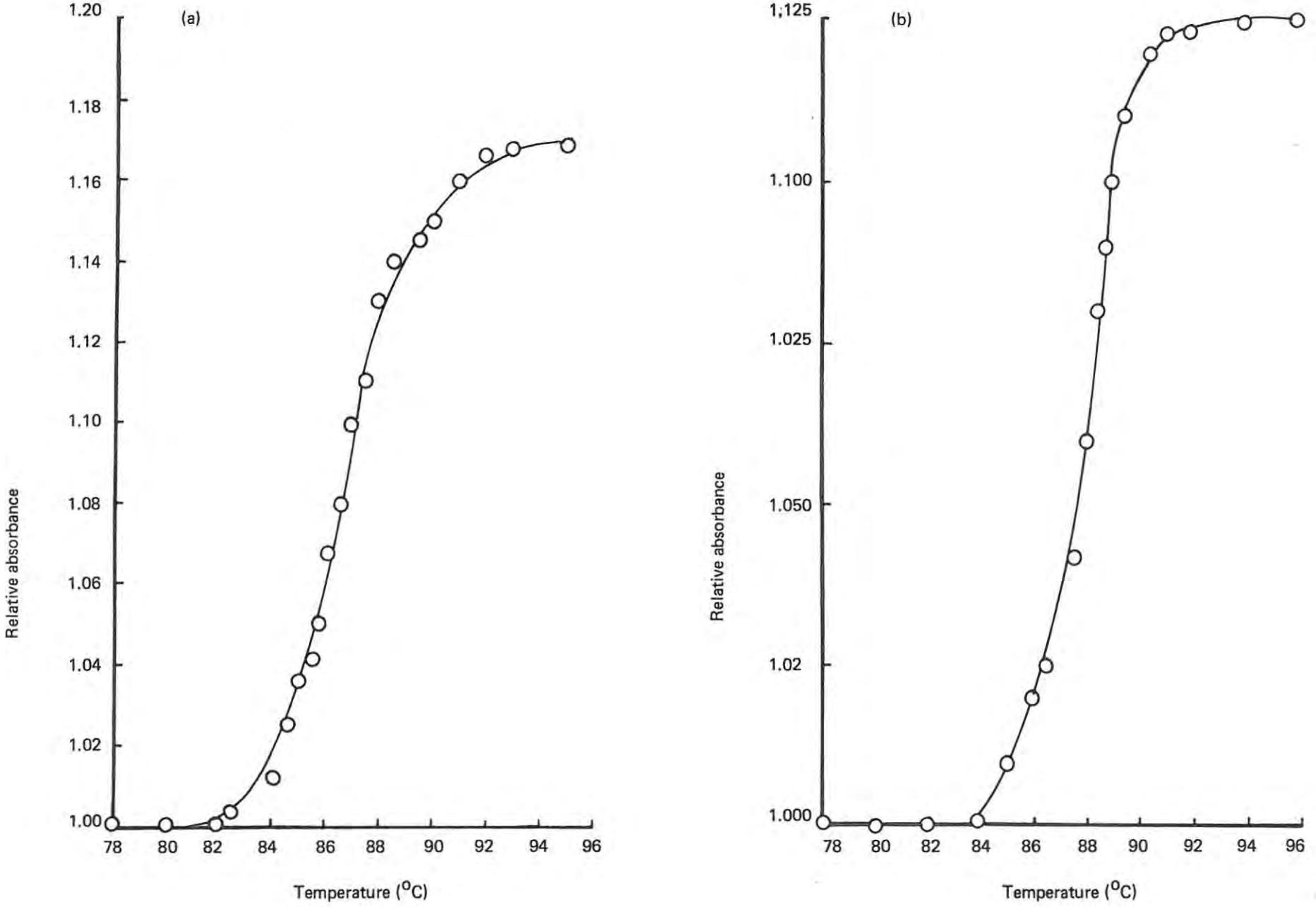


Fig. 2–6. Thermal denaturation of phage DNA. (a) ∝ 1. (b) ∝ 3b.

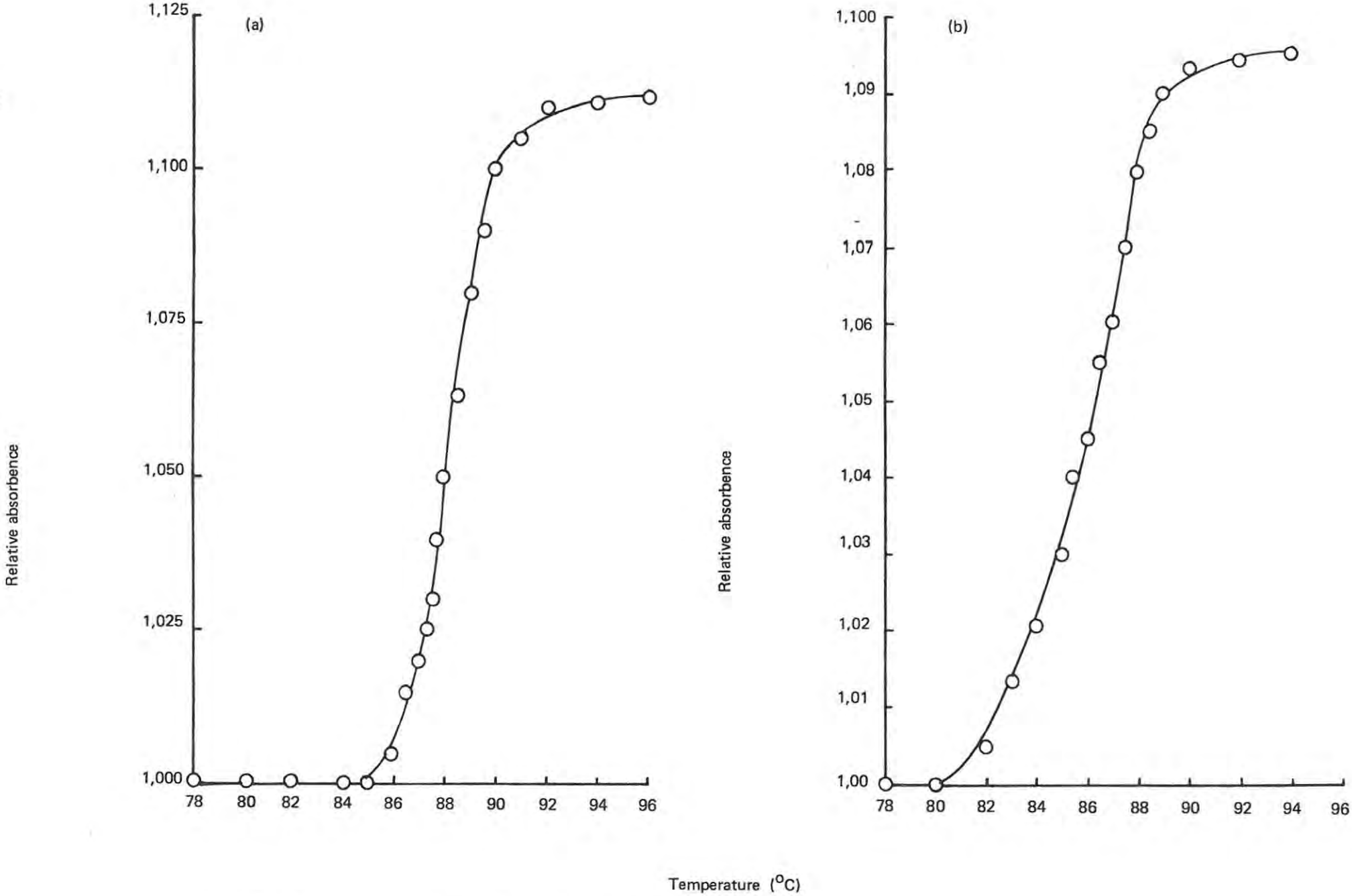


Fig. 2–7. Thermal denaturation of phage DNA. (a) ∝ 3a. (b) ∝ 2.

Determination of the base composition of phage DNA

The DNA was shown to be double-stranded by the melting curves (Figs. 2-6 and 2-7). The Tm's of the different phage DNA were α1 = 86.7 °C, α2 = 86.2 °C, α3a = 88.2 °C and α3b = 88.0 °C. The corresponding molar compositions of guanine and cytosine were calculated (Table 2-2).

Serology

Antiserum against phage α3a neutralized all the α phages at comparable rates (Table 2-2), which is indicative of a close serological relationship between them.

Single-step growth curves

The phages were characterized by long latent periods (Table 2-2) and large average burst sizes (Fig. 2-8). When single-step growth experiments of α3a were performed at 37 °C without aeration the latent period was increased to 130 min. and the average burst size reduced to 35. At 30 °C the latent period of α3a was 100 min. and the average burst size 180.

Phage adsorption

Initial adsorption of phages to Achromobacter sp. 2 was rapid and by 10 min. more than 99% of phages were adsorbed (Fig. 2-9). Adsorption constants are similar for the 4 phages (Table 2-2).

Ultraviolet inactivation

Compared with T2 (Fig. 2-10), the α phages are relatively resistant to u.v. inactivation (Fig. 2-11 and Table 2-2). Inactivation kinetics are biphasic. Further study of the response to u.v. light by α3a showed that free phage was more resistant to irradiation than vegatative phage (Fig. 2-12). In both cases kinetics were one-hit and biphasic.

Thermal inactivation

Kinetics of inactivation at 60 °C were biphasic suggesting the presence of two components of different heat sensitivity (Fig. 2-13).

Table 2-2. Phage characteristics

Phages	k value, $min.^{-1}$ ★	G+C(%)	Latent period (min.)	Average burst size	Ultraviolet irradiation †	Adsorption to *Achromobacter* sp. 2 ($ml.\ min.^{-1}$)
α1	247	42.56	85	380	950	3.1×10^{-9}
α2	230	40.97	100	300	1195	5.0×10^{-9}
α3a	300	45.73	100	230	1152	1.4×10^{-9}
α3b	300	45.58	90	300	1317	3.2×10^{-9}

★ Neutralization constants with α3a antiserum

† U.V. dose ($ergs.\ mm.^{-2}$) required for 75% inactivation

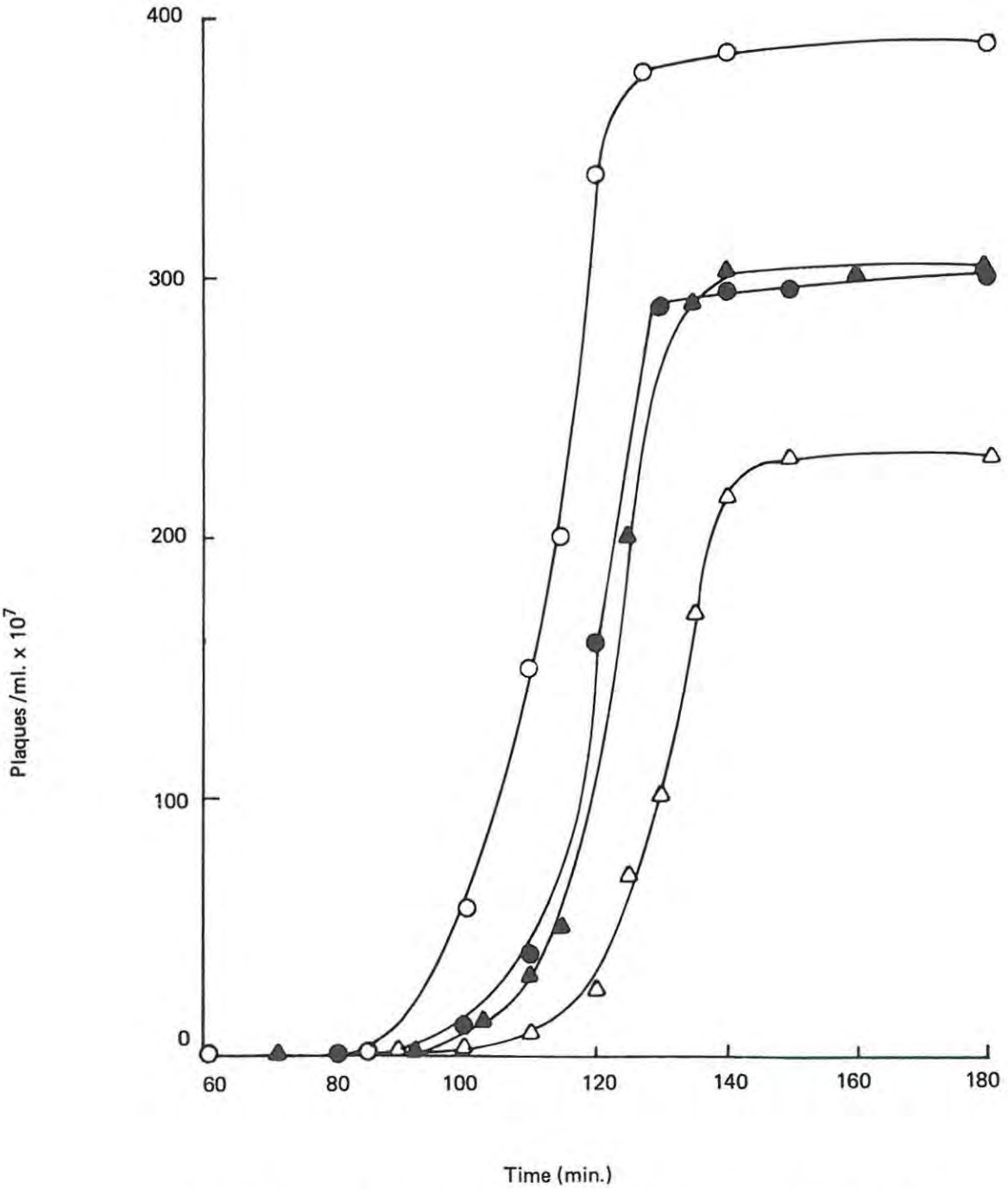


Fig. 2–8. Single step growth curve. ○—○ , ∝1; ▲—▲ , ∝2; △—△ , ∝3a; ●—● , ∝3b.

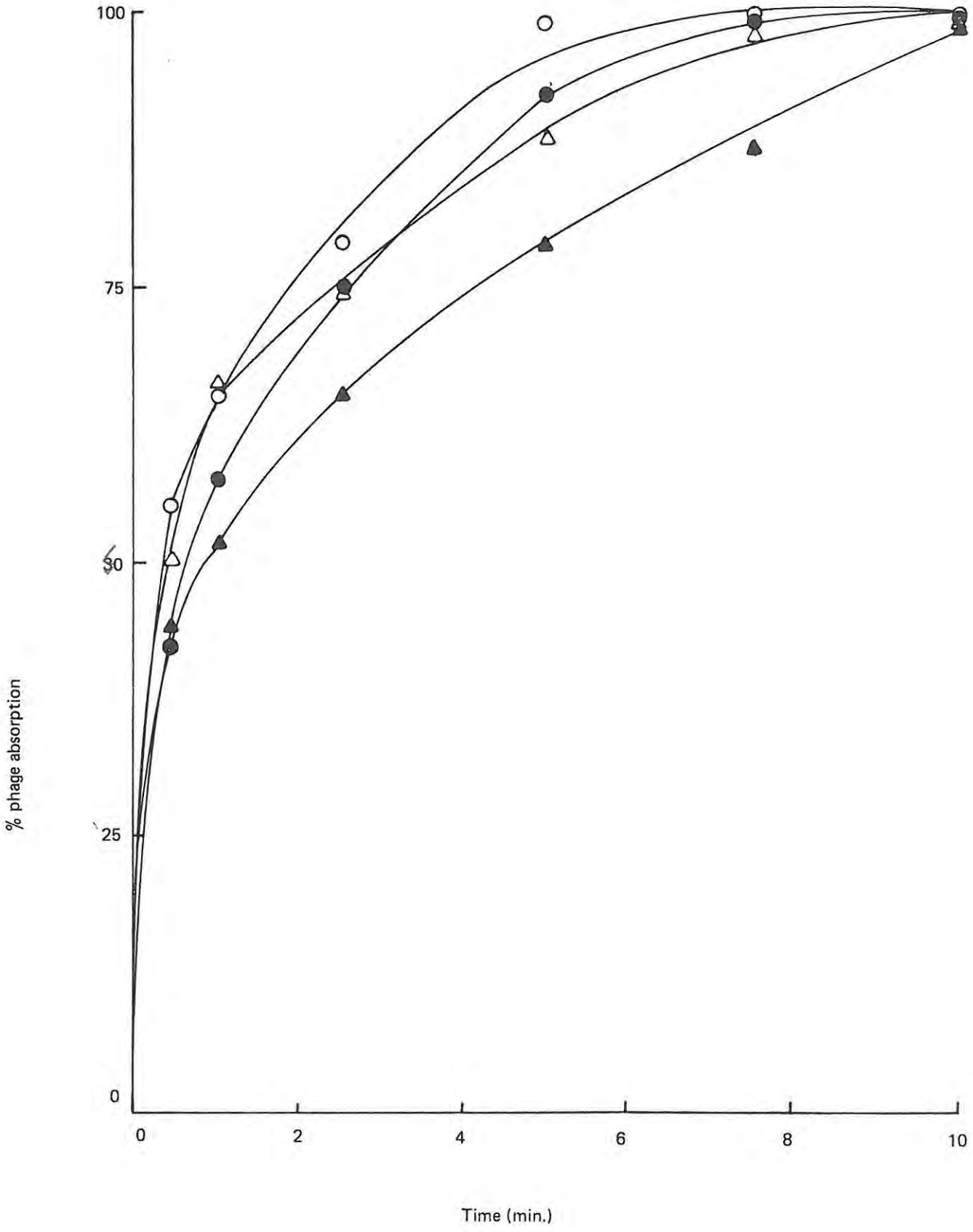


Fig. 2–9. Phage absorption to Achromobacter sp. 2.
○—○ , ∝1; ▲—▲ , ∝2; △—△ , ∝3a;
●—● , ∝3b.

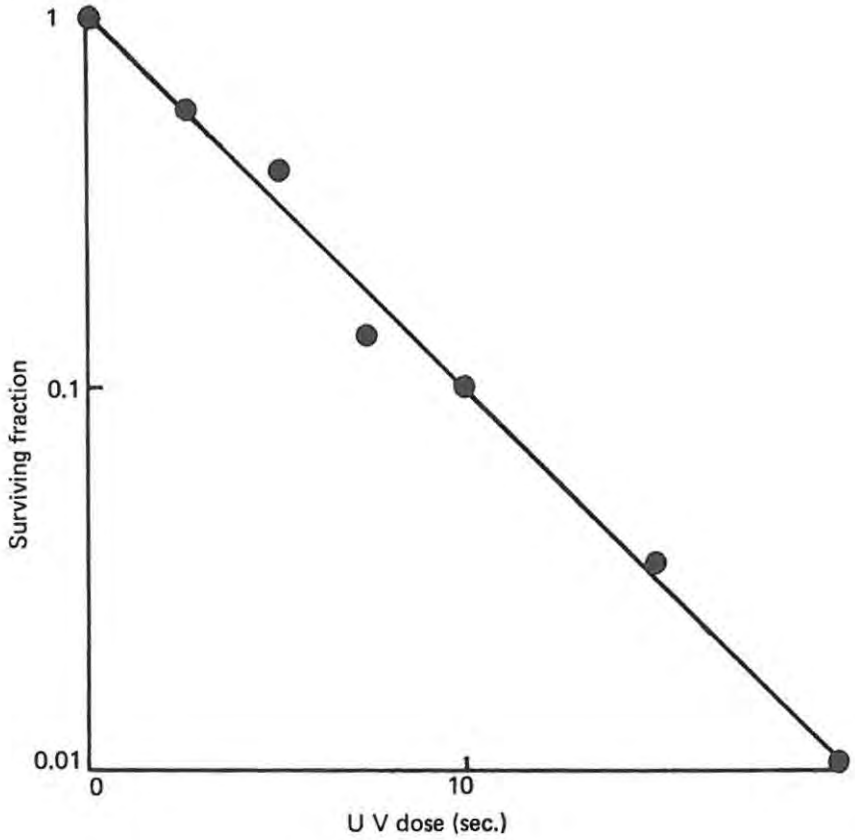


Fig. 2–10. UV inactivation of phage T2.

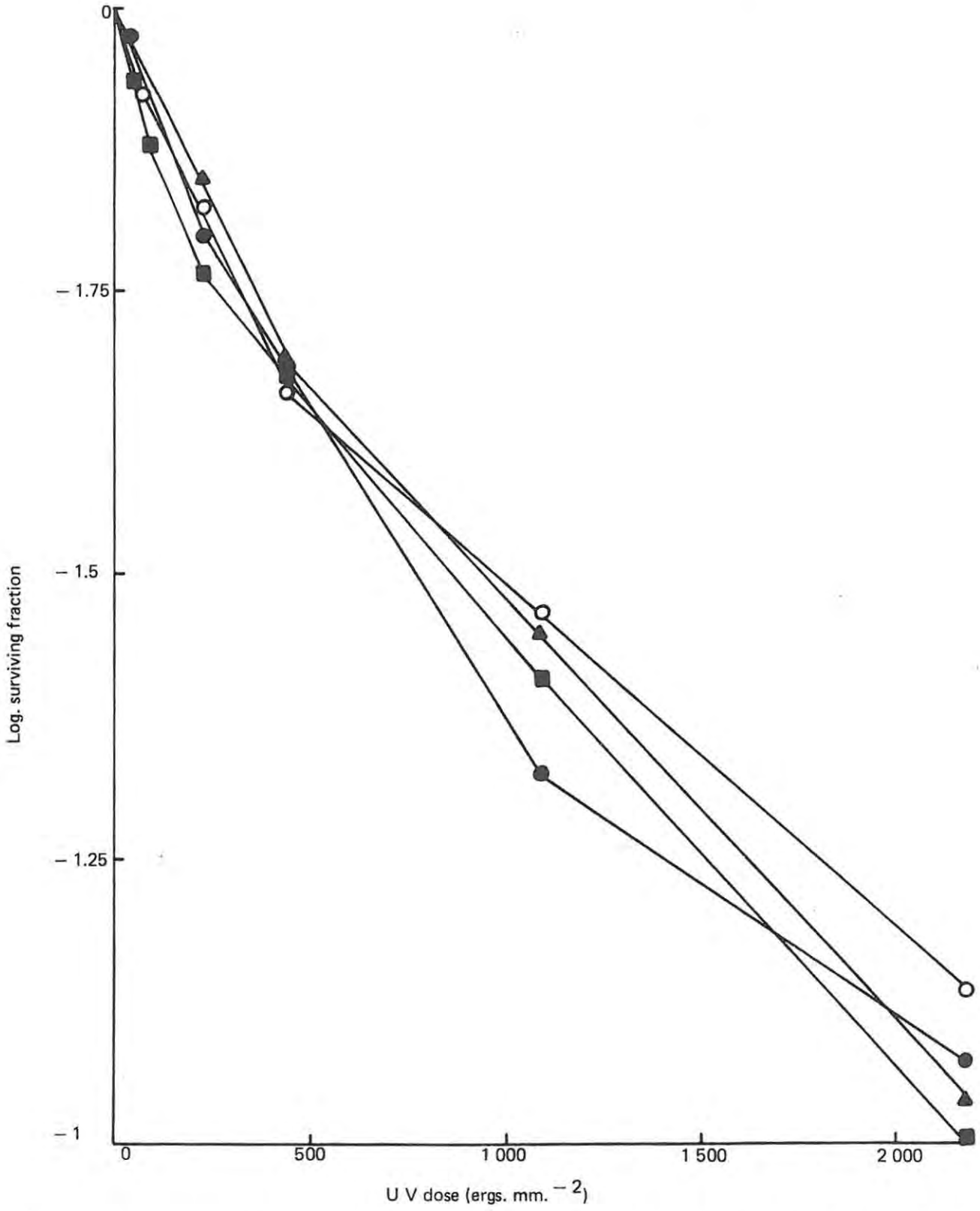


Fig. 2–11. UV inactivation of phages. ●—●, α 1; ▲—▲, α 2; ■—■, α 3a; ○—○, α 3b.

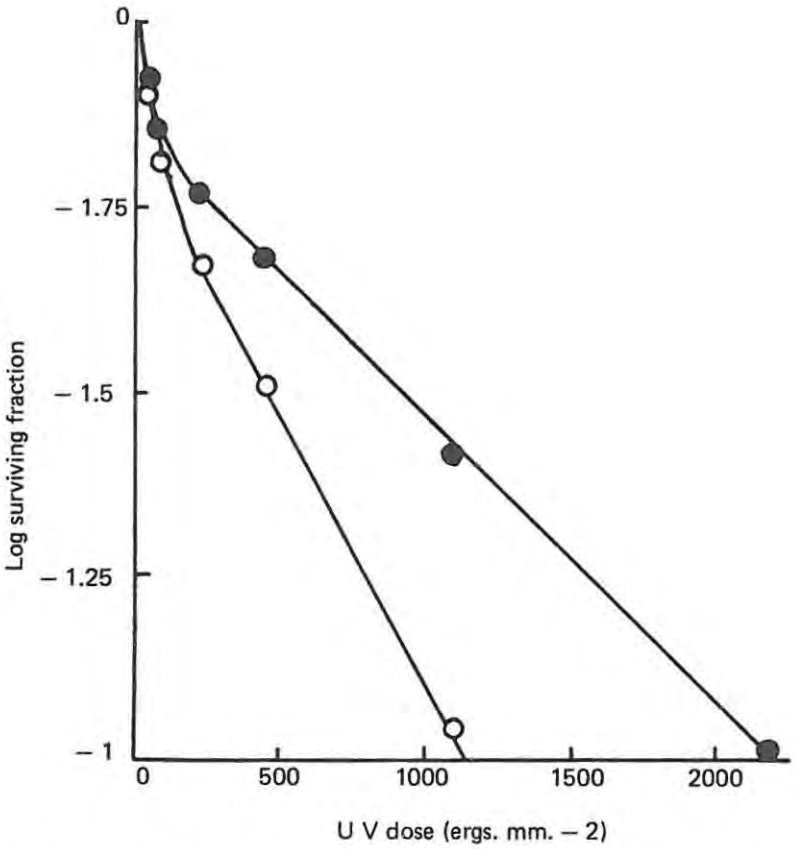


Fig. 2–12. UV inactivation of free phage ∝ 3a (●—●) and vegetative phage ∝ 3a (○—○).

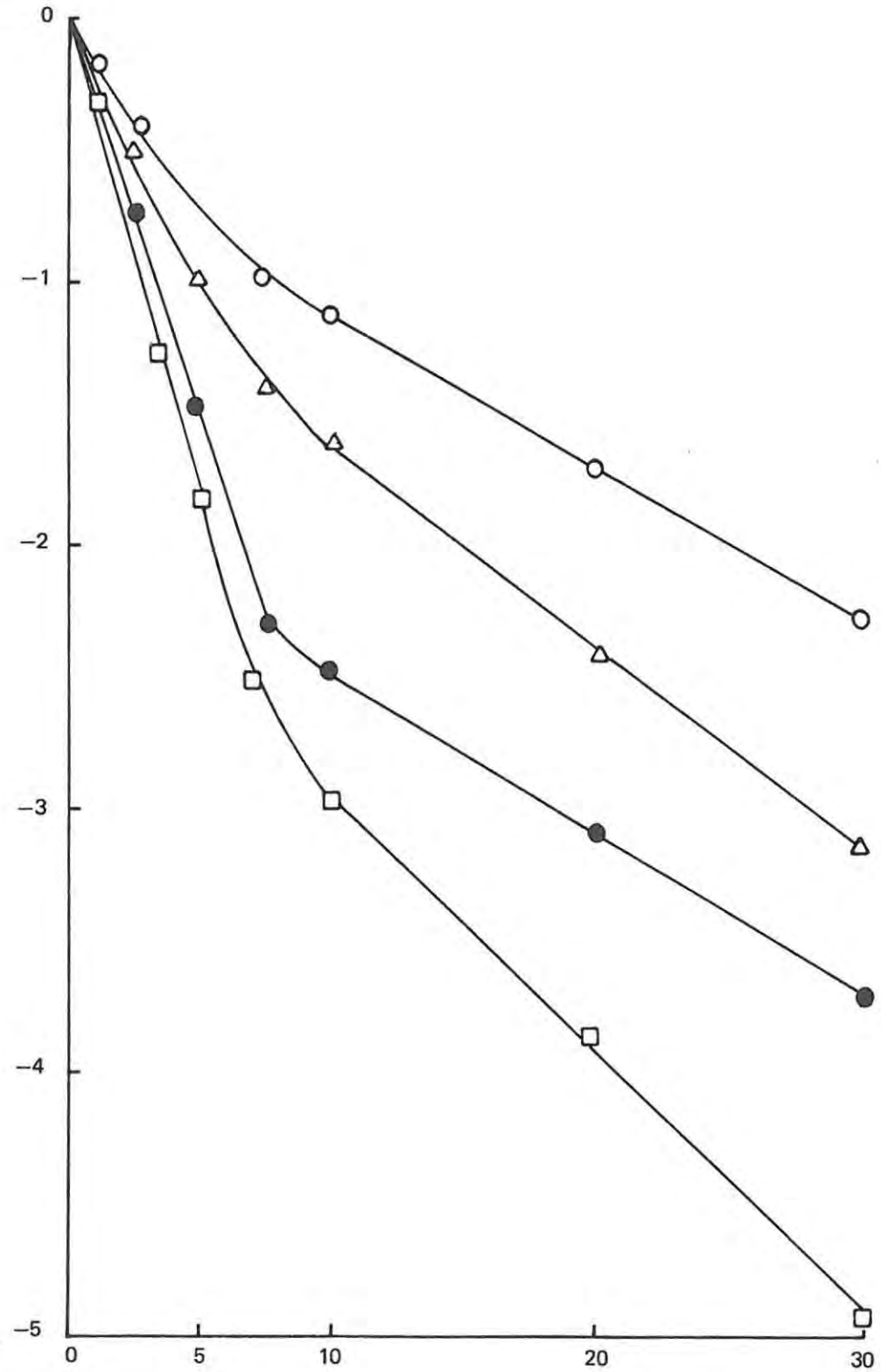


Fig. 2–13. Thermal inactivation of the ∝ phages at 60°C.
○—○ , ∝ 1; △—△ , ∝ 2; ●—● , ∝ 3a;
□—□ , ∝ 3b.

DISCUSSION

The α phages, while closely related serologically and morphologically, show differences in base ratios, ultraviolet inactivation, single-step growth characteristics and rate of adsorption to their host. They also have different sensitivities to thermal inactivation. Heat inactivation has been shown to be largely dependent upon the DNA content of a phage particle and hence can be used in phage characterization (Parkinson & Huskey, 1971). Biphasic inactivation patterns similar to the α phages have been shown for a Vibrio phage (Smith & Kreuger, 1952), φ 80 (Yamagishi & Ozeki, 1972) and phages of Streptomyces lactis where non-linearity is attributed to more resistant particles in the phage population (Wilkowske, Nelson & Parmelee, 1954). Heat inactivation of λ is multiphasic (Parkinson & Huskey, 1971) while others, for example Streptomyces griseus (Alexander & McCoy, 1956) and Proteus phages (Coetzee, 1958), are monophasic.

Vegetative α3a is more sensitive to u.v. inactivation than free phage. This is similar to phage P22 (Garen & Zinder, 1955) but different from Proteus mirabilis phages (Krizsanovich, 1973). The partial genetic homology between P22 and its host Salmonella typhimurium LT2 which causes this response (Yamamoto & Anderson, 1961) may also be the cause of the α3a response. The G+C(%) of Achromobacter sp. 2 is 40.00 (p. 100) which is very similar to that of α3a, 45.73.

THE PHAGE-HOST RELATIONSHIP OF ACHROMOBACTER SP. 2

SUMMARY

Achromobacter sp. 2 had a very low rate of spontaneous and ultraviolet induction and was not immune to superinfection by homologous phage. It was shown to be a cryptic lysogen for phage α3. The rate of phage induction could be increased by treatment with mutagens, precluding the possibility that the rare appearance of plaques was due to phage contamination. Cryptic lysogeny of this strain may be maintained by a defective excision mechanism and the mode of prophage integration in the host chromosome. Double lysogens were isolated which were immune to superinfection and were inducible by ultraviolet light. They were subject to lysogenic conversion by the temperate phages resulting in their inability to adsorb phage.

INTRODUCTION

Lysogeny is the hereditary property whereby bacteria can produce bacteriophage without infection with external particles, a property which lysogenic bacteria transmit to their progeny (Jacob & Wollman, 1959). Even after prolonged growth in antiphage serum culture filtrates contain phage. These phages can only be detected, however, by a suitable indicator strain e.g. a cured lysogen, since lysogens are immune to superinfection by homologous phage. Phages capable of lysogenizing the host are termed temperate and in the lysogenic state are known as prophages.

The phage-host relationship breaks down in a small proportion of a lysogenic population each generation. The lytic cycle ensues and spontaneous phage release occurs. The probability of phage liberation by a cell is about 10^{-2} to 10^{-5} per generation depending on the lysogenic system (Six, 1959).

As temperate phages can either lysogenize or lyse the host there must be a stage in phage development when the choice between the two is made. The factors affecting this choice are many and varied and the exact nature of the "effectors" and their targets are unknown (Echols, 1972). In general it appears that lysogeny will predominate when there is a low energy level in the host cell i.e. in the stationary phase. Hong, Smith & Ames (1971) have correlated lysogeny with a low energy level and a high level of cyclic AMP. The physiological usefulness of this regulation may be to prevent lytic development under unfavourable cellular conditions. Lysogeny is also more frequent at high multiplicities of infection (Fry, 1959). High multiplicities appear to activate repressor synthesis (Echols, 1972) possibly by a gene dosage effect.

It was discovered in 1950 (Lwoff & Gutmann, 1950) that u.v. could induce the development of phage in lysogens. Many other physical and chemical agents have since been shown to have inducing abilities e.g. mitomycin C (MC) (Shiba, Terawaki, Taguchi & Kawamata, 1958), X-rays and thymine deprivation (Melechen & Skaar, 1962). All have in common the selective inhibition of bacterial DNA synthesis (Otsuji, Sekiguchi, Iijima & Takagi, 1959). U.v. induction is due to inactivation of phage repressor, but this is an indirect consequence of bacterial DNA damage (Tomizawa & Ogawa, 1967).

Kirby, Ruff & Goldthwait (1972) showed that there was a close association between cell division and prophage induction by repressor inactivation in E. coli. Induction was accompanied by the production of long filaments (Kirby, Jacob & Goldthwait, 1967).

The maintainance of stable lysogeny and immunity to superinfection are both due to the presence of phage repressor protein (Kaiser & Jacob, 1957; Jacob & Monod, 1961). Repressors act on operators to switch off the activity of adjacent structural genes by preventing transcription of mRNA. Phage λ mRNA increases from very low to very high levels following induction of lysogens (Sly, Echols & Adler, 1965).

The establishment and maintainance of lysogeny in E. coli by phage λ has been the subject of extensive research. Kaiser (1957) showed that λ repressor protein (a dimer of 2 identical subunits (Pirrotta, Chadwick & Ptashne, 1970) produced by the cI gene) was the only phage protein needed to maintain lysogeny. Evidence now shows, however, that the repressor protein is itself subject to regulation (Eisen, Brachet, da Silva & Jacob, 1970; Heinemann & Spiegelman, 1970). It appears that after infection with λ, the cII and cIII gene products act at a promotor pre in the cy region to establish repressor synthesis. Mutants in cI, cII or cy do not give a burst of repressor after infection nor do they establish efficient lysogeny. However these mutants can lysogenize normally if supplied with repressor (Kourilsky, 1971). Once synthesized, the repressor protein (cI gene product) acts at a second promotor prm to ensure its own continued synthesis and the maintainance of lysogeny (Echols & Green, 1971; Reichardt & Kaiser, 1971). The cI repressor is also antagonized by an antirepressor, the cro product, (Oppenheim, Neubauer & Calef, 1970) which acts to increase the efficiency of induction.

The cI repressor prevents synthesis of lytic phage gene products by acting at two operators. These sites were identified by mutants V_2 and V_1V_3 which together prevent the establishment of lysogeny and render the phage virulent (Ptashne & Hopkins, 1968).

Most prophages are linearly inserted into the host chromosome and are thus replicated under the control of the bacterial cell. The model of Campbell (1962) is generally considered as the method of prophage integration. The phage genome circularizes upon entry into the cell and a recombination event occurs between a specific region of phage DNA and an homologous host region, the attachment region. The prophage genetic map is a circular permutation of the vegetative map (Campbell, 1963).

Phage-specific integrase catalyzes the integrative recombination in λ (Gingery & Echols, 1967; Zissler, 1967), P22 (Smith & Levine, 1967) and P2 (Choe, 1969). This is also required for the recombination event which mediates excision (Smith, 1968; Bertani, 1970; Echols, 1970) but an additional gene product, xis for λ (Echols, 1970; Guarneros & Echols, 1970; Kaiser & Masuda, 1970) and cox for P2 (Lindahl & Sunshine, 1972) are required for excision. It has been found in λ (Weisberg & Gottesman, 1971) that the in vivo xis activity is much less stable than that of int. This helps to explain why integration is so much more efficient than excision.

Integration in most systems is site-specific and each phage has a defined site on the bacterial chromosome with which it recombines (Weil & Signer, 1968). An exception is phage Mu-1 which inserts at random in the host chromosome (Taylor, 1963) regardless of the host nucleotide sequence (Bukhari & Zipser, 1972). With a phage such as P2 which has a wide host range, there are a number of possible integration sites

(Sunshine & Kelly, 1967). Occasional errors in the reverse recombination event leading to excision can occur due to "illegitimate" pairing between non-homologous regions of phage and bacterial DNA. Where this occurs transducing particles carrying phage and bacterial DNA are formed (Campbell, 1962).

An unusual prophage is P1 where stable lysogens occur despite the fact that the phage is not integrated but exists as an autonomous replicon in the cytoplasm (Ikeda & Tomizawa, 1968). That the prophage is under strict control is evidenced by the rarity of non-lysogenic segregants. Prophage N15 also appears to have an extrachromosomal location (Ravin & Shulga, 1970).

In both λ and P22 the int genes are under phage control (Protass & Korn, 1966; Botstein, Chan & Waddell, 1972). In P2, however, integrase is synthesized constitutively in vegetative phage while the prophage, even in the absence of repressor, cannot synthesize integrase (Bertani, 1970). Therefore the int gene belongs to a constitutive operon which is physically disrupted by phage integration. Mutants of P2 have been isolated which are temperature inducible (Calendar, Lindahl, Marsh & Sunshine, 1972). They were found to contain a nip mutation in the immunity region which in some way allows the int product to be formed even while P2 is in the prophage state.

When a lysogen is superinfected with homoimmune phage, replication of the incoming phage is inhibited. In λ this appears to be due to the fact that replication requires transcription of the X-O-P region (Dove, Inokuchi & Stevens, 1971) which is under repressor control. In P22 a superinfection exclusion system operates whereby the membrane association necessary for vegetative replication is prevented (Darlington & Levine, 1971).

A few temperate phages, notably P2, are noninducible. Double lysogens of P2 are located at different attachment sites although occasional tandem lysogens have been isolated when the prophage carries an *int* mutation (Bertani, 1971). Tandem lysogeny by λ is a common occurrence (Campbell, 1963).

Among the more unusual phage-host relationships is one in which the phage DNA is not integrated but, unlike P1, there is no mechanism to prevent its segregating out. Thus a proportion of the cells will be non-lysogenic. This is called the "carrier state" or "pseudolysogeny" (Baess, 1971). The lysogen can be cured by growth in phage antiserum. Bott & Strauss (1965) showed that SP10 existed in a noninducible carrier state in *B. subtilis* and therefore generalised SP10 transducing particles were not formed by genetic recombination between phage and host DNA.

Another phage-host relationship is that of cryptic lysogeny where the lysogens do not liberate viable phage and are not immune to superinfection by homologous phage (Fischer-Fantuzzi & Calef, 1964). Irradiation with u.v. does not produce active phage or promote cell lysis. Cryptic λ is a fragment of about one half of the λ map and is recoverable by superinfection. The deleted fragment includes the cI region whose absence is demonstrated by the lack of immunity. The λ*cry* is obtained from all λ lysogens but with lower frequencies compared with λ*crg* lysogens which are variants of normal λ lysogens (Marchelli, Pica & Soller, 1968). The genes deleted from the λ*cry* cover the *int* to *end* region (Adhya & Campbell, 1970).

Recombination between a *Staphylococcus aureus* penicillinase plasmid and temperate phage P11 has caused the formation of a high-frequency transducing element for erythromycin resistance, P11*de* (Novick, 1967).

This phage is cryptic as it has lost part of its genome including the immunity region.

A P1 cryptic prophage has been isolated from a P1 phage which had acquired a chloramphenicol resistance marker by recombining with an R factor. This P1cry, unlike P1, has a chromosomal site and has many genes deleted including vir, the site of action of the immunity repressor. It is suggested that P1cry stability is conferred by an excision mutation (Scott, 1970). The chromosomal site of P1cry is due to the R factor-host genome integration mechanism and is not directed by the P1 moeity (Scott, 1973). While P1cry cannot express immunity it can express P1 modification and restriction. These lysogens are therefore unable to plate mutant phages which plate efficiently on nonlysogens (Rosner, 1973).

Recently Krizsanovich (1973) reported cryptic lysogeny in Proteus mirabilis due to a defective excision mechanism and disruption of the repressor gene by the mode of phage integration. While isolating Achromobacter phages, one spontaneous plaque on a lawn of Achromobacter sp. 2 was noted (p. 29). It was thought possible that this strain might be a cryptic lysogen similar to that found in P. mirabilis. An investigation of the phage-host relationship of Achromobacter sp. 2 was therefore instigated.

METHODS

Bacteria. Achromobacter sp. 2 was used.

U.v. induction. Forty exponential phase cultures in minimal medium and phage broth were tested for induction by u.v. irradiation as described on p. 23.

MC and NG induction. This was performed in 2 ways:

(i) Exponential phase cultures in minimal medium were diluted to 1×10^5/ml. in fresh minimal medium. To 0.8 ml. cells 0.2 ml. MC or NG was added to final concentrations of 5 μg./ml. and 3 μg./ml. respectively. Cultures were incubated for 1 hr., 9 ml. phage broth added and incubation continued with shaking for 2 hr. After centrifugation the supernatants were tested for phage.

(ii) Overnight broth cultures were diluted 1:10 in 400 ml. phage broth containing 2.5 μg. MC/ml. or 3 μg. NG/ml. and incubated with aeration for 5 hr. and 9 hr. respectively. Supernatants were purified and concentrated by cycles of differential centrifugation at 12,000 g for 10 min. and 35,000 g for 90 min. and tested for phage. Controls were carried out without the addition of MC or NG.

Temperature induction. Temperature sensitive inducible prophages were investigated by incubating lawns of bacteria at 25 °C and 42 °C and screening for phages after 2 and 4 days.

Induction by ageing. Induction by ageing was tested by incubating cultures in phage broth and nutrient broth for 3 weeks at 30 °C. Cultures were centrifuged at 2,000 g for 20 min. and supernatants tested for phage.

Electron microscopy. Culture supernatants were prepared for electron microscopy as on p. 24.

Isolation of lysogenic bacteria. Colonies from the centre of turbid plaques were streaked twice for single colonies on plates spread with antiphage serum (k = 10) (Levine, 1957). Suspected lysogens were tested for superinfection immunity by spotting phage on lawns of the bacteria. The ability of lysogens to adsorb phage was tested as on p. 28. The supernatants of overnight cultures of the lysogens were tested for phage.

U.v. induction of lysogens. Cell samples less than 2 mm. deep were irradiated in Petri dishes with a Hanovia lamp (p. 23). One ml. aliquots were removed at time intervals, 9 ml. broth added and cultures incubated for 90 min. After centrifugation the SNF was assayed for phage titre.

Stability of lysogens. The method of Choe (1969) was used. Overnight cultures of lysogens were diluted 1:100 in broth containing antiphage serum (k = 5) and allowed to grow to saturation. The serum cultures were again diluted 1:100 in serum-broth and the cycle repeated a number of times. Finally cells were plated to obtain single colonies, a number of which were tested for phage production in broth cultures.

RESULTS

Induction of Achromobacter sp. 2

No induction was obtained by u.v. irradiation, growth at different temperatures, ageing or by MC and NG treatment of 10 ml. cultures. Only treatment of 400 ml. cultures with MC and NG resulted in the induction of phage (Table 3-1). The high titres obtained were due to the phage being able to re-infect its parent strain. Controls of untreated 400 ml. cultures were shown to be free of phage when assayed and examined electron microscopically.

Isolation of lysogens

Not all colonies isolated from turbid plaques of the α phages were lysogenic (Table 3-2). Only colonies immune to superinfection by all 4 phages were lysogenic, having phage in the supernatant of broth cultures and being u.v. inducible. Ultraviolet induction of Achromobacter sp. 2 and the different lysogens were compared (Table 3-3).

Adsorption of phage to lysogens

The percentage phage adsorption to Achromobacter sp. 2 strains lysogenic for the α phages is shown in Table 3-4. All 4 phages caused lysogenic conversion which resulted in the inability of lysogens to re-adsorb phage. Lysogenization by any one of the phages caused resistance to all four.

Stability of lysogens

As no non-lysogenic segregants were found it was concluded that the 4 lysogens were stable.

Table 3-1. Induction of Achromobacter sp. 2

Technique	No. of experiments	Phage titre
Ultraviolet irradiation	40	0
Mitomycin C (10 ml.)	20	0
Mitomycin C (400 ml.)	3	$1.2 \times 10^2 - 5.3 \times 10^5$
Nitrosoguanidine (10 ml.)	20	0
Nitrosoguanidine (400 ml.)	3	$4 \times 10^1 - 1.4 \times 10^2$
Temperature	20	0
Ageing	20	0

Table 3-2. Isolation of lysogens

Phage plaque from which colony isolated	No. colonies tested	e.o.p. with phages				Ultraviolet induction*
		α1	α2	α3a	α3b	
α1	2	0	0	10^{-6}	10^{-6}	-
	4	0	0	0	0	+
α2	22	$0-10^{-4}$	0	$0-10^{-4}$	$0-10^{-6}$	-
	1	0	0	0	0	+
α3a	5	0	0	0	0	+
α3b	20	$0-10^{-4}$	0	$10^{-2}-10^{-6}$	$0-10^{-6}$	-
	1	0	0	0	0	+

* - not inducible

\+ inducible

Table 3-3. Ultraviolet induction of lysogens

Strains	U.v. dose (ergs. mm.$^{-2}$)					
	0	18	36	54	72	216
Achromobacter sp. 2	0	0	0	0	0	0
A. sp. 2 (α1)	7.0×10^4*	8.0×10^4	9.8×10^4	1.8×10^5	1.0×10^5	4.0×10^4
A. sp. 2 (α2)	1.7×10^6	3.5×10^6	5.4×10^6	4.0×10^6	2.5×10^6	8.0×10^5
A. sp. 2 (α3a)	4.5×10^5	6.4×10^5	1.9×10^6	2.0×10^6	1.7×10^6	9.6×10^5
A. sp. 2 (α3b)	5.0×10^3	6.9×10^3	9.1×10^3	6.0×10^3	5.5×10^3	1.0×10^3

* Phage titre

Table 3-4. Phage adsorption to lysogens

Lysogen	Phage	% adsorption after 10 min.
A. sp. 2 (α1)	α1	0
	α2	0.1
	α3a	0
	α3b	0.6
A. sp. 2 (α2)	α1	0.4
	α2	0
	α3a	0.1
	α3b	0.5
A. sp. 2 (α3a)	α1	0.5
	α2	0
	α3a	0
	α3b	0
A. sp. 2 (α3b)	α1	0
	α2	0
	α3a	0.6
	α3b	0

DISCUSSION

The rate of spontaneous phage liberation by Achromobacter sp. 2 is lower than previously described for any lysogen as only one spontaneous plaque has been observed. This plaque could not have been due to a dismune variant (Cohen, 1959; Meynell, 1962) of continually liberated phage as no phage particles were present in culture supernatants examined

electron microscopically; nor could it have been due to a virulent mutant of a prophage as the plaque was turbid. As phage could be isolated by MC and NG induction of large volumes of bacterial culture the possibility of phage contamination was eliminated. As this lysogenic Achromobacter strain had a very low frequency of spontaneous and u.v. induction and was not immune to superinfection the lysogeny may be termed cryptic (Fischer-Fantuzzi & Calef, 1964). Strains Achromobacter sp. 2 (α1), A. sp. 2 (α2), A. sp. 2 (α3a) and A. sp. 2 (α3b) may thus be referred to as double lysogens. This phage-host relationship is very similar to that described in Proteus mirabilis by Krizsanovich (1973).

Phages α3a and α3b both originated from Achromobacter sp. 2, the former spontaneously and the latter by u.v. induction. Both were very rare events which could not be repeated. The induction of Achromobacter sp. 2 by MC and NG yielded phages which were not distinguished as either α3a or α3b and are, therefore, referred to as α3. Phages α3a and α3b are very similar and it is concluded that they are variants of the same cryptic prophage of Achromobacter sp. 2.

Cryptic lysogens have been isolated from a number of bacterial species including Pseudomonas putida (Chakrabarty & Gunsalus, 1969), Staphylococcus aureus (Novick, 1967), Shigella (Scott, 1970) and Escherichia coli (Adhya & Campbell, 1970). Unlike cryptic lysogens of Proteus mirabilis (Krizsanovich, 1973) these lysogens all owe their crypticogenicity to extensive deletions including the repressor gene or the site of action of the immunity repressor. They are not inducible but can be recovered by superinfection with related phage.

Crypticogenicity in Achromobacter sp. 2 must have a different cause for although the cryptic prophage does not synthesize repressor, vegetative α3 can be recovered and forms turbid plaques on the parent strain. As some of these resistant bacteria have been shown to be lysogenic, the repressor gene must be present but only expressed by the vegetative phage. A functional repressor gene can also be rescued from P1cry by superinfection with clear plaque mutants (Scott, 1970). The Achromobacter sp. 2 cryptic lysogen fails to express the gene for lysogenic conversion. This is similar to the cryptic lysogens of P. mirabilis (Krizsanovich, 1973).

The MC and NG inducibility of the cryptic lysogen argues against an extensive prophage deletion which is the usual cause of crypticogenicity. A simple explanation is that of Krizsanovich (1973) whereby a defective enzyme necessary for prophage excision and/or vegetative phage development is produced by the cryptic prophage. Scott (1970) also favours defective excision causing stability in P1cry. Induction by MC and NG could be due to reversion or suppression of this mutation. Nitrosoguanidine is a powerful mutagen (Adelberg, Mandel & Chein Ching Chen, 1965) and MC is thought to cause cross-linkages in DNA strands. Repair of these cross-linkages could result in mutations (Boyce & Howard-Flanders, 1964).

Lack of functional repressor in the cryptic lysogen could be due to the phage integration site lying within the repressor gene and causing its disruption (Krizsanovich, 1973). According to Campbell's model of prophage integration (1962) the phage genome circularizes and lysogenization results from a reciprocal recombination between the chromosite and episite. Bertani (1970) has shown that stability of P2 lysogens is

due to integration within the *int* gene resulting in a split-operon which prevents prophage excision.

Because double lysogens are subject to lysogenic conversion by the phages it was not possible to determine whether they produce repressor. However, their stability and normal inducibility makes it probable that they are under repressor control as in normal lysogens (Krizsanovich, 1973). The reconstitution of the repressor gene in double lysogens may be caused by superinfecting phage lysogenizing in tandem with the cryptic prophage. Superinfecting P2 can integrate in tandem and reconstitute the *int* operon (Bertani, 1971). Alternatively, the superinfecting phage may integrate at a different chromosite which does not split the repressor gene.

TRANSFECTION OF ACHROMOBACTER SP. 2 SPHEROPLASTS

SUMMARY

Achromobacter sp. 2 was converted to spheroplasts by growth in the presence of penicillin in sucrose-supplemented medium. The spheroplasts could be stabilized by sucrose and $CaCl_2$ and supported phage growth. Spheroplasts formed from bacteria incubated for 90 min. were competent for transfection with DNA isolated from phage α3a. A linear relationship between infective units and DNA concentration was obtained. Prolonged incubation of spheroplasts with DNA caused a drop in phage titre.

INTRODUCTION

Spheroplasts are osmotically sensitive, spherical bodies surrounded by a partially degraded, non-rigid cell wall (Martin, 1963). This is in contrast to protoplasts where dissolution of the cell wall is complete, resulting in a body enclosed only by the cytoplasmic membrane. Cell wall rigidity is conferred by a mucopeptide layer which is present in all bacteria, both Gram-positive and Gram-negative (Cummins & Harris, 1956) and is often referred to as the rigid layer (Weidel, Frank & Martin, 1960). The mucopolymer is a heteropolymer of amino sugars and amino acids. It contains units of N-acetyl glucosamine and N-acetyl muramic acid with short peptides attached to the muramic acid residues. The peptides characteristically contain L-alanine, D-alanine, D-glutamic acid

and a dibasic amino acid e.g. lysine, diaminopimelic acid (DAP), ornithine or 2,4-diaminobutyric acid. The peptide chains are covalently linked to form a net-like polymer which confers wall rigidity (Park, 1968).

Conversion of an organism to a spheroplast or protoplast involves depolymerization of the rigid layer. Weibull (1953) isolated naked protoplasts from Bacillus megaterium by treatment with lysozyme. This enzyme hydrolyzes the β-1,4 linkages between the N-acetylglucosamine and muramic acid residues (Salton & Ghuysen, 1960). If the bacteria are exposed to lysozyme in hypotonic medium they will rapidly lyse but treatment in hypertonic medium e.g. 20% sucrose yields stable protoplasts. The lysozyme-protoplasts could not form colonies in the presence of the enzyme (Weibull, 1953) and cell wall damage is irreparable even after removal of the lytic agent (McQuillen, 1960). Because the wall of Gram-positive bacteria consists mainly of mucopeptide these cells are more susceptible to lysozyme than are the Gram-negative organisms where the cell wall is more complex.

Protoplasts are soft, fragile, pleomorphic bodies whose structure can only be maintained in a hypertonic milieu such as sucrose which does not penetrate the protoplast (Weibull, 1953). They retain their flagella, confirming the opinion that these have a cytoplasmic origin. Morphological studies on protoplasts were confirmed chemically when no traces of DAP, a typical cell wall constituent, could be detected (McQuillen, 1960). Protoplasts can synthesize protein, nucleic acids and lipids and can have as much as a ten-fold increase in mass (Weibull, 1968). Brenner et al. (1958) laid down criteria for the classification of protoplasts. They must be osmotically sensitive, retain no residual cell wall as evidenced by the absence of cell wall antigens and phage receptor activity,

and an enzyme which induces protoplasting should cause complete dissolution of isolated cell walls.

The breakdown of the rigid layer of cell walls will only yield naked protoplasts in a limited number of Gram-positive organisms which possess simple mucopolymer walls. Gram-negative bacteria, as also a large number of Gram-positive bacteria, have cell wall components which cannot be completely removed. These bodies with cell wall remnants are called spheroplasts. They can have an increased surface area of as much as seventy times that of the normal cell (Gebicki & James, 1960). Although Gram-negative bacteria normally only form spheroplasts, these may occasionly burst open and release truly naked protoplasts surrounded by one membrane (Martin, 1963).

The first clear description of the transformation of bacteria to spheroplasts was in 1895 by Pfeiffer (see Davis, Gemsa, Iannetta & Wedgwood, 1968) who injected Vibrio cholera into the peritoneal space of guinea pigs and examined the fluid under a microscope. It was later determined that the bacteria had been killed by an antibody-complement system and converted to spheroplasts by lysozyme (Gemsa, Davis & Wedgwood, 1966).

Although Gram-negative bacteria are relatively resistant to lysozyme, treatment with ethylenediaminetetra-acetic acid (EDTA) in tris buffer allows penetration of the wall (Repaske, 1956). EDTA probably removes ionic cross-linkages to make larger spaces for the passage of lysozyme (Martin, 1963). Spheroplasts of Gram-negative bacteria can also be formed by treatment with penicillin (Lederberg, 1956; Park & Strominger, 1957). Penicillin acts on the mucopeptide layer (murein) by inhibiting its biosynthesis. Synthesis of this layer occurs by a polymerization

reaction forming polysaccharide chains with short peptides attached to each muramic acid residue (Anderson, Matsuhashi, Haskin & Strominger, 1965). This is followed by a crosslinking reaction whereby neighbouring chains are linked (Wise & Park, 1965). Penicillin interfered with the crosslinking reaction, causing the cell to make a defective wall and at the same time allowing lytic enzymes to be formed which can attack the existing murein. Antibiotics which prevent the polymerization reaction do not form spheroplasts (Park, 1968). Because penicillin interferes with the synthesis of the cell wall it affects growing bacteria only (Bayer, 1967).

Spheroplasts can also be formed if mutants which are unable to synthesize the essential mucopeptide component DAP are grown in a hypertonic medium (Bayer, 1967). Treatment of Gram-negative bacteria with glycine can also yield spheroplasts (Brown, Drummond & North, 1962). Park (1968) postulated that glycine, like penicillin, inhibited cell wall synthesis.

Spheroplasts produced by these techniques resemble one another morphologically but differ in their ability to divide and multiply. Lysozyme-EDTA spheroplasts do not divide but penicillin spheroplasts are revertible (Martin, 1963) and can adapt to life in a milieu where cell wall synthesis is impossible by conversion and multiplication in the osmotically labile L-form (Landman, Altenbern & Ginoza, 1958).

The osmotic fragility of spheroplasts can be decreased by treatment with certain aliphatic amines e.g. spermine and putrescine. As little as 10^{-3} - 10^{-5} M spermine protects E. coli spheroplasts against dilution of the osmotic stabilizer. Spermine also prevents lysis of halophiles in hypotonic media. The stabilization is presumably due to an interaction

between these cationic compounds and some negatively charged molecule on the cell surface (Mager, 1959). Stabilization is greater for lysozyme spheroplasts than for penicillin ones. Streptomycin, polylysine and Ca^{++} are also partially effective (Tabor, 1962).

Naked protoplasts are resistant to phage (Weibull, 1953) but if infection is carried out on whole cells immediately before protoplasting, phage development can occur (Brenner & Stent, 1955; Salton & McQuillen, 1955). However, phages can adsorb to and develop in spheroplasts as in normal cells (Dénes & Polgár, 1959) although the rate of adsorption to spheroplasts may be lower than to whole cells (Hines, Freeman & Pearson, 1964). The burst size may also be somewhat reduced (Ray & Burma, 1970). Removal of the lipopolysaccharide-containing phage receptor sites renders spheroplasts resistant to phage infection (Watson & Paigen, 1972).

The different synthetic capabilities of spheroplasts may be decreased in unequal amounts and Rubenstein, Nass & Cohen (1970) found a greater decrease in DNA and phospholipid synthesis than in RNA and protein synthesis. The cell wall thus appears to hold the membrane in the optimal conformation for cell synthesis.

Spheroplasts of Achromobacter were studied with a view to developing a transfection system.

The term transfection was first used by Földes and Trautner (1964) to describe the "infection of cells by the isolated nucleic acid from a virus resulting in the production of a complete virus." The term also covers infection of protoplasts and spheroplasts by phage DNA. Purified viral nucleic acid was shown to be infective by itself when RNA from tobacco mosaic virus caused lesions in leaves of Nicotiana glutinosa

(Gierer & Schramm, 1956). The infectivity of the RNA was 2% of that of a native virus. Since then there have been many examples of nucleic acid infectivity including plant and animal virus DNA and RNA as well as bacteriophage nucleic acid. Kaiser & Hogness (1960) first demonstrated transfection in bacteria by infecting intact E. coli K-12 cells with DNA isolated from phage λdg. This could only be achieved if infection with DNA was accompanied by simultaneous infection with "helper" phage λ. Using this procedure fragments of λ DNA could be assayed by recombination.

The discovery of transformation in Bacillus subtilis (Spizizen, 1958) prompted the investigation of the productive infection of competent cells of this organism with DNA from some of its phages. Romig (1962) showed that DNA from phage SP3 could transfect competent cells. Treatment of the infective nucleic acid with RNAse, trypsin and SP3 antiserum did not affect infectivity which was, however, destroyed by DNAse. Only 8×10^2 p.f.u./ml. were recovered when maximally competent bacteria were incubated with 100 μg. of SP3 DNA/ml., whereas 1.7×10^6 transformants/ml. were obtained using 2 μg. bacterial DNA/ml.

This system established a tenuous relationship between transfection and transformation which was supported by several further observations. Harm & Rupert (1963) showed that transforming DNA competed with transfecting DNA of phage HP1 to substantially decrease the number of plaques obtained in Haemophilus influenzae. Moreover, transfection and transformation decreased concomitantly when competent cells were maintained at 37°C. They also observed that both transfection and transformation showed a linear dependence on DNA concentration and saturation at high levels. The relationship was further emphasized when studies by Green (1964), Okubo, Strauss & Stodolski (1964) and Reilly & Spizizen (1965)

showed that cells which were transfectable or transformable occurred at about the same frequency in the cell population. Moreover, it appeared that the same subpopulation of cells was involved in both processes (Nester, 1964; Reilly & Spizizen, 1965). Bott & Wilson (1967) and Riva & Polsinelli (1968) showed little, if any, difference in the development of competence of B. subtilis for phage or bacterial DNA. It thus appears that in this strain the initial stages in transformation and transfection are identical. Harm & Rupert (1963) and Lindberg, Sjöström & Johansson (1972) reached the same conclusion for H. influenzae and Staphylococcus aureus. Transfection may therefore be viewed as a simple model of transformation. The advantage of using phage DNA is that it enables the establishment of competence criteria without the requirement for integration of incoming DNA. Phage DNA can be more easily used to estimate the fraction of competent cells in a culture. In B. subtilis this amounts to $1 - 7 \times 10^{-2}$ (Riva & Polsinelli, 1968).

In general transfection is characterized by a lower efficiency than transformation. Using a DNA concentration sufficient to transform all competent cells present, Young & Spizizen (1963) showed that only 1 - 4% of added DNA was irreversibly taken up, and less than one phage equivalent of DNA per $10^4 - 10^5$ added could form an infectious centre (Spizizen, Reilly & Evans, 1966). For transfection with SPP1 DNA the efficiency is $5 \times 10^3 - 6 \times 10^3$ phage equivalents per p.f.u., the highest reported for B. subtilis (Riva & Polsinelli, 1968).

Transfection in B. subtilis is characterized by two different dependencies on phage DNA concentration. SP02 and ϕ29 transfect linearly with DNA concentration (Okubo & Romig, 1965; Reilly & Spizizen, 1965) and SP82G (Green, 1964), SP3 (Romig, 1962), SP50 (Földes & Trautner, 1964),

SP01 (Okubo, Strauss & Stodolsky, 1964) and ϕ1 and ϕ25 (Reilly & Spizizen, 1965) as the second, third or fourth power of the concentration. Interaction of several infective DNA molecules is required to establish an infective centre in these latter cases.

In the case of SP82G the incoming phage DNA is inactivated making genetic recombination necessary to produce functional phage molecules (Green, 1966). Recombination-defective mutants have a marked reduction in transfectibility (Okubo & Romig, 1966). As SP82G DNA differs from host DNA by containing hydroxymethyluracil instead of thymine (Kahan & Kahan, quoted by Green, 1964) it acts as "foreign" DNA susceptible to defensive cellular Ca^{++}-activated nucleases (Epstein, 1971).

SP02 is a temperate phage and its DNA has the same buoyant density in CsCl as host DNA (Okubo & Romig, 1965). In view of the heterologous nature of SP82G DNA it is surprising to find that in the same population of competent B. subtilis cells the apparently homologous SP02 DNA is markedly less efficient in transfection than is SP82G DNA. This is despite the fact that SP02 DNA has a molecular weight about 25% that of SP82G DNA (Epstein, 1968) making it less susceptible to damage during extraction. An explanation proposed by Epstein is that replication of SP02 DNA is decreased due to the sequestering of the required host enzymes in competent cells (McCarthy & Nester, 1967). During the arrest of synthesis the SP02 DNA would be degraded.

Williams & Green (1972) showed that sequential attachment of both ends of phage DNA to the cell precedes its entry. Attachment is end- and time-specific, the establishment of irreversible binding being similar to that in transformation (Dubnau & Cirigliano, 1972). Only after both ends are attached does entry begin and this lasts 3 - 4 minutes. The polarity

of entry is the reverse of that observed in normal phage injection (McAllister, 1970). There is an eclipse period after entry when single-stranded breaks appear in the DNA (Levner, 1972).

Hirokawa (1972) discovered that the transfecting activity of ϕ29 DNA was sensitive to pronase. Protein is therefore associated with this transfecting DNA. It probably maintains the infective structure of ϕ29 DNA and appears to be bound to the ends of the DNA involved in DNA conformational stability.

An interesting development in transfection of B. subtilis reflects the universality of the genetic code (Crick, 1967). Competent cells have been successfully transfected with DNA from polyoma virus (Bayreuther & Romig, 1964) and vaccinia virus (Abel & Trautner, 1964).

In transfection studies on E. coli using the helper assay of Kaiser & Hogness (1960) it was established that the infecting DNA must have at least one free single-stranded end (Kaiser & Inman, 1965). As in B. subtilis transfection, the molecule need not be intact as half-molecules (Radding & Kaiser, 1963) and even terminal sixth-molecules (Kaiser & Inman, 1965) can donate markers to progeny phage by recombination with the helper genome. The helper assay, however, is complicated by the presence of two different phage genomes and transfection in E. coli was greatly improved when Guthrie & Sinsheimer (1960) and Hofschneider (1960) infected protoplasts with urea-treated ϕX174 phage and purified DNA. Spheroplasts, but not whole cells, could be transfected despite resistance to ϕX174 phage. Infection was destroyed by DNAse but not by trypsin or phage antiserum. Guthrie & Sinsheimer (1963) showed a linear relationship between infective centres produced and DNA concentration. Meyer, Mackal, Tao & Evans (1961) transfected E. coli spheroplasts with λ DNA, showing

that not only single-stranded DNA was infective. There is no eclipse of λ DNA infectivity during transfection (Young & Sinsheimer, 1967a).

Although transfection is a useful tool for studying the biological activity of DNA the main disadvantage is the low efficiency. This can be improved by using spheroplasts prepared from competent cells. Conversion of bacteria to spheroplasts fixes the state of competence (Iliashenko, Dityatkin & Danileichenko, 1968). Efficiency can be enhanced by using recB and recC spheroplasts, as nuclease degradation of the phage DNA does not occur (Barbour & Clark, 1970). As λ and P2 DNA uptake is dependent on Ca^{++}, infectivity can be increased by adding this to the medium (Mandel & Higa, 1970).

Early attempts to transfect with T4 DNA were unsuccessful due to the effect of endonuclease I (Spizizen, 1957), but Baltz (1971) overcame this by limiting the Mg^{++} concentration, precipitating the enzyme with protamine sulfate or by using a nuclease mutant strain. Hotz & Mauser (1969) also showed that the efficiency of transfection with T1 DNA was increased by protamine sulfate. A more efficient assay for biological activity of T4 DNA is by phage transformation (Baltz & Drake, 1972) which was first developed by Van de Pol, Veldhuisen & Cohen, (1961). They used disrupted phage and pure T4 DNA to recombine in spheroplasts and the technique has been used i.a. to measure the amount of DNA between markers, to assay gene-specific mRNA, to determine the origin and direction of replication of T4 and assay mutations induced in vitro (Goldberg, 1966; Jayaraman & Goldberg, 1969; Marsh, Breschkin & Mosig, 1971; Baltz & Drake, 1972).

Staphylococcus aureus excretes a nuclease which hydrolyzes DNA and this has been considered responsible for the failure to transform this species (Omenn & Friedman, 1970). Despite this, however, transfection was achieved (Riggs & Rosenblum, 1969). The yield, however, was one phage per infected protoplast. Sjöström, Lindberg & Philipson (1972) achieved a normal phage burst when transfecting competent cells. It was obligatory to use cells lysogenized with phage P11 for the expression of competence (Sjöström, Lindberg & Philipson, 1973). Cells harbouring cryptic phage P11de (Novick, 1967) could not induce competence. Transfection efficiencies of 6×10^5 p.f.u./μg. DNA were obtained. The requirement for lysogeny is not yet clear. The prophage may have a helper function to enhance DNA penetration similar to the helper phage effect in E. coli transfection (Kaiser & Hogness, 1960).

Transfection of H. influenzae by phage HP1c1 DNA has a similar requirement for a cellular recombination mechanism (Boling, Setlow & Allison, 1972) to that found in B. subtilis transfection by SP82G DNA (Green, 1964). The low efficiency is due to DNA fragmentation prior to reassembly as concatemers generated by recombination (Notani, Setlow & Allison, 1973).

In order to investigate the biochemical and genetic basis of thermophily, Streips & Welker (1969, 1971a) have studied transfection in Bacillus stearothermophilus to facilitate the development of a transformation system. Transfection in this organism is facilitated by a competence factor (Streips & Welker, 1971b).

When studying the transfection of protoplasts of Streptomyces kanamyceticus with DNA from actinophage PK-66 (Okanishi, Utahara & Okami, 1966) it was noted that protoplasts from organisms resistant

to phage could be transfected. This opened up a new method for the study of interspecies transmission of genetic materials. In the light of this it is of interest to note that Salmonella phage P22 DNA will transfect both E. coli and S. typhimurium spheroplasts (Benzinger, Kleber & Huskey, 1971; Benzinger & Kleber, 1971). A particular advantage of this system is that E. coli mutants defective in phage-supporting capacity are available. As such mutants are not available in other genera, essential host functions for phage production can be identified. Wais & Goldberg (1969) also demonstrated that urea-treated T4 can infect and grow in spheroplasts of Aerobacter aerogenes, S. anatum, Proteus vulgaris and Serratia marcescens. Thus only the initial adsorption step and not genome injection, replication or protein synthesis prevents T4 phage replication in these bacteria.

Thus it can be seen that while transfection is in itself an interesting phenomenon, its main importance lies in its use as a tool to study many aspects pertinent to the biology of bacteria and their viruses. Table 4-1 lists reports in which penetration of viral nucleic acid into bacteria has been claimed.

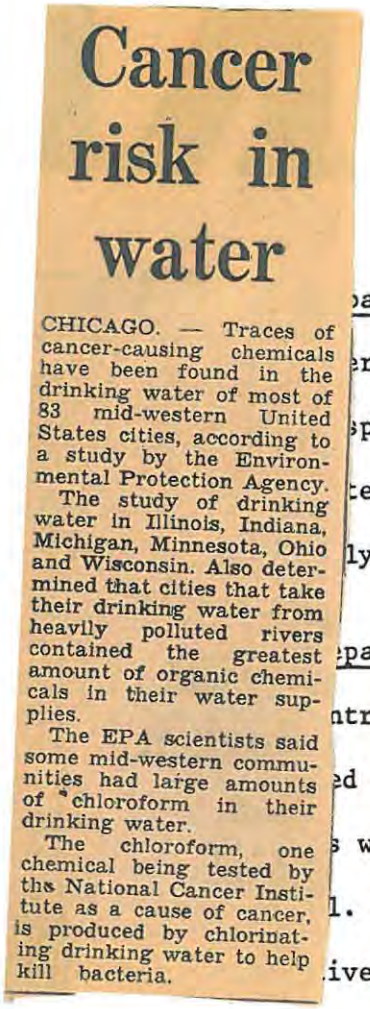
Transfection of Achromobacter spheroplasts was studied with a view to developing a transformation system to be used in the genetic mapping of this organism.

Table 4-1. Penetration of viral nucleic acid into bacterial cells

Recipient	Nucleic acid	Reference
E. coli cells	λdg DNA + helper phage	Kaiser & Hogness (1960)
E. coli spheroplasts	Urea-disrupted T2	Fraser, Mahler, Shug & Thomas (1957)
E. coli spheroplasts	NaCl-shocked T2	Spizizen (1957)
E. coli spheroplasts	ϕX174 DNA	Sekiguchi, Taketo & Takagi (1960)
E. coli spheroplasts	T4 DNA	Pouwels, Veldhuisen, Jansz & Cohen (1963)
E. coli spheroplasts	T1 DNA	Evans, Mackal & Coleman (1962)
E. coli spheroplasts	λ DNA	Meyer, Mackal, Tao & Evans (1961)
E. coli spheroplasts	ϕX174 & ϕ7 DNA	Iliashenko (1964)
E. coli spheroplasts	M12 RNA & M13 DNA	Benzinger, Delius, Jaenisch & Hofschneider (1968)
E. coli spheroplasts	R17 RNA	Paranchych & Graham (1962)
E. coli cells + Ca^{++}	P2 & λ DNA	Mandel & Higa (1970)
E. coli spheroplasts	ft5 RNA	Knolle & Kaudewitz (1962)
E. coli spheroplasts	λ & T7 DNA	Iliashenko, Dityatkin & Danileichenko (1968)
E. coli spheroplasts	P22 DNA	Benzinger & Kleber (1971)
B. subtilis cells	SPP1 DNA	Riva & Polsinelli (1968)
B. subtilis cells	ϕ29 DNA	Bott & Wilson (1967)
B. subtilis cells	SP3 DNA	Romig (1962)
B. subtilis cells	Polyoma virus DNA	Bayreuther & Romig (1964)
B. subtilis cells	Vaccinia virus DNA	Abel & Trautner (1964)
B. subtilis cells	SP50 DNA	Földes & Trautner (1964)
B. subtilis cells	SP8 DNA	Bayreuther & Romig (1964)
B. subtilis cells	SP82G DNA	Green (1964)
B. subtilis cells	SP01 DNA	Okubo, Strauss & Stodolsky (1964)
B. subtilis cells	ϕ1, ϕ25 & ϕ29 DNA	Reilly & Spizizen (1965)
B. subtilis spheroplasts	SP50 DNA	Tichy & Landman (1969)

Table 4-1 (cont.)

Recipient	Nucleic acid	Reference
Staphylococcus aureus spheroplasts	44AHJD & 53 DNA	Riggs & Rosenblum (1969)
S. aureus cells	80α DNA	Sjöström, Lindberg & Philipson (1972)
Proteus mirabilis spheroplasts	13vir DNA	Van Rensburg (1969)
P. morganii spheroplasts	13vir DNA	Van Rensburg (1969)
P. vulgaris spheroplasts	13vir DNA	Van Rensburg (1969)
B. stearothermophilus cells	TP-1C DNA	Streips & Welker (1969)
Aerobacter aerogenes	Phage DNA	Spizizen (1957)
Streptomyces kanamyceticus	PK-66 DNA	Okanishi, Utahara & Okami (1966)
Providence spheroplasts	PL25 DNA	Pitout & Van Rensburg (1969)
Haemophilus influenzae cells	HP1c1 DNA	Harm & Rupert (1963)
Shigella spheroplasts	φX174 DNA	Iliashenko (1964)
Mycobacterium smegmatis cells	D29 DNA	Tokunaga & Sellers (1964)
Acholeplasma laidlawii cells	Viral DNA	Liss & Maniloff (1972)
Salmonella typhimurium spheroplasts	P22 DNA	Benzinger & Kleber (1971)
Streptococci cells	Phage DNA	Parsons & Cole (1973)
Agrobacterium tumefaciens cells	LR-4 DNA	Milani & Heberlein (1972)
Achromobacter sp. 2 spheroplasts	α3 DNA	Thomson & Woods (1973)



Cancer risk in water

CHICAGO. — Traces of cancer-causing chemicals have been found in the drinking water of most of 83 mid-western United States cities, according to a study by the Environmental Protection Agency.

The study of drinking water in Illinois, Indiana, Michigan, Minnesota, Ohio and Wisconsin. Also determined that cities that take their drinking water from heavily polluted rivers contained the greatest amount of organic chemicals in their water supplies.

The EPA scientists said some mid-western communities had large amounts of chloroform in their drinking water.

The chloroform, one chemical being tested by the National Cancer Institute as a cause of cancer, is produced by chlorinating drinking water to help kill bacteria.

METHODS

paration of penicillin spheroplasts. Achromobacter sp. 2 was rnight in nutrient broth with aeration. Cells were diluted spheroplasting medium containing penicillin (500 to 5000 units/ml.) ted at 37 °C. Samples were removed at intervals, examined micro- ly and counted on a haemocytometer.

paration of lysozyme-EDTA spheroplasts. Overnight broth cultures ntrifuged, washed twice in 0.1 M tris buffer (pH 8.0) and re- d at 10^9/ml. in the lysozyme spheroplasting medium. To 1 ml. s was added 0.1 ml. lysozyme at concentrations of 1 mg./ml., 1. and 16 mg./ml. and 1% EDTA (0.2 ml., 0.3 ml. and 0.5 ml. ively). The mixture was swirled gently for 2 min., incubated at 37 °C and examined at intervals.

Preparation of glycine spheroplasts. Overnight broth cultures were diluted 1:10 in nutrient broth supplemented with 0.5 M sucrose, 0.2% $MgSO_4.7H_2O$ and 2% glycine. Cultures were aerated at 30 °C and 37 °C and examined microscopically at intervals.

Spheroplast photography. A drop of spheroplast culture was mounted on a microscope slide in 2% aqueous methyl cellulose containing a drop of Gram's saffranin. Spheroplast development was observed under a light microscope.

Osmotic sensitivity of spheroplasts. Duplicate 10 ml. samples of spheroplasts were collected by centrifugation at 2000 g for 20 min. The pellets were resuspended in 10 ml. volumes 0.5 M sucrose and

deionized water. Extinction at 660 nm. was monitored in an EEL colorimeter (Evans Electroselenium Ltd.) for 15 min. Controls were bacteria in 0.5 M sucrose and deionized water.

Spheroplast stabilization. Spheroplasts collected as above were resuspended in equal volumes of 0.5 M sucrose, deionized water, $CaCl_2$ (10^{-2}M), $MgCl_2$ (10^{-2}M), NaCl (10^{-2}M, 0.4 M), spermidine (10^{-2}M, 10^{-4}M, 10^{-5}M) nutrient broth and transfection medium. Extinction at 660 nm. was measured after 10 min.

Phage techniques. Phage lysates and high titres of phage were prepared as described on p. 24. Phage was purified as for electron microscopy (p. 24) and resuspended in standard SC buffer pH 7.0.

Phage growth in spheroplasts. After incubation with penicillin for 5 hr. a spheroplast culture was diluted 1:10 in spheroplast medium containing 1000 units penicillin/ml. Phage was added at a titre of 10^4 p.f.u./ml. to spheroplasts and bacterial cultures (10^7/ml.) and incubated at 37°C. Samples were removed at 10 min. intervals and diluted in 0.4 M NaCl. Samples (0.1 ml.) were added to 0.1 ml. volumes of indicator bacteria in 2.5 ml. of top-layer agar at 45°C prior to pouring on salt agar plates. Plaques were counted after incubation for 24 hr.

Preparation of phage α3a DNA. The method described on p. 26 was used.

Transfection. Competence for transfection was determined by inoculating 10 ml. of an overnight culture into 100 ml. of nutrient broth followed by incubation at 37°C with aeration. At 30 min. intervals 3 ml. samples of bacteria were removed and converted to penicillin

spheroplasts. After 5 hr., aeration of the spheroplasts was stopped and after a further 7 hr. of incubation they were centrifuged at 2000 g for 15 min. and resuspended in transfection medium (1×10^8/ml.). To 0.5 ml. volumes of spheroplast suspension, phage DNA was added to a final concentration of 20 μg./ml. The mixture was incubated for 2 hr. at 37 °C and assayed for infective centres. Dilutions for the assay were made in transfection medium to prevent lysis of spheroplasts. For all further transfection experiments, bacteria were incubated for 120 min. prior to conversion to spheroplasts. In control experiments, spheroplasts and DNA alone were each assayed for infective centres. The effect of pre-treatment of DNA with 1 μg./ml. DNAse, 0.1 μg./ml. pronase and 0.1 μg./ml. trypsin on transfection was determined.

RESULTS

Penicillin spheroplasts

Incubation with penicillin caused the rod-shaped cells to swell terminally forming pear-shaped bodies (Fig. 4-1). After 5 hr. of incubation these intermediate forms were converted to large, round spheroplasts. One hundred per cent conversion to spheroplasts was obtained with 500 to 1000 units penicillin/ml. but higher concentrations produced aberrant forms. Most of the aberrant forms were very long rod-shaped bodies. Spheroplasts were not produced by treatment with lysozyme + EDTA in tris buffer, nor with glycine (2%) even after overnight incubation at 37 °C.

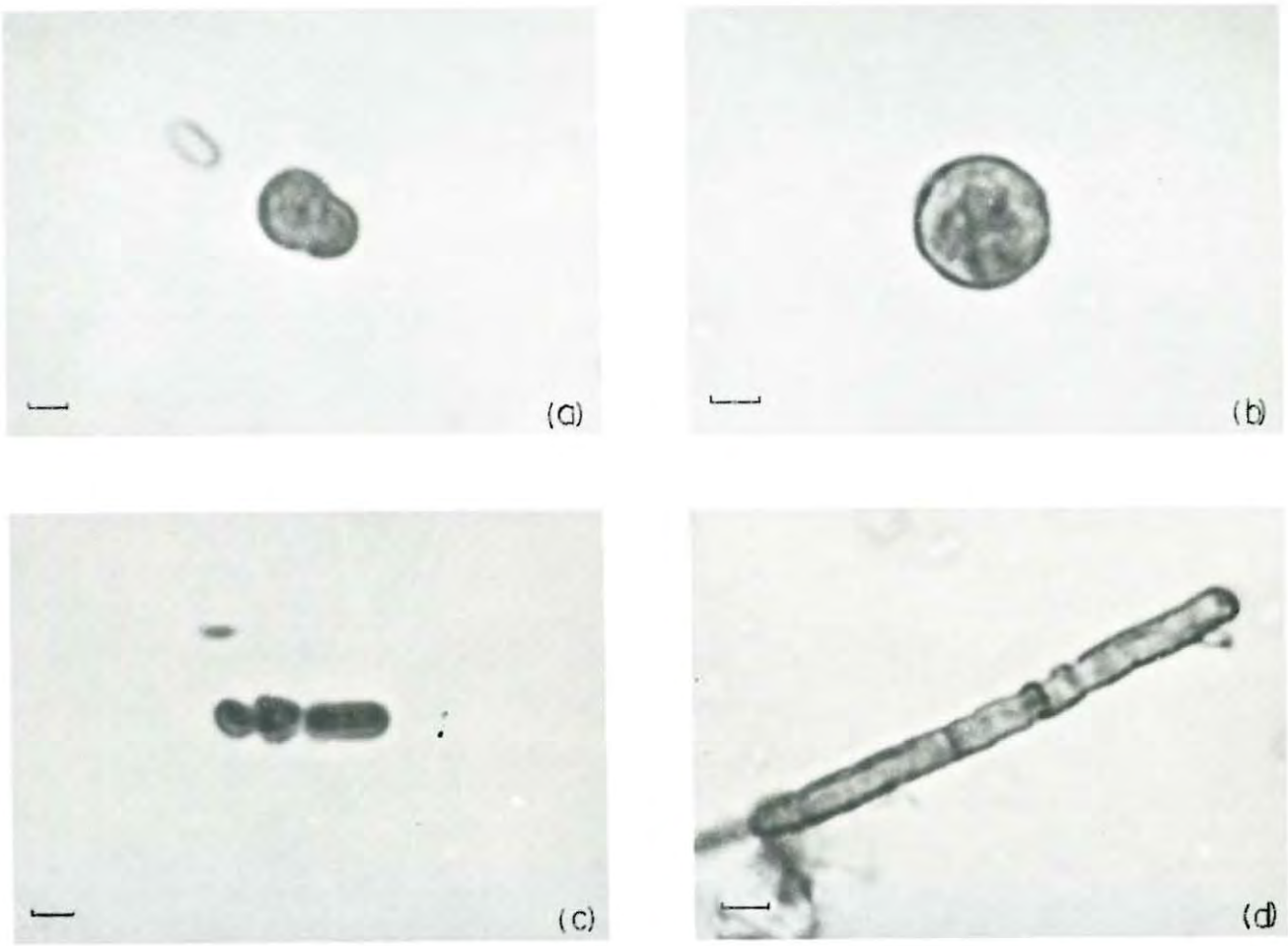


Fig. 4–1. Penicillin spheroplasts of Achromobacter sp. 2. (a) Intermediate pear-shaped body formed during conversion to spheroplasts. (b) Spheroplast formed after 5 hr. of incubation with 1 000 units penicillin/ml. (c) and (d) Aberrant forms. Scale bar represents 1 μm.

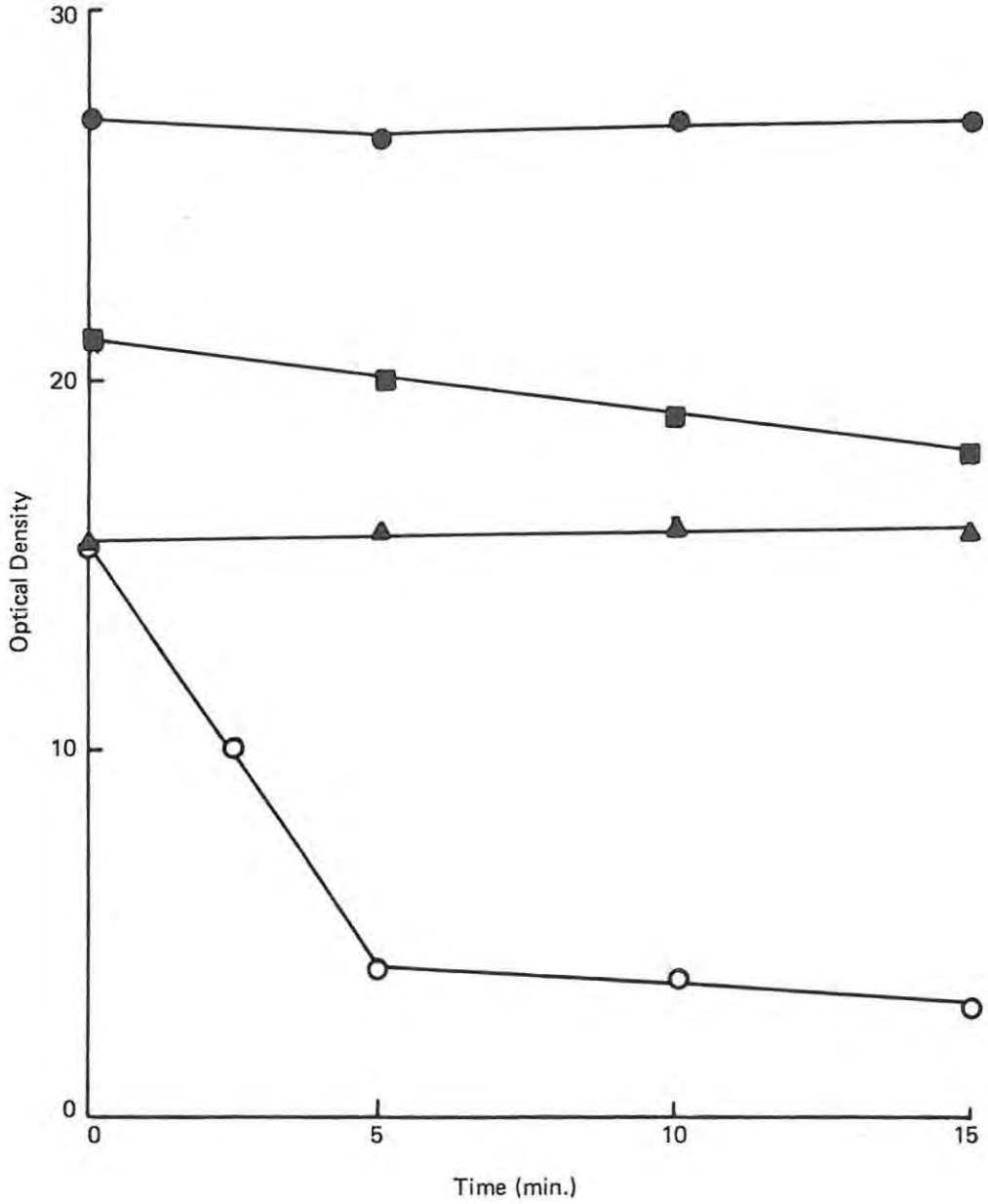


Fig. 4–2. Osmotic sensitivity of Achromobacter sp. 2 cells and spheroplasts. ●—● , cells in 0.5M sucrose; ■—■ , cells in deionised water; ▲—▲ , spheroplasts in 0.5M sucrose; ○—○ , spheroplasts in deionised water.

Osmotic sensitivity of spheroplasts

Spheroplasts were stabilized by 0.5 M sucrose but lysed in water (Fig. 4-2), a characteristic of all spheroplasts. Achromobacter cells were stable in 0.5 M sucrose but showed a gradual decrease in extinction in water.

Stability of spheroplasts

Spheroplasts were stabilized when suspended in transfection medium, nutrient broth + 0.4 M NaCl, sucrose (0.5 M) and $CaCl_2$ (10^{-2} M). NaCl (0.4 M, 10^{-2} M) and $MgCl_2$ (10^{-2} M) provided little support (Table 4-1). Spermidine did not stabilize spheroplasts and suspension in water caused rapid lysis.

Table 4-1. Stability of spheroplasts

Suspending medium	Extinction at 660 nm.
Spheroplast medium	25
Transfection medium	24
Nutrient broth + 0.4 M NaCl	21
Sucrose, 0.5 M	20
$CaCl_2$, 10^{-2} M	19
NaCl, 0.4 M	15
$MgCl_2$, 10^{-2} M	12
NaCl, 10^{-2} M	8
Spermidine, 10^{-2} M	8
Spermidine, 10^{-4} M	7
Spermidine, 10^{-5} M	7
Deionized water	4

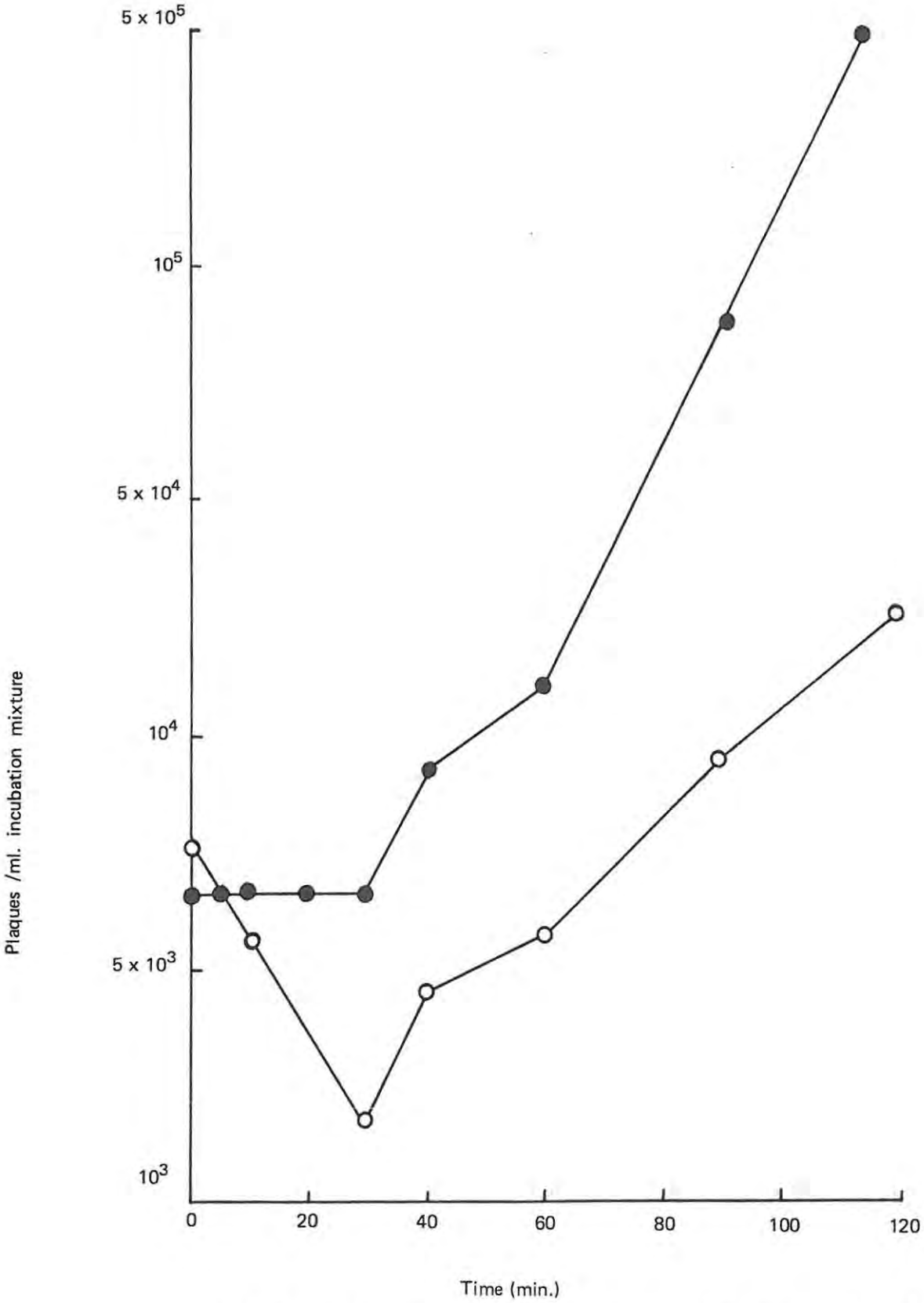


Fig. 4–3. Phage ∝ 3a multiplication in Achromobacter sp. 2 spheroplasts (O—O) and cells (●—●).

Multiplication of bacteriophages in spheroplasts and bacteria

Immediately after phages were added to spheroplasts there was a decrease in phage titre (Fig. 4-3). No decrease was observed when phages were added to bacteria. The decrease obtained with spheroplasts was presumably due to the release of non-infective vegetative phages by premature lysis of the spheroplasts which were removed from the stabilizing medium and diluted in 0.4 M NaCl prior to plating on salt agar (Hayes, 1968).

Transfection

Spheroplasts prepared immediately after dilution of cells into nutrient broth were resistant to infection with α3a DNA. Optimal competence for transfection was obtained when bacteria were incubated in nutrient broth for 120 min. before conversion to spheroplasts (Fig. 4-4). The state of competence was transient and there was a rapid fall-off in the ability of spheroplasts to be transfected after the optimal time of 120 min. Incubation of cells in minimal medium prior to conversion to spheroplasts did not result in the development of competence. Thus a step-down medium is not required to render cells competent for DNA uptake.

Over the concentration range 0 to 50 μg. DNA/ml. a linear relationship between DNA concentration and infective centres was obtained (Fig. 4-5). Length of incubation of competent spheroplasts with DNA affected the phage titre obtained (Fig. 4-6). The optimal time of incubation was 60 min.

Pretreatment of α3a DNA with pronase and trypsin did not affect transfection, but infectivity of the DNA was destroyed by incubation with DNAse for 30 min. at 37 °C.

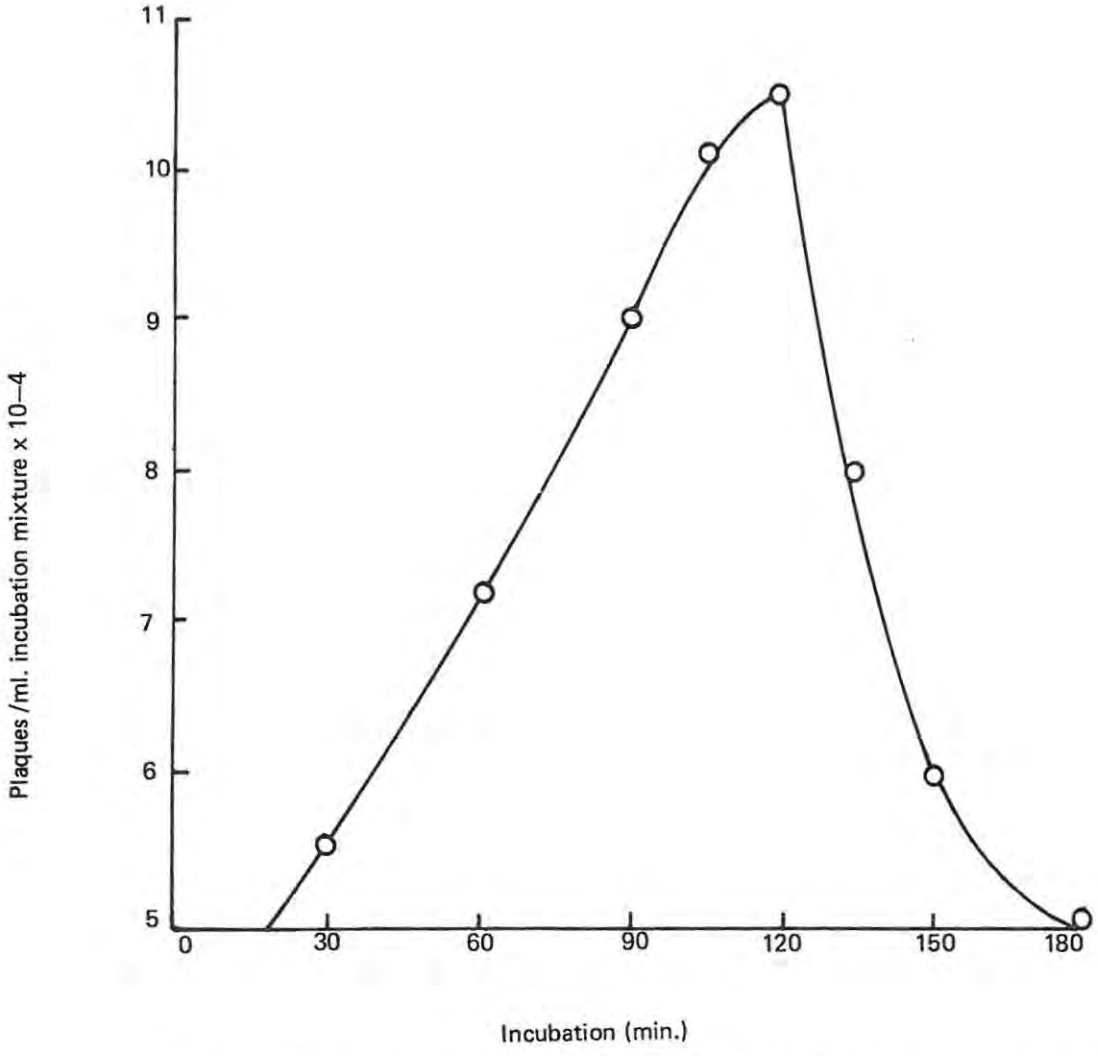


Fig. 4–4. Development of competence for transfection in Achromobacter sp. 2 spheroplasts. Concentrations of spheroplasts (1 x 10^8/ml.) and ∝3a DNA (20 μg/ml.) and time of incubation of spheroplasts with DNA (60 min.) were kept constant. Abscissa represents time of incubation of bacteria in nutrient broth before conversion to spheroplasts.

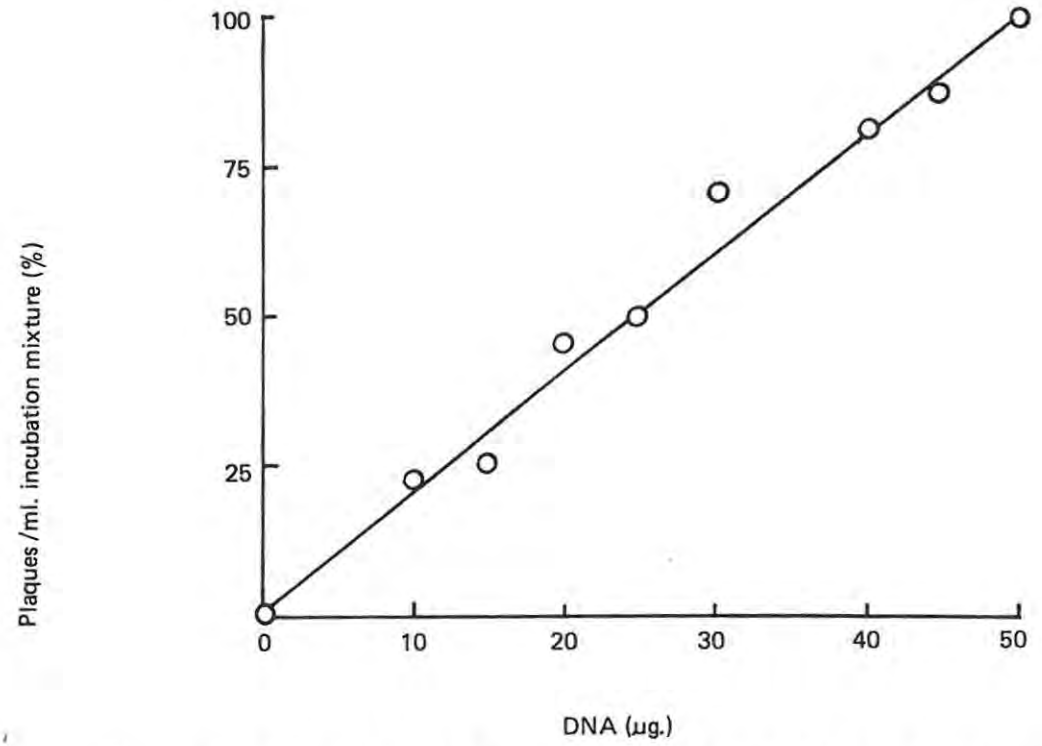


Fig. 4–5. Relationship between concentration of ∝3a phage DNA added and infective units produced after 90 min. incubation.

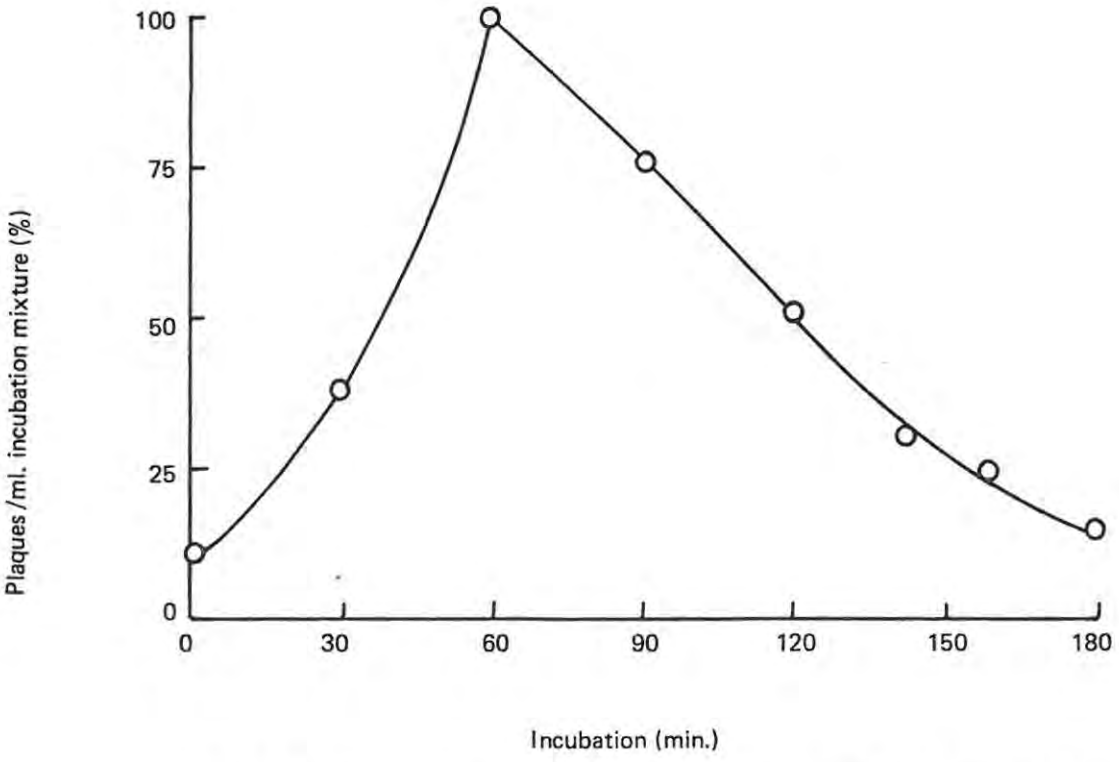


Fig. 4–6. Release of phage by spheroplasts treated with ∝ 3a phage DNA at 20 μg/ml. Bacteria were grown in nutrient broth for 120 min. before conversion to spheroplasts. Abscissa represents time of incubation of spheroplasts with phage DNA.

DISCUSSION

The spherical bodies produced by penicillin treatment correspond to spheroplasts on grounds of morphology, osmotic sensitivity and phage infection laid down by Brenner et al. (1958). Stages in the development of penicillin spheroplasts were similar to those reported by McQuillen (1960) for Bacillus megaterium and by Lederberg (1956) for E. coli. The aberrant forms produced by penicillin concentrations higher than 1000 units/ml. appear to be due to end-to-end adhesion of cells with partial or total degradation of adjacent cell walls. Highton & Hobbs (1971) showed that penicillin inhibited the separation of Bacillus licheniformis cells after cross wall formation. As a result long chains of cells grew whose appearance were similar to those in Fig. 4-1 (d).

Although glycine is thought to inhibit cell wall synthesis in the same way as penicillin (Park, 1968), glycine spheroplasts of Achromobacter sp. 2 could not be formed. This may reflect a slight difference in the activity of the two agents.

Phage multiplication in spheroplasts indicates the retention of receptor sites for bacteriophages. As these sites are contained in the lipoprotein and lipopolysaccharide layers (Oram & Reiter, 1968) this confirms that penicillin affects only the mucopeptide layer (Park & Strominger, 1957).

The initial rapid increase in phage titre (Fig. 4-6) suggests the rapid uptake of DNA by spheroplasts. The decrease in effective transfection after 60 min. may be due to the α3a DNA being partially degraded by host nucleases. Infective phage DNA appears to be subjected to

nuclease attack in a number of transfection systems (Green, 1966; Epstein, 1968) and the time of incubation of competent spheroplasts with phage DNA is therefore critical. When the effect of increasing α3a DNA concentration on the efficiency of transfection was determined after 60-90 min. incubation of spheroplasts with DNA the relationship was linear. This was no longer obtained when incubation was for longer periods. Thus in the presence of nuclease attack on the DNA more than one infective molecule is required for the formation of a plaque forming unit.

That the infective agent was DNA was shown by the abolition of transfection by DNAse but not by pronase or trypsin. Conversion of bacteria to spheroplasts results in the fixation of the competent state (Iliashenko, Dityatkin & Danileichenko, 1968). As competence for transfection can be associated with competence for transformation (Bott & Wilson, 1967), it was decided to use competent spheroplasts of Achromobacter sp. 2 to investigate transformation in this strain.

STUDIES ON TRANSFORMATION IN ACHROMOBACTER SP. 2

SUMMARY

A method of DNA extraction was devised which gave a high yield of purified, undegraded DNA from Achromobacter sp. 2. Electron micrographs of the DNA were taken and the G+C (%) determined. Despite the use of various techniques no transformation of Achromobacter was achieved.

INTRODUCTION

Transformation of bacterial cells occurs when genetically marked donor DNA is taken up and integrated into the recipient genome conferring the genetic marker on the recipient. This process has been reported in several genera and can be used in the mapping of bacterial genomes although linkages obtained may be rather unstable (Kelly & Pritchard, 1965). The ability of recipient bacteria to be transformed depends on their physiological state (McCarty, Taylor & Avery, 1946). Transformable cells are called "competent" and the state of competence is transitory. In Bacillus subtilis 0.1 to 0.5% of a population were found to be competent while in Haemophilus influenzae 5% were competent (Nester & Stocker, 1963).

The conditions under which bacteria develop competence vary between organisms (see Spizizen, Reilly & Evans, 1966) and the efficiency of transformation also depends on the efficiency of DNA integration and expression (Vermeulen & Venema, 1971). Some transformation systems require cells to be grown in a "step-down" medium for the development

of competence (Spizizen & Prestidge, 1969) and competent cells are marked by a general biosynthetic latency (Nester & Stocker, 1963). Competence appears to occur under conditions of high internal concentrations of cyclic AMP e.g. in late log. to early stationary phase (Wise, Alexander & Powers, 1973).

DNA greater than a minimal molucular weight is required for transformation (Litt, Marmur, Ephrussi-Taylor & Doty, 1958) and there is a linear progression of DNA markers through the cell wall (Strauss, 1965). B. subtilis appears to have 20-53 DNA entry sites per cell (Singh, 1972).

Extracellular competence factors whose synthesis is required for the development of competence have been found in H. influenzae (Barnhardt, 1967), pneumococci (Kohoutová, Braná & Holubová, 1968), B. stearothermophilus (Streips & Welker, 1971a) and streptococci (Leonard & Cole, 1972). There appears to be some controversy as to whether the factor which stimulates competence development in B. subtilis (Akrigg & Ayad, 1969) is a true extracellular competence factor (Goldsmith, Havas & Kallenbach, 1970; Joenje, Gruber & Venema, 1972).

As Achromobacter sp. 2 could be transfected (Thomson & Woods, 1973) it was decided to use spheroplasts competent for transfection in an attempt to develop a transformation system. Transformation would then be investigated in intact cells.

METHODS

Isolation of drug resistant mutants. Overnight broth cultures of Achromobacter sp. 2 were resuspended in 1/5 volume 0.4 M NaCl and plated on gradient plates containing 1,000 μg. streptomycin/ml. or 30 μg. nalidixic acid/ml. Resistant colonies were streaked onto plates containing the maximal drug concentration.

Isolation of auxotrophic mutants. Log. phase broth cultures of Achromobacter sp. 2 were resuspended in fresh pre-warmed nutrient broth and NG added to a final concentration of 45 μg./ml. After 15 min. incubation at 37°C the cells were washed twice in 0.4 M NaCl, diluted 1:10 in nutrient broth and incubated overnight. Dilutions were plated on slightly enriched minimal agar and incubated at 37°C. Colonies were stabbed onto salt agar and after incubation were replicated onto minimal agar. Colonies which were unable to grow on minimal agar were transferred from the salt agar onto minimal agar supplemented with different amino acids. Auxotrophs were retested for growth on minimal agar.

Extraction of bacterial DNA.

1) Chloroform-isoamyl alcohol method. A modification of the method of Marmur (1961) was used. Cells from an overnight broth culture were harvested by centrifugation at 2,000 g, washed in SC and resuspended in 40 ml. SC. Lysis was effected by the addition of 2 ml. 25% sodium dodecyl sulphate (SDS), the mixture incubated at 50°C for 15 min. and cooled to room temperature. Sodium perchlorate was added to a final concentration of 1 M. To the viscous lysate an equal volume of chloroform-isoamyl alcohol (24:1 v/v) chilled to 4°C was added and the mixture rotated on a tilted turntable at 45 r.p.m. for 30 min. The milky solution was centrifuged at 2,000 g for 10 min. causing separation into two layers.

The bottom layer was removed with a Pasteur pipette and the chloroform-isoamyl alcohol treatment repeated on the aqueous layer until it was clear and there was no protein at the interface. The nucleic acids were precipitated by gently layering 2 vol. chilled 95% ethyl alcohol on the aqueous phase. These layers were gently mixed with a glass rod causing the nucleic acids to "spool" onto the rod. The precipitate was dissolved in dilute SC. The ethyl alcohol mixture was subjected to a 15 min. centrifugation at 2,000 g, the sediment of nucleic acids dissolved in dilute SC and added to the previously obtained nucleic acid solution. RNA was removed by incubation for 30 min. at 37°C with RNAse at a final concentration of 50 μg./ml. The RNAse was first incubated at 80°C and pH 5.0 for 10 min. to destroy any DNAse activity which might have been present in the enzyme preparation. DNA was precipitated by the addition of 2 vol. chilled isopropanol and the precipitate collected by spooling and centrifugation, and dissolved in dilute SC. If the DNA solution was not clear a final chloroform-isoamyl alcohol precipitation was performed. The salt concentration was increased to SC with concentrated SC and the DNA solution stored over chloroform at 4°C.

2) Phenol method. An overnight broth culture was centrifuged and the cells washed three times with SC. Cells were resuspended in SC at a concentration of 10^9 cells/ml. Pronase (1 mg./ml.) and SDS (0.1%) were added to the suspension which was incubated for 4 hr. at 37°C to facilitate lysis. An equal volume of freshly prepared water-saturated phenol was added to the lysate and the mixture rotated on a tilted turntable at 45 r.p.m. for 30 min. The mixture was cooled to 4°C and centrifuged at 2,000 g for 15 min. to separate the two layers. The bottom layer was removed with a Pasteur pipette and the phenol extraction repeated on the aqueous layer until no protein was seen at the interface.

The final aqueous layer was dialysed for 48 hr. at 4 °C against three changes of 3 ℓ. SC. Nucleic acid precipitation, RNAse treatment and DNA precipitation were carried out as above. The dissolved DNA was dialysed for 24 hr. at 4 °C against 3 ℓ. SC. DNA was stored over chloroform at 4 °C.

3) Pronase method. A modification of the method of Massie & Zimm (1965) was used. Stationary phase cells (400 ml.) were harvested as above and resuspended in 40 ml. NaCl-EDTA. SDS was added to a final concentration of 1% and the mixture incubated at 37 °C for 2 hr. A 10 mg./ml. solution of pronase was prepared and the pH adjusted to 5.0 with 1 N HCl. The solution was heated to 80 °C for 10 min. to denature any DNAse present and allowed to cool. The pH was readjusted to 7.0 with 1 N NaOH and NaCl added to a concentration of 1 M (Hotta & Bassesl, 1965). Pronase was added to the lysate for a final concentration of 1 mg./ml. and the mixture poured into a dialysis bag. This was suspended in buffer (NaCl-EDTA pH 8.0) in a water bath at 50 °C. The mouth of the dialysis bag was held open with a glass tube and the pronase replenished at 1 mg./ml. every 8 hr. The buffer was changed every 12 hr. and dialysis continued for 48 hr. The protein was precipitated by the addition of an equal volume of chloroform-isoamyl alcohol as above. The aqueous layer was dialysed again for 3 hr. RNAse was added to the dialysis bag to give a final concentration of 100 μg./ml. and dialysis continued for 12 hr. with one change of buffer. The solution was chilled and two vol. chilled isopropanol layered on. The DNA was spooled onto a glass rod and dissolved as above.

Lysis by SDS. To determine the optimal growth phase of cells for maximal lysis by SDS, 100 ml. nutrient broth was inoculated with a loopful of bacteria and incubated with aeration. At intervals samples

were removed and the optical density in an EEL colorimeter measured. SDS was added at a final concentration of 1%, the mixture incubated and the decrease in optical density measured over a period of 2-3 hr.

DNA concentration. This was determined by the diphenylamine reaction (p. 27) and ultraviolet absorption at 260 nm. (μg. DNA = 0.0205 absorbency units) in a Unicam spectrophotometer. The equation $\mu g.\ DNA/ml. = \frac{A_{254\ nm.} + 0.0048}{0.021}$ was also used to calculate the concentration of DNA.

Presence of RNA and protein in the DNA preparations. The concentration of RNA was determined by the orcinol reaction. One ml. of nucleic acid solution was added to 2 ml. acid orcinol and 0.1 ml. alcohol orcinol. The mixture was placed in a boiling water bath for 20 min. and held in an ice bucket with agitation for 5 min. The optical density at 660 nm. was compared with values obtained from standard solutions of RNA and a blank containing SC and the reagents. Protein was determined by the Lowry procedure (Lowry, Rosebrough, Farr & Rabdall, 1951). One ml. solution was mixed with 3 ml. 2 M tris buffer. One ml. of diluted (1:40) alkaline copper solution was added. After 10 min. 1 ml. Folin-Ciolcateu reagent was added. A blue colour indicated the presence of protein.

Transformation of spheroplasts. Spheroplasts (10^8/ml.), competent for transfection (p. 79) were incubated with 25 μg./ml. pronase-extracted DNA from Achromobacter sp. 2 resistant to streptomycin and nalidixic acid in transfection medium containing 10 ml. $CaCl_2$/ml. After incubation for time intervals of 30 min. to 2 hr. DNAse (2 μg./ml.) was added and incubation continued for 15 min. The mixture was plated on salt agar

containing either streptomycin (25 μg./ml.) or nalidixic acid (30 μg./ml. and incubated for 48 hr. The percentage of spheroplasts which reverted to cells when plated was determined by plating serial dilutions of a known concentration of spheroplasts on salt agar. Experimental controls consisted of spheroplasts incubated without the addition of DNA. DNA was examined for sterility by plating 0.1 ml. aliquots on salt agar.

Transformation of intact cells. Overnight 10 ml. cultures were centrifuged at 2,000 g for 10 min., washed and resuspended in 100 ml. of the following media:

(i) Achromobacter minimal medium

(ii) Achromobacter minimal medium supplemented with 0.5% casein hydrolysate and 0.13% lactose

(iii) Nutrient broth with and without 10^{-2} M $CaCl_2$

(iv) Nutrient broth containing 10^{-2} M $CaCl_2$ and 2×10^{-4} M EDTA

(v) Glucose broth

(vi) E. coli minimal medium with and without 2×10^{-4} M EDTA

(vii) B. subtilis minimal medium

(viii) Alcaligenes minimal medium

(ix) Pasteurella minimal medium

(x) Tryptone broth with and without 10^{-2} M $CaCl_2$

Aliquots (1 ml.) were removed at half-hourly intervals for 5 hr. and incubated with 10-100 μg. DNA/ml. for 30-90 min. with and without aeration. The reaction was terminated by the addition of DNAse (2 μg./ml.) and the mixture incubated for 15 min. Cells were plated on salt agar with and without streptomycin and nalidixic acid for transformation of drug resistance and on Achromobacter minimal agar for transformation of prototrophy. Plates were incubated for 48 hr. Controls were cells incubated without added DNA. Sterility of DNA was determined by plating 0.1 ml. aliquots on salt agar.

Determination of base composition of DNA. This was calculated from the thermal denaturation temperature (Tm) as on p. 27. Pronase-extracted DNA was used.

Electron microscopy of DNA. The method described by Van Rensburg (1970) was used. Pronase-extracted DNA was diluted to a final concentration of 10 μg./ml. in 1 M ammonium acetate. An equal volume of 0.04% cytochrome c in 1 M ammonium acetate was added to the DNA. A steel trough (80 cm. × 10 cm. × 1 cm.) was filled with 0.1 M ammonium acetate in deionized water. The surface was cleaned with a glass spreader. A clean wet glass ramp was inserted in the ammonium acetate solution and 0.4 ml. of the DNA-cytochrome c was allowed to run down the ramp to form a surface layer on the ammonium acetate. The movement of DNA was followed by sprinkling a trace of talcum powder slightly ahead of the DNA-protein film. After 10 min. the surface film was touched with a carbon-coated electron microscope grid. The water droplet adhering to the grid was removed by touching the specimen to the surface of absolute alcohol for 30 sec. The specimen was shadowed by small angle (8°) deposition of 40% gold-palladium from two directions by means of a rotating device. The gold-palladium was vapourized by heating on tungsten wire in a Hitachi Vacuum Evaporator HUS-3B apparatus. The grids were examined under a Hitachi BU electron microscope.

RESULTS

Isolation of mutants

Mutants resistant to 1000 μg. streptomycin/ml. and 30 μg. nalidixic acid/ml. and a trp auxotroph were isolated.

Extraction of bacterial DNA

Difficulties were encountered in separating protein from nucleic acids by the chloroform-isoamyl alcohol and phenol methods. Diphenylamine reactions carried out during the procedures showed that DNA was being precipitated together with the protein. Marmur (1961) reported that if the concentration of cells being lysed was too high lumps of denatured protein and occluded DNA formed a voluminous middle layer during the DNA extraction procedure. However, even when more dilute concentrations of cells were used DNA continued to be precipitated with protein. This could not have been due to the time of cell lysis with SDS as the efficiency of lysis was independent of the age of the culture (Fig. 5-1). This is in contrast to results published by Adachi, Nakano, Inuzuka and Tomoeda (1972) who showed an age dependence for lysis of E. coli. The final yield by the above methods was less than 100 μg. Moreover, the DNA was denatured as heating did not cause a hyperchromic shift at 260 nm.

The pronase method proved highly efficient yielding a total of 1 - 1.4 mg. DNA. No protein could be detected with the Lowry procedure but the preparations were usually contaminated with 10 - 15% RNA. This could not be removed even by prolonged treatment with RNAse.

Ultraviolet absorption spectrum

The clear solutions of DNA obtained by the pronase method showed maximal absorbance at 258 nm. and a trough at 232 nm. (Fig. 5-2). The ratios of A_{258}/A_{232} and A_{258}/A_{280} were 1.5 and 1.96. These ratios are criteria for the purity of the DNA.

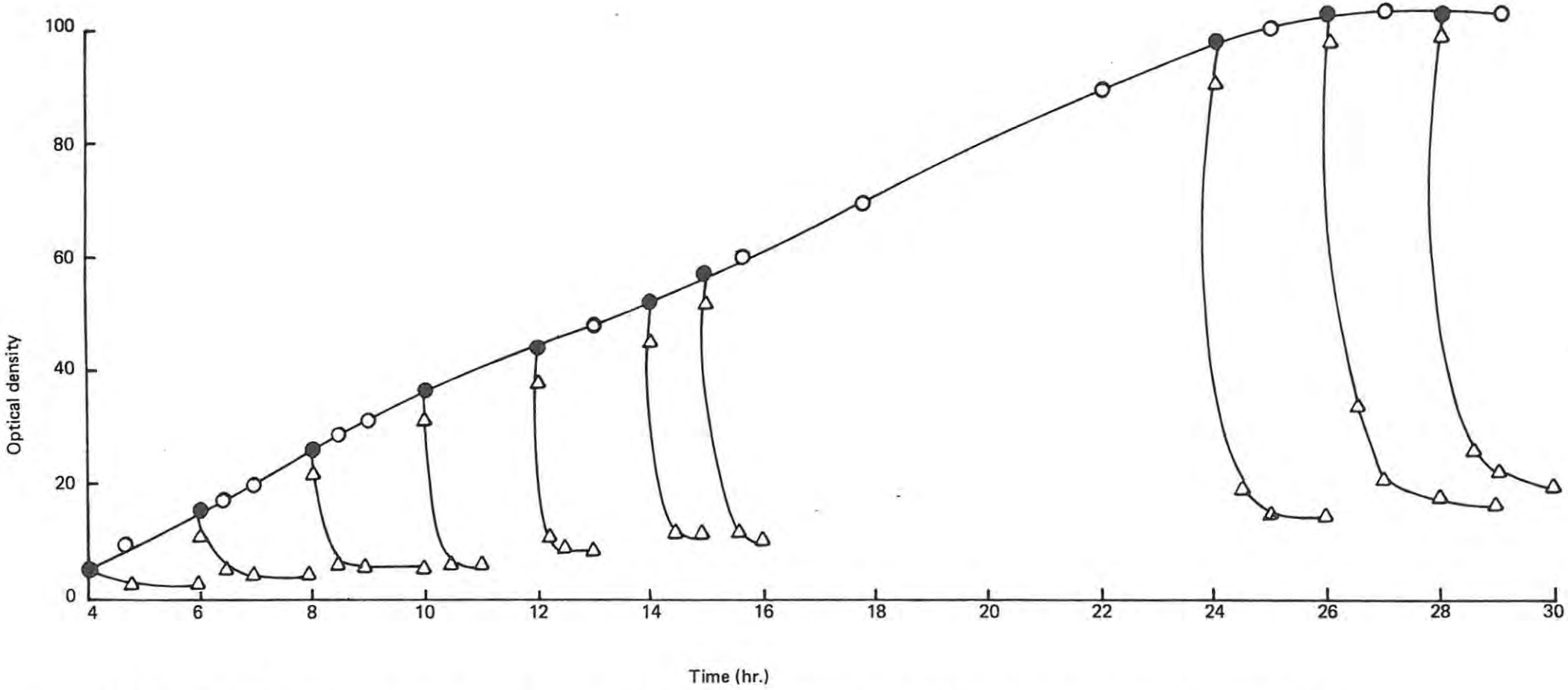


Fig. 5–1. Lysis of Achromobacter sp. 2 by sodium dodecyl sulphate. ○—○ , Achromobacter sp. 2 growth curve; —●— , addition of SDS; △—△ , SDS-induced lysis.

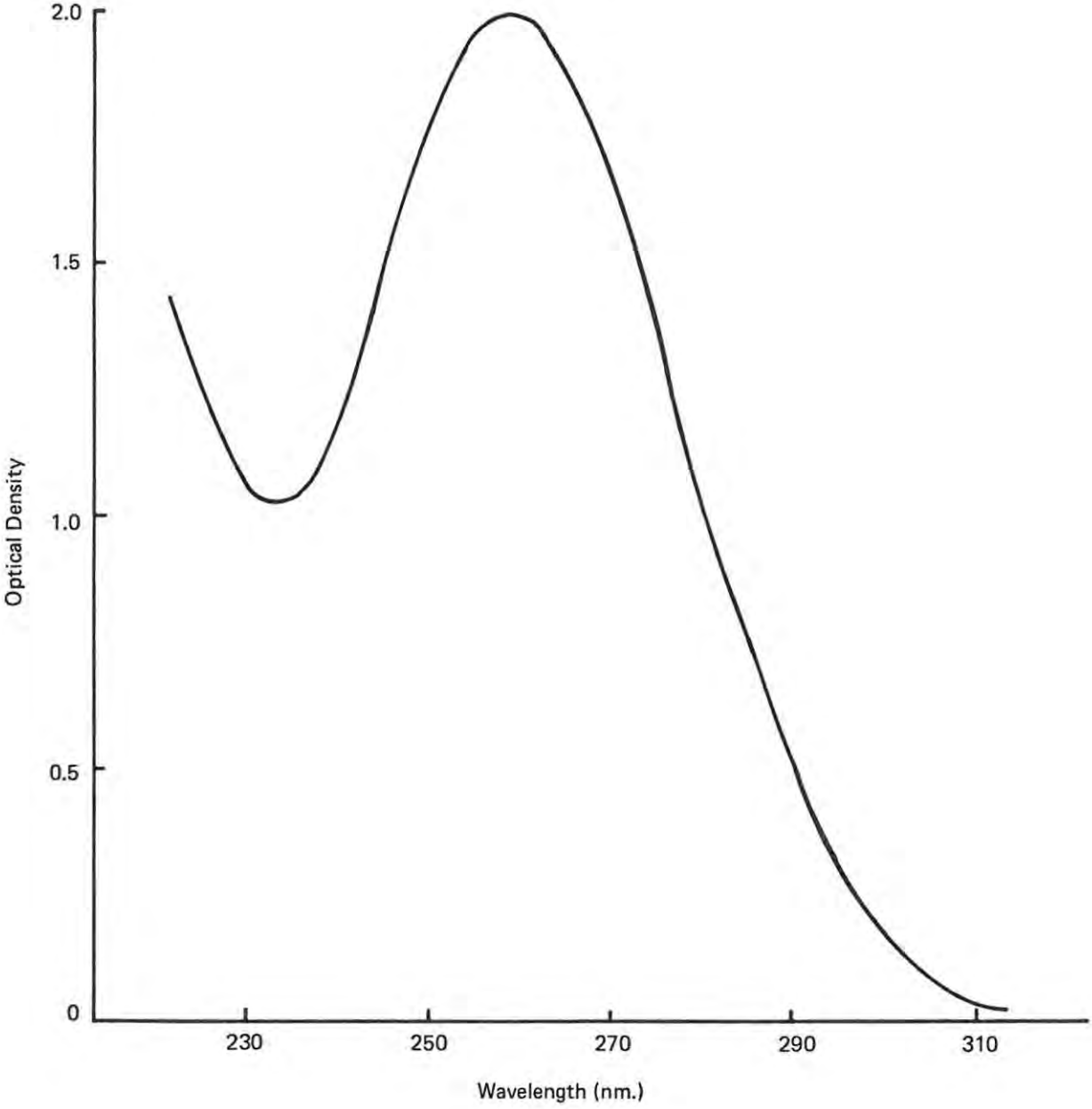


Fig. 5–2. Ultraviolet absorption spectrum of Achromobacter sp. 2 DNA. Maximal absorbance was at 258 nm. and a trough occurred at 232 nm.

Base composition of DNA

The melting curve of the DNA is shown in Fig. 5-3. The hyperchromic shift proves it to be double-stranded. Tm was 85.7 °C which corresponds to a molar guanine + cytosine composition of 40.0%.

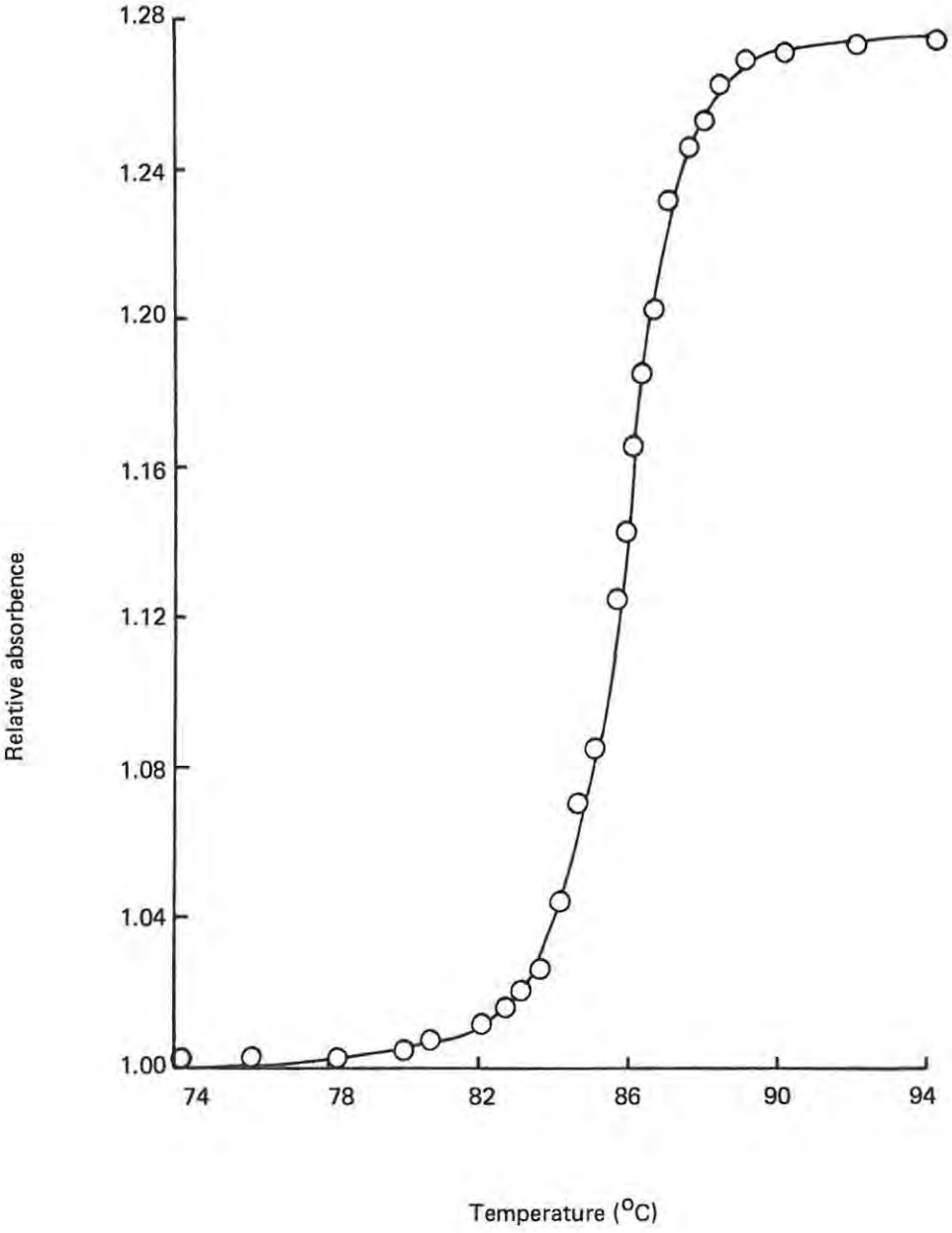


Fig. 5–3. Thermal denaturation of Achromobacter sp. 2 DNA.

Transformation

It was not possible to transform Achromobacter sp. 2 by any of the methods used.

Electron microscopy of DNA

An electron micrograph of the DNA is shown in Fig. 5-4.

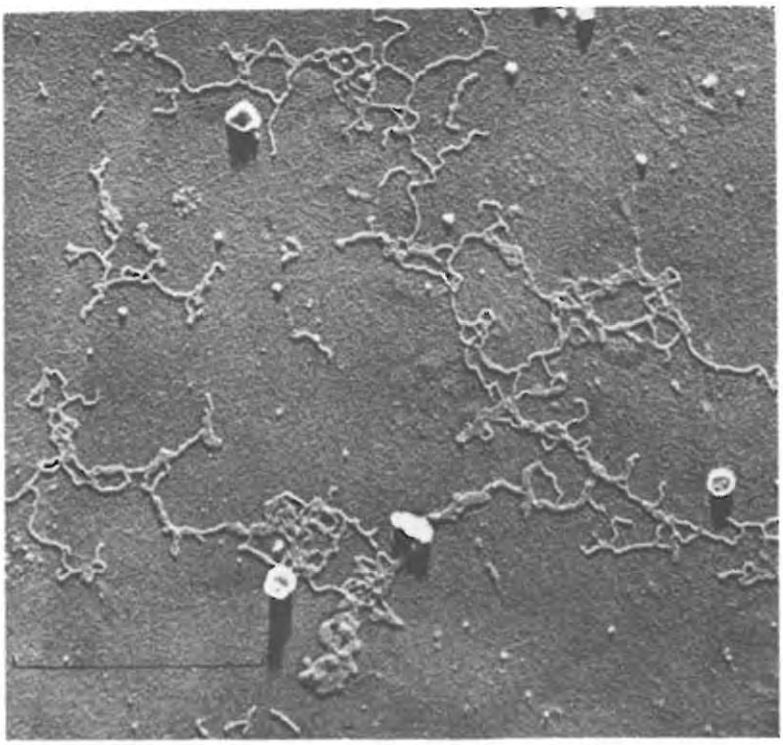


Fig. 5–4. Electron micrograph of Achromobacter sp. 2 DNA. The bar represents 1 μ.

DISCUSSION

Despite the use of spheroplasts competent for transfection and the variety of media and techniques used it was not possible to transform Achromobacter sp. 2. Thus, although the criteria for transfection and transformation are often similar (Riva & Polsinelli, 1968) there are significant differences between the two processes in Achromobacter. It was not determined whether bacterial DNA was taken up by recipient cells and it was therefore not possible to decide whether the limiting step was uptake or incorporation of donor DNA. Yasbin & Young (1972) also found significant differences between transformation and transfection.

The idea of using transformation as a genetic mapping technique was therefore abandoned and attention turned to the development of a transduction system.

TRANSDUCTION BY α PHAGES

SUMMARY

Six auxotrophic mutants of Achromobacter sp. 2 were isolated by treatment with NG. All four α phages were capable of generalised transduction of the markers. The transducing particles were shown to contain both phage and bacterial DNA as transduction of the cryptic lysogen resulted in complementation between the prophage and the residual phage function in the transducing particles. This caused the transductants to release phage at a high rate. The majority of transductants were not lysogenic but infection at high multiplicities led to multiple lysogeny. The mechanism of generalised transduction is unusual in that transductants were unstable, characteristic of transduction by lysogeny. It is proposed that a single cross-over event in regions of homology between recipient and transducing DNA yields unstable transductants which are not immune to superinfection due to the lack of repressor function of the residual phage DNA in the transducing particles.

INTRODUCTION

While examining auxotrophic strains of Salmonella typhimurium for conjugation, Lederberg, Lederberg, Zinder & Lively (1951) discovered that a cell-free extract of a wild-type strain could restore prototrophy to the mutant. They called this process transduction, and later found that transduction was mediated by temperate phage P22 (Zinder & Lederberg, 1952). A large number of bacterial genes could be transduced though with different frequencies (Zinder, 1955) and each donor marker could be independently transduced. There was perfect correlation between phage

and transducing particles in all respects other than that transduction was independent of the ability of phage to lyse or lysogenize the recipient bacteria (Zinder, 1953). This could be shown by the different effects of ultraviolet irradiation which inactivated plaque-forming ability but could at low doses increase the transduction frequency. Therefore, as the transducing particles were not identical to the P22 infective particles, the simplest explanation was that fragments of host DNA were occasionally incorporated into phage particles which thus acted as vectors of bacterial DNA.

Transduction has also been reported i.a. in Escherichia coli by phages λ (Morse, 1954), P1 (Lennox, 1955) and 363 (Jacob, 1955), Shigella by phage P1 (Lennox, 1955), Pseudomonas by a number of phages including PPP1C (Holloway & Van de Putte, 1968) and pf16h2 (Chakrabarty, Gunsalus & Gunsalus, 1968), Staphylococcus (Morse, 1959; Lacey, 1972), Proteus (Coetzee & Sacks, 1960), Bacillus subtilis by PBS1 (Takahashi, 1961), SP-10 (Thorne, 1961) and SP-15 (Taylor & Thorne, 1963) and in Salmonella typhimurium by phage MG40 (Grabnar & Hartman, 1968). Phage P1 can mediate transduction between S. typhimurium and E. coli and as it is able to carry much larger segments of genetic material than P22 (Watanabe & Fukasawa, 1961) it proves useful for mapping bacterial genes and comparing the chromosomes of E. coli, Salmonella and Shigella.

There are two types of transduction, generalised and specialised, depending on whether a number of genes from different parts of the bacterial chromosome or merely a limited cluster adjacent to the prophage location on the host chromosome can be transduced. A distinction between the two is that only preparations of phage obtained by lysogen induction can mediate restricted transduction while phage from lytic infection can perform generalised transduction (Zinder, 1955; Morse, Lederberg & Lederberg, 1956a; Wing, 1968a).

During restricted transduction e.g. by λ, the circular phage chromosome is integrated into the bacterial chromosome by a single recombination event in the attachment region (Campbell, 1962). During induction occasional illegitimate pairing between host DNA adjacent to the prophage and λ DNA yield a circular chromosome incorporating bacterial DNA (e.g. gal region) and part of the λ genome. These defective phages are known as λdg particles and occur at a frequency of 10^{-5} or less per wild-type phage (Signer, 1968). The usual event during specialised transducing phage formation is the interaction between a specific region on the bacterial chromosome and an homologous prophage region. This was shown by Deeb, Okamoto & Hall (1967) who found that trp-transducing particles of φ80 usually carried all the trp genes except that for anthranilate synthetase. Specialised transducing particles when infecting an auxotroph will lysogenize it yielding transductants which are partial diploids. They are thus unstable and can segregate auxotrophic progeny. Partial diploids of this type are called heterogenotes (Morse, Lederberg & Lederberg, 1956a & 1956b). They are normally lysogenic and on induction produce a lower than normal yield of phage. However, about half of these emergent particles can now transduce gal$^+$ and are called high frequency transducing lysates (HFT).

In theory all prophages with a chromosomal location are potentially transducing. The transduction would naturally only be seen if the host DNA fragment carried an observable marker. However, the degree of homology between prophage and bacterial DNA, required for the illegitimate recombination, is a limiting factor and it is possible that not all temperate phages can transduce (Jacob & Wollman, 1961).

The distinguishing features of generalised transduction are that any gene can be transferred, gene pick-up can occur during lytic infection

as well as after induction and the transferred donor DNA usually becomes integrated into the recipient chromosome. There are two general models to explain generalised transduction. The first proposes that, as in specialised transduction, a fragment of bacterial DNA is incorporated into the phage genome yielding defective phages. However, in the case of generalised transduction, it is proposed that the phage genome has affinities for many regions on the host chromosome. Recombination between phage and bacterial DNA can therefore occur at many sites on the host chromosome. This model can explain the findings of Luria, Adams & Ting (1960) that P1 transduction of *lac*$^+$ from *E. coli* to *Shigella* can yield heterogenotes carrying defective phage from which HFT preparations can be obtained. A wide range of variability exists in this transduction system. The transducing phage may be hardly defective at all and on induction may be able to multiply without the help of superinfecting phage to yield particles which can lyse as well as transduce. At the other extreme the transducing particles may lack all phage activities including immunity. The only function retained is the ability to transduce.

It appears that whether generalised transduction by P1 yields stable recombinants or unstable heterogenotes depends on the extent of homology between the bacterial DNA fragment and the recipient chromosome as well as the degree of residual phage function in the transducing particles. Extensive homology and little residual phage increases the probability of integration (Luria et al., 1960).

Further evidence in support of this theory comes from a study of transduction of mandelate genes in *Pseudomonas putida* by phage pf16h2 (Chakrabarty & Gunsalus, 1969). The transducing particles (pf*dm*) could yield stable (transduction by integration) or unstable (by lysogenization)

transductants depending on whether the recipient was the same or a different strain. Thus the degree of homology between incoming and recipient DNA determined the type of transduction. During interstrain transduction, the mandelate genes segregated at about 10^{-3} per cell per generation. As in the P1 system, the pfdm particles contained different amounts of phage genome (35 - 70%) which might or might not include an immunity-controlling region.

Although generalised transduction can yield heterogenote transductants a different mechanism appears to be more usual and does not involve recombination between phage and host DNA. During lytic infection when phage DNA is replicated, the bacterial chromosome is fragmented at random and incorporated into phage heads (Zinder, 1953). Evidence in support of this theory comes from transducing particles of P1 (Ikeda & Tomizawa, 1965), P22 (Ebel-Tsipis, Botstein & Fox, 1972), SP10 (Okubo, Stodolsky, Bott & Strauss, 1963) and PBS1 (Yamagishi & Takahashi, 1968). These have all been shown to contain bacterial DNA synthesized before infection and little or no phage DNA. The transduced DNA is integrated into the host chromosome, displacing allelic genes, and the transductants are therefore stable and non-lysogenic. This integration requires a double cross-over event whereas transduction by lysogeny only involves a single one.

Not all transduced fragments of bacterial DNA become integrated into the recipient chromosome. Fragments which are not integrated are unable to replicate and are inherited unilinearly. The inherited fragment is functionally active, however, and every cell through which it passes contains the gene products. These products can be inherited cytoplasmically but will eventually be diluted out. This abortive transduction yields minute colonies when the recipient is an auxotroph

(Ozeki, 1956). The number of abortive transductants always exceeds that of complete transductants by a factor of between five and twenty (Ozeki, 1959). The effect of u.v. in increasing transduction frequency is thought to be due to a shift from abortive transduction to stable integration as u.v. stimulates recombination and increases the efficiency of stable integration (Arber, 1960).

Wing (1968a) demonstrated that P22 can carry out both generalised and specialised transduction. As integration of the incoming bacterial DNA is mediated, during generalised transduction, by the bacterial recombination system, it is possible to study specialised P22 transduction by using a rec$^-$ recipient. This system is selective for transduction by lysogenization as prophage integration but not bacterial recombination can occur in a rec$^-$ strain (Wing, 1968b). During specialised transduction by P22 only proA and proB loci, located to one side of the prophage attachment sit, are transduced. The int$^+$ function of the phage is necessary for this transduction by lysogenization.

During a study of generalised transduction by P22, Ebel-Tsipis et al. (1972) showed that the transducing particles were formed independently of the known bacterial or phage recombination systems. They state that only 5% of the total host DNA appears in transducing particles. As more than half of intracellular phage DNA is encapsulated during lytic infections (Botstein, 1968) they conclude that the phage encapsulation system packages host DNA less efficiently than phage DNA.

The mechanism of integration of transducing DNA during generalised transduction by P22 is different from that found during transformation where single-stranded fragments of donor DNA are integrated in Pneumococcus

(Fox & Allen, 1964) and B. subtilis (Dubnau & Davidoff-Abelson, 1971). Ebel-Tsipis, Fox & Botstein (1972) found that 12 - 15% of the incoming DNA was integrated as double-stranded fragments by a recombination event that displaced recipient DNA. The rest of the transducing DNA persisted as unreplicated fragments. The difference may well be due to the fact that during transformation the donor DNA may be converted to single-stranded molecules during entry (Piechowska & Fox, 1971).

During a study on HFT lysates, Watanabe, Ogata, Chan & Botstein (1972) transduced tetracycline resistance in Salmonella typhimurium with P22 and showed the mechanism to be that of specialised transduction. The tet^R marker was integrated in the proline region of the Salmonella chromosome adjacent to the prophage site and HFT lysates could be obtained from the transductants. The efficiency of transduction by HFT lysates increased with the m.o.i. However, co-infection with non-transducing P22 resulted in maximal efficiency of transduction at all m.o.i. of the HFT. Therefore the factor accounting for increased efficiency at high multiplicity could be provided by ordinary phage.

Chan, Botstein, Watanabe & Ogata (1972) showed that the HFT lysate contained defective P22 particles. Co-operation between two particles was necessary for growth and lysogenization and thus for transduction. It was proposed that when the tet^R genes were inserted into P22 genomes and "headfuls" cut (Streisinger, Emrich & Stahl, 1967), the phage particles lacked the terminal repetitious sequences and adjacent phage genes. As phage particles without the terminally repetitious DNA sequences would not be able to circularize, grow or lysogenize (Botstein & Matz, 1970), double infection was necessary for these functions and therefore for transduction. As the DNA in the defective phage particles

was still circularly permuted (Rhoades, MacHattie & Thomas, 1968) the missing region would be different in different particles which could result in complementation.

Transduction is one of the most useful methods for chromosome mapping (Tyeryar, Taylor, Lawton & Goldberg, 1969) and has been widely used in the study of the genetic control of operons e.g. the *E. coli trp* operon using $\phi 80$ (Sato & Matsushiro, 1965) and the *gal* operon using λ (Attardie, Naono, Rouvière, Jacob & Gros, 1963). Transduction can also be used to study the genetic relationships between organisms by comparing gene loci (Coetzee, Smit & Prozesky, 1966). The possibilities for this type of study are increased by the fact that phages can transduce strains on which they do not form plaques (Taylor & Thorne, 1963).

A striking use of transduction was demonstrated by Merril, Geier & Petricciani (1971) when they transduced *gal* genes from *E. coli* to human fibroblasts from a patient suffering from galactosemia. As this disease was due to the absence of galactose-1-phosphate uridyl transferase activity the success of these experiments underlines the similarity of the genetic material in organisms as different as *E. coli* and man.

Phages which have turbid plaques are often transducing (Boyd, 1951). As the α phages all have turbid plaques it was considered likely that they would be able to transduce *Achromobacter*. A study on the transducing abilities of the four phages was therefore carried out with a view to establishing an efficient genetic mapping technique.

METHODS

Isolation of auxotrophic mutants. The method described on p. 91 was used.

Preparation of phage lysates. These were prepared as described on p. 24.

Transduction. Overnight broth cultures of auxotrophs were diluted 1:10 and aerated at 30°C for 5 hr. (1×10^9 cells/ml.). Two ml. of culture were resuspended in 4 ml. phage lysate prepared on the wild-type strain. The transduction mixture was incubated for 1 hr. for phage adsorption. Cells were harvested by centrifugation, resuspended in 1 ml. saline and spread onto minimal agar. Plates were scored for prototrophs after incubation for 2 - 3 days. Controls consisted of cells incubated with 4 ml. 0.4 M NaCl and 4 ml. phage propagated on the homologous strain. Phage sterility was tested by spreading 0.1 ml. onto salt and minimal agar. Control transduction experiments were also carried out using phage pre-incubated with antiphage serum (k = 10) for 15 min.

Transductant lysogeny. Transductants were inoculated into nutrient broth and incubated for 24 - 48 hr. Cultures were tested for sensitivity to phage by spotting high titres of phage onto double-agar-layer plates seeded with the transductants. Cultures were tested for the presence of phage in the supernatant by centrifuging and spotting the SNF onto plates seeded with the wild-type strain.

Induction of transductants. Transductants which were resistant to superinfection and had phage in the supernatant fluid were tested for induction by u.v. as described on p. 55.

Transductant stability. Transductants were suspended in nutrient broth and their titres calculated from the optical density (Fig. 1-3). Suspensions were diluted to a titre of c. 5×10^4 cells/ml. in nutrient broth and incubated for 18 hr. Dilutions were plated onto salt and minimal agar and the rate of segregation per cell per generation calculated after incubation for 48 hr. To determine whether segregation of prototrophy was correlated with the loss of the ability of transductants to release α3cry spontaneously, transductants were streaked onto salt agar. The isolated colonies were then streaked onto minimal agar and inoculated into nutrient broth. After 24 hr. the broth cultures were tested for spontaneous phage release by spotting the supernatants after centrifugation onto plates seeded with the wild-type strain.

Preparation of HFT lysates. Log. phase cultures of transductants were centrifuged and the supernatants sterilized with 10% (v/v) chloroform. The cells were resuspended in 0.4 M NaCl and treated with u.v. (p. 23) for 15 sec., 30 sec., 1 min., 2 min., 3 min. and 4 min. The irradiated cultures were pooled, incubated for 2 hr. and cells removed by centrifugation. The spontaneous and induced supernatants were tested for transducing ability.

Abortive transduction. The very small colonies formed by transduction at high multiplicities of phage (m.o.i. c. 50 - 100) were streaked onto minimal agar and incubated. Isolated colonies were cut out on their agar base, resuspended in 1 ml. 0.4 M NaCl and assayed on both salt and minimal agar. The titre of cells in these small colonies was also compared with that of wild-type colonies on minimal agar. Growth of

mutants, wild-type and transduced strains were compared in minimal medium and nutrient broth at 30 °C and 37 °C.

RESULTS

Mutant isolation

Auxotrophs isolated were trp, met, arg, pro and cys. Another cys mutant was shown to be temperature sensitive, the permissive temperature being 30 °C and the non-permissive temperature 37 °C. This mutant was called cys A and the non-temperature sensitive one, cys B.

Transduction

All four α phages were able to transduce all the auxotrophs to prototrophy (Table 6-1). Only those experiments where controls showed no growth, and thus no spontaneous reversion, were recorded. No transductants were formed when the transducing lysate was pre-treated with antiphage serum or when phage lysates propagated on the homologous strain were used. At high m.o.i. (50 - 100) of transducing lysate, transductant colonies were small, c. one quarter the size of wild-type colonies and transductants produced at lower m.o.i.

Transductant sensitivity to phage

The majority of transductants tested were sensitive to phage (Table 6-2). An unexpected result was the appearance of spontaneous plaques on lawns of transductants (Fig. 6-1). Often the rate of spontaneous phage release was so great that total lysis occurred and the titre of phage in an overnight culture of such transductants was c. 10^{10} p.f.u./ml. The titre of phage in overnight cultures of transductants

resistant to phage was $5 \times 10^2 - 10^3$ p.f.u./ml. Spontaneous phage release could also be seen in streaks of transductants grown on salt agar (Fig. 6-2).

Among the small transductant colonies there was a higher proportion of colonies resistant to phage but whose supernatants carried phage. These phages had an increased tendency to lysogenize the wild-type strain when compared with the phage released from sensitive colonies, as evidenced by the extreme cloudiness of the phage spots (Fig. 6-3). A few transductant colonies were sensitive to phage but did not release phage. These were probably revertants. A small number of transductants were resistant to phage but not inducible (Table 6-2).

Induction of transductants

Transductant colonies which were resistant to superinfection by homologous phage and showed phage in the SNF (c. 10^3 p.f.u./ml.) could be induced by u.v. (Fig. 6-4).

Transductant stability

The average rate of segregation of *trp*$^-$ in *trp* transductants when α3a was used as the transducing lysate was 3.6×10^{-3} per cell per generation. This segregation was not correlated with the loss of the ability of transductants to produce spontaneous plaques. After two cycles of single cell isolation of a total of 85 *trp* transductants, none could produce spontaneous plaques. They all showed resistance to superinfection by α3a and were u.v. inducible. The other markers showed similar rates of segregation.

HFT lysates

It was not possible to prepare HFT lysates. Phage lysates in the supernatant of transductants which could form spontaneous plaques (c. 10^{10} p.f.u./ml.) were unable to transduce.

Abortive transduction

The small transductants obtained when the transducing lysate used was at a m.o.i. of 50 - 100 were not abortive as every cell in the colony could grow on both minimal and salt agar. The number of cells in a small transductant colony on minimal agar was 2.4×10^5 compared with 6.0×10^8 in wild-type colonies on minimal agar. The colonies derived from the cells of the original small colony were likewise of the same small size. Growth patterns of mutant, wild-type and transduced strains (Fig. 6-5) showed that the small colonies were slow-growing. As cys A was temperature sensitive, transduction of this marker was carried out at 37 °C but the optimal temperature for transduction of all the other markers was 30 °C.

Table 6-1. Transduction by Achromobacter phages

Phage	Marker	Transduction rate ($\times 10^{-7}$) per p.f.u. adsorbed
α1	arg	0.13
	cys A	0.9
	cys B	1.5
	met	1.77
	pro	1.6
	trp	1.7

Table 6-1 cont.

Phage	Marker	Transduction rate ($\times 10^{-7}$) per p.f.u. adsorbed
α2	arg	0.0
	cys A	3.1
	cys B	0.0
	met	1.85
	pro	0.74
	trp	2.0
α3a	arg	3.3
	cys A	3.2
	cys B	6.5
	met	4.3
	pro	4.8
	trp	7.3
α3b	arg	3.5
	cys A	4.6
	cys B	6.0
	met	4.0
	pro	5.0
	trp	7.6

Table 6-2. Phage sensitivity of transductants

Marker	Phage	m.o.i.	Colony size	Cols. sensitive. Spontaneous plaques.	Cols. sensitive. No spontaneous plaques.	Cols. resistant. Phage in SNF.	Cols. resistant. No phage in SNF.	Total cols. tested
trp	α1	50 - 100	small	12	0	1	0	13
		1 - 5	w.t.	11	1	0	0	12
trp	α2	50 - 100	small	12	0	2	0	14
		1 - 5	w.t.	9	2	0	0	11
trp	α3a	50 - 100	small	51	0	3	0	54
		1 - 5	w.t.	100	3	0	2	105
trp	α3b	50 - 100	small	10	0	2	0	12
		1 - 5	w.t.	7	4	0	0	11
met	α3a	50 - 100	small	17	0	11	3	31
		1 - 5	w.t.	10	0	0	0	10
arg	α3a	50 - 100	small	7	0	1	0	8
		1 - 5	w.t.	10	0	0	0	10
pro	α3a	50 - 100	small	13	2	4	1	20
		1 - 5	w.t.	78	4	0	2	84
			Total small	122	2	24	4	152
			Total w.t.	225	14	0	4	243

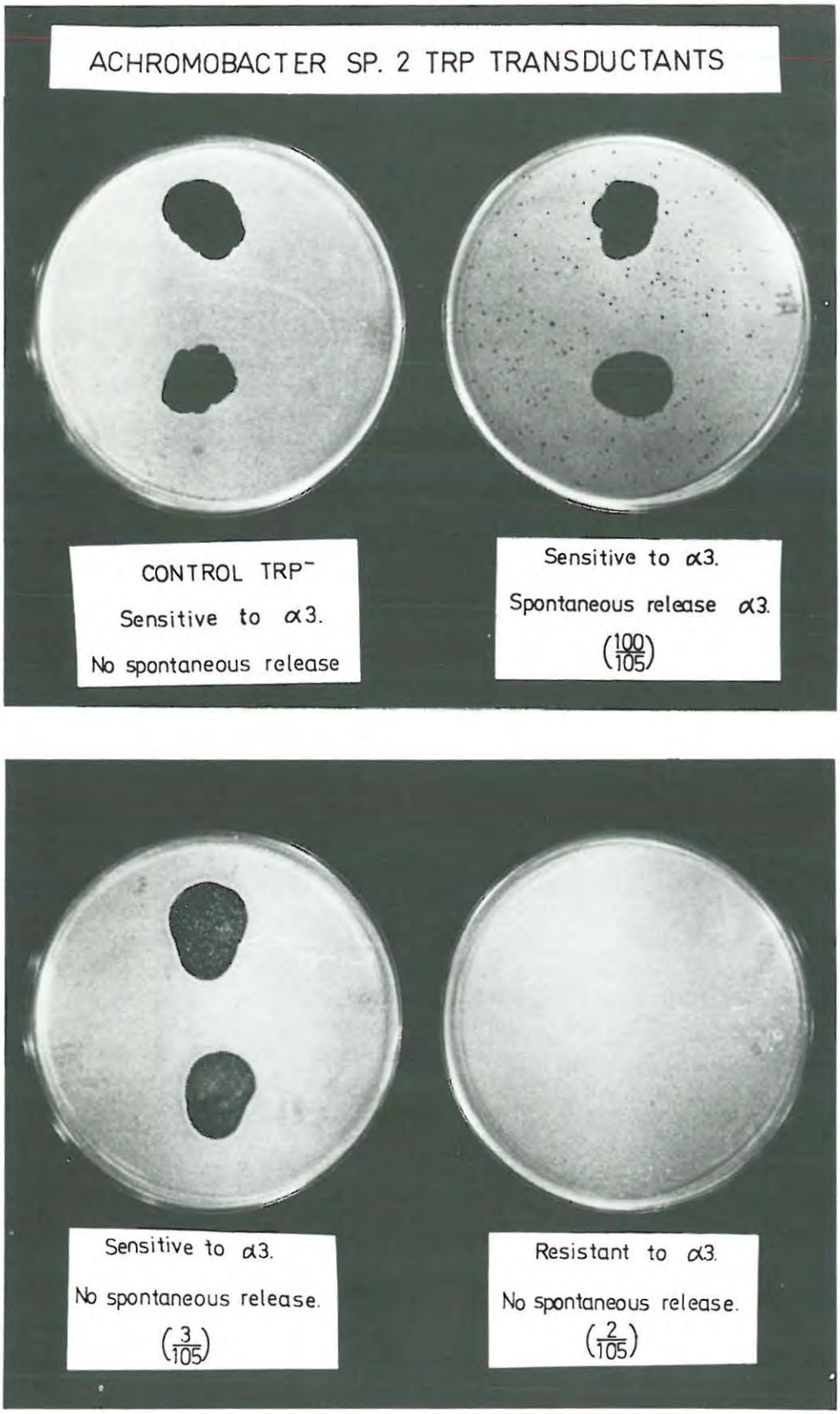


Fig. 6–1. Phage sensitivity of Achromobacter sp. 2 trp transductants. (See Table 6–2).

Fig. 6–2. Spontaneous release of ϕ 3 phage by Achromobacter sp. 2 trp transductants streaked onto salt agar.

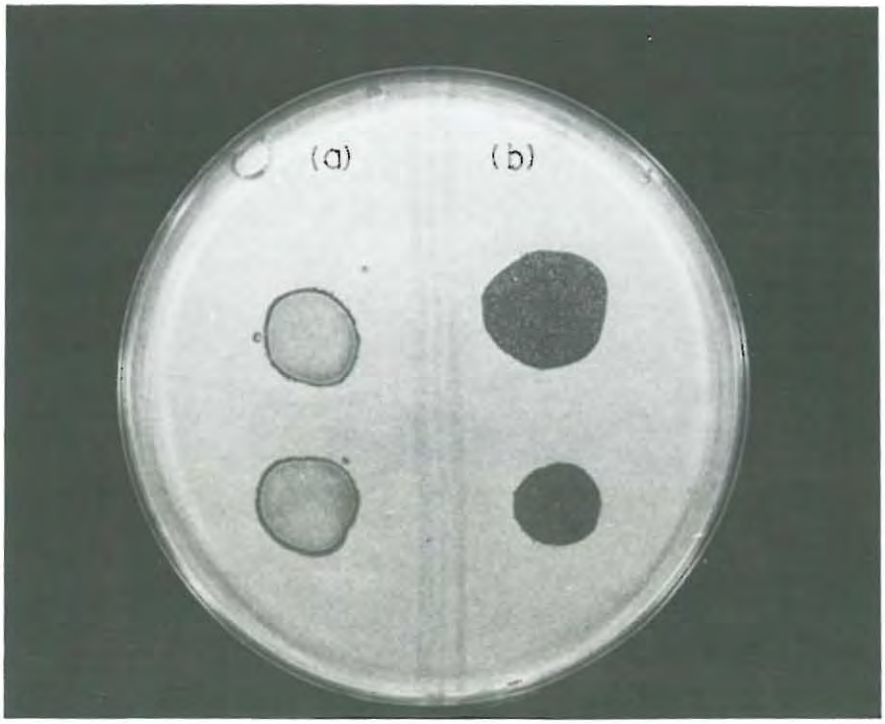


Fig. 6–3. Achromobacter sp. 2 sensitivity to phage liberated by (a) lysogenic transductants and (b) non-lysogenic transductants.

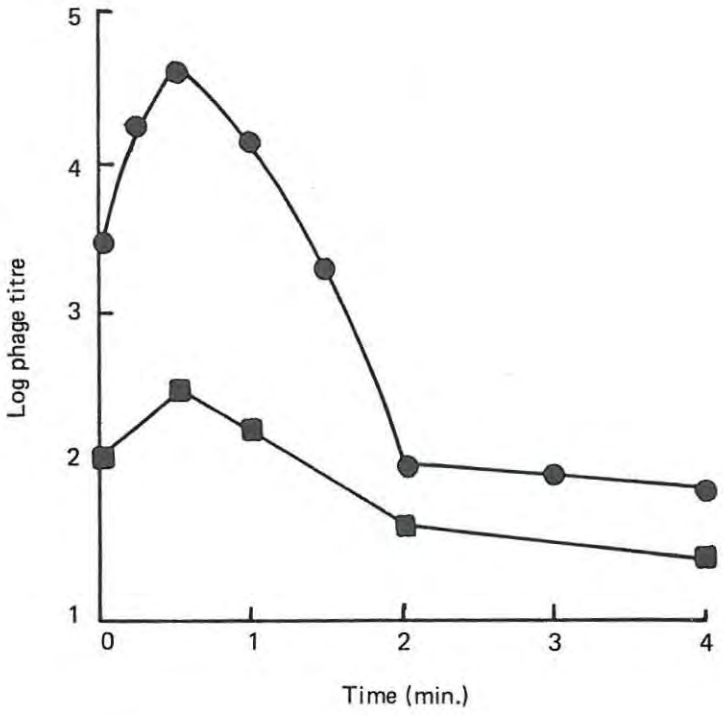


Fig. 6–4. UV induction of transductants resistant to superinfection with α 3a. Transduction was with phage α 3a at m.o.i. 50. ●—● , trp. transductant; ■—■ , met. transductant.

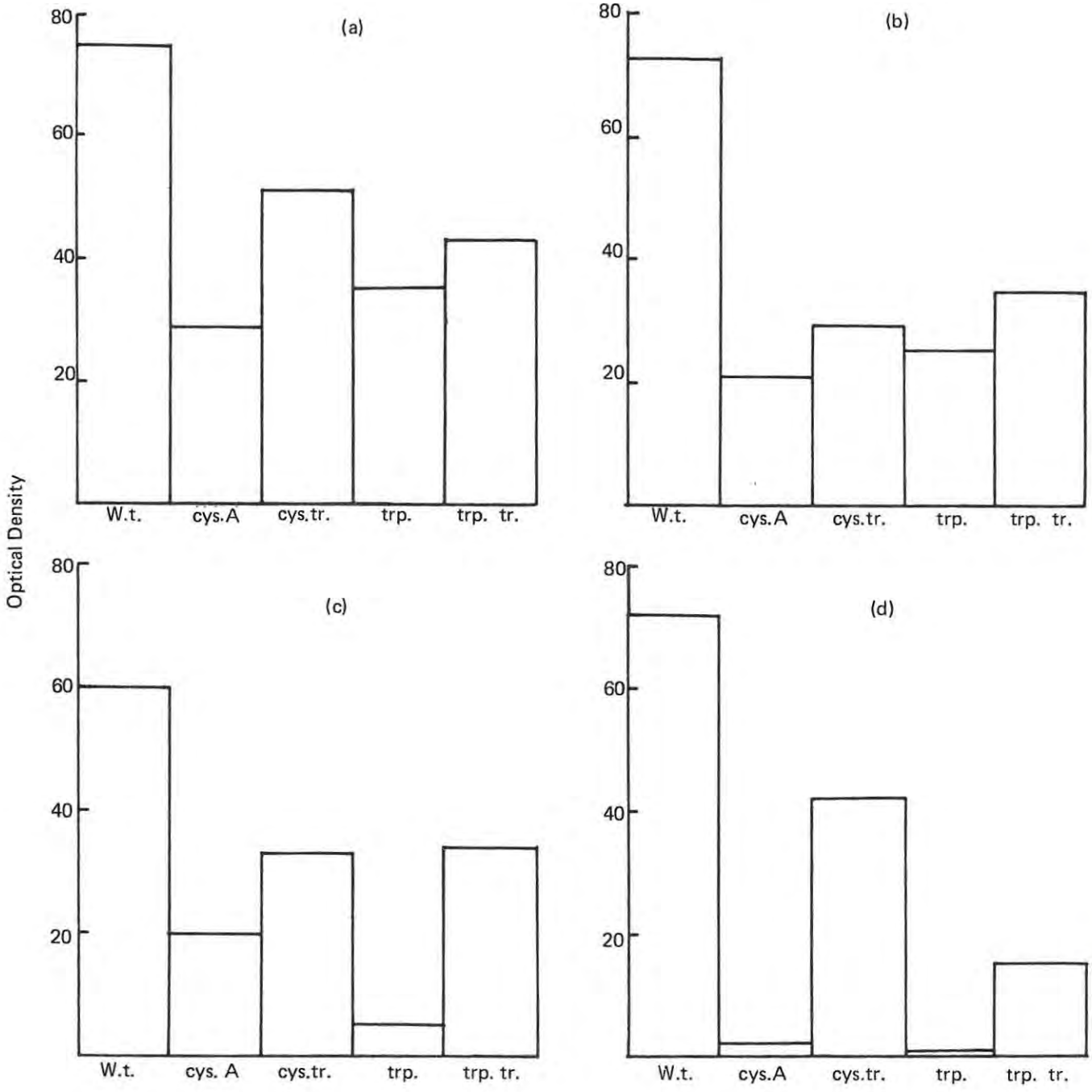


Fig. 6–5. Growth of Achromobacter sp. 2 wild type (w.t.), cystein A (cys. A), tryptophan (trp.), cystein A transductants (cys. tr.) and tryptophan transductants (trp. tr.). (a) In nutrient broth at 30°C. (b) In nutrient broth at 37°C. (c) In minimal medium at 30°C. (d) In minimal medium at 37°C. Growth in nutrient broth recorded after 60 hr. Growth in minimal medium recorded after 3 days.

DISCUSSION

As the α phages all transduced a number of auxotrophic markers at a fairly high frequency (c. 10^{-7}/p.f.u.) they were capable of generalised transduction. Generalised transducing particles usually contain little or no phage DNA (Ebel-Tsipis et al., 1972) and transduction is by a double-crossover integration event yielding stable transductants which are still sensitive to phage. Segregation of auxotrophy does not occur under these conditions. Achromobacter transductants are clearly not of this nature as segregation of auxotrophy occurred with a probability of c. 10^{-3} per cell per generation. However, as the majority of transductants were still sensitive to phage the transduction cannot be by normal lysogenization. Lysogenic transductants, while segregating auxotrophs, are normally resistant to superinfection by homologous phage (Morse et al., 1956a & 1956b).

The clue to the problem lies in the fact that α-sensitive transductants released phage spontaneously at an extremely high rate. Evidence of this is the high titre (c. 10^{10} p.f.u./ml.) obtained from overnight broth cultures, the appearance of spontaneous plaques on transductant lawns (Fig. 6-1) and in streaks of transductants on salt agar after overnight incubation (Fig. 6-2).

It therefore seems likely that the process of transduction was causing the liberation of the cryptic prophage which has been shown to be present in Achromobacter sp. 2 (Thomson & Woods, 1973b). It was proposed that the cryptic state of this phage is due to integration occurring within the repressor gene, resulting in a split repressor, and a mutation in the gene(s) involved in prophage excision and/or vegetative phage development. If the transducing particles carry phage

genes which can provide the necessary excision functions, the lack of functional repressor in the cryptic lysogen will result in very rapid prophage induction. As expected, the cryptic prophages induced by this transduction process were themselves unable to transduce.

Thus it is clear that the transducing particles must carry phage as well as bacterial genes. Luria et al. (1960) and Chakrabarty & Gunsalus (1969) have isolated transducing P1 and pf16h2 particles containing varying amounts of phage genes which may or may not include an immunity-controlling region. As the majority of the Achromobacter transductants were sensitive to phage the majority of transducing particles could not contain the repressor gene(s).

At high multiplicities of infection of the transducing lysate (m.o.i. 50 - 100) there was an increase in the number of transductants which behaved like normal lysogens, being resistant to phage and u.v. inducible. It is likely that at high m.o.i. there is an increased probability that transducing particles contain the repressor gene(s) yielding multiply lysogenic transductants. These transductants will, as shown, be immune to superinfection and show the induction characteristics of normal lysogens. The colonies of these transductants were small and their growth rate slow indicating an interference with bacterial function by multiple lysogeny.

The finding that after cycles of single cell isolation transductants which were formerly sensitive to phage and released the cryptic prophage at a rapid rate became resistant and u.v. inducible can also be explained by multiple lysogenization. As the rate of cryptic prophage release is so rapid there is a high probability that cells in the presence of these

will be doubly lysogenized. Double lysogenization was shown to occur in the cells in the centres of turbid plaques (Thomson & Woods, 1973b).

From the above evidence it is concluded that phage lysates contain generalised transducing particles carrying phage and bacterial DNA. This is in contrast to the theory that generalised transducing particles incorporate bacterial DNA by a mechanism like phenotypic mixing. In the Achromobacter system recombination between phage and host DNA must occur. Schmieger (1970) has proposed, for generalised transducing particles of P22, a similar recombination event during phage replication between newly synthesized phage DNA and the host chromosome.

As the recipient strains are cryptic lysogens they carry DNA homologous to the phage DNA in the transducing particles as well as to the bacterial markers. Integration by a single cross-over event in these homologous regions will yield unstable prototrophic transductants which, owing to the defective nature of the phage DNA, will not be immune to superinfection. Moreover, complementation between the cryptic phage and the residual phage functions in the transducing particles results in the spontaneous release of the cryptic phage.

There have been reports of generalised transducing phages causing transduction either by integration or lysogenization. The choice depends on the degree of homology between the bacterial DNA in the transducing particles and the recipient strain as well as on the amount of residual phage DNA in the transducing particles (Luria et al., 1960; Chakrabarty & Gunsalus, 1969). However, although the phages worked with, P1 and pf16h2, are capable of generalised transduction, DNA incorporation by lysogenization was in both cases shown only for a specific set of genes. With P1, lac and with pf16h2, mandelate genes

were transduced. Thus the α system is the first to show generalised transduction by a method other than the normal integration viz. by a defective lysogenization for a number of different markers.

If transduction of Achromobacter is by a type of lysogenization it should be possible to isolate HFT lysates. Although no such lysate was isolated, only one transductant has been tested. Watanabe et al. (1972) obtained only 3 HFT lysates by u.v. induction of 26 independently isolated transductants. The screening of more transductants might therefore possibly enable the isolation of an HFT lysate.

This interpretation of the mechanism of transduction of a cryptic Achromobacter lysogen by the α phages is being confirmed by differential labelling. It should be possible to determine by this method the origin of the DNA in the transducing particles. The isolation of rec$^-$ mutants will also indicate whether the incorporation of transducing DNA is under bacterial or phage control. This should help to clarify whether the recombination event occurs between regions of phage or bacterial DNA homology.

CONCLUSION

The aim of this study, to isolate collagenolytic bacteria from hides and to establish a genetic system for the investigation of the control of collagenase production, has been achieved. Although transformation does not appear to be possible, the α phages may now be used to transduce many different genes and so establish a genetic map of Achromobacter. Collagenase mutants will be isolated and likewise transduced to determine their number and location on the chromosome before studying control systems. From a practical point of view the understanding of conditions resulting in collagenase production or its inhibition could have far-reaching effects for the hide and skin industry in the prevention of leather decay.

The unusual system of generalised transduction by the α phages is to be further investigated by determining the amount of bacterial and phage DNA present in the transducing particles. The isolation of rec$^-$ mutants will help to further elucidate the mechanism of DNA incorporation into the recipient genome.

The unexpected discovery of cryptic lysogeny in Achromobacter sp. 2 is also open to further investigation using phage mutants to determine the nature of the excision defect in the cryptic prophage. Genetically marked phage can be used to assay for recombination with the cryptic prophage. The unforeseen manner in which transduction confirmed the presence of the cryptic prophage brings to mind the words of Sir Alexander Fleming: "Never neglect any appearance or any happening which seems to be out of the ordinary: more often than not it is a false alarm, but it may be an important truth."

APPENDIX 1

PROPHAGE INDUCTION IN ESCHERICHIA COLI (λ) BY CARCINOGENIC N-NITROSAMINES

SUMMARY

The carcinogenic N-nitrosamines dimethylnitrosamine, di-n-propylnitrosamine, methyl-n-propylnitrosamine and N-nitrosopiperidine were shown to be inducers of phage development in E. coli EMG 21. No induction was obtained by the non-carcinogenic diphenylnitrosamine nor by the related non-carcinogenic substance diethylamine. The N-nitrosamines were unable to revert his⁻ mutants of S. typhimurium.

INTRODUCTION

An apparent correlation between the ability of a substance to induce lysogenic bacteria and its mutagenic and carcinogenic activities was reported in 1953 by Lwoff. It was subsequently shown that azaserine, a potent mutagen and carcinostatic agent (Gots, Bird & Mudd, 1955), mitomycin C, an antineoplastic compound (Otsuji, Sekiguchi, Iijima & Takagi, 1959) and aminopterin, a carcinostat (Ben-Gurion, 1962) were all inducers in the E. coli K-12 (λ) system. Since then an ever-increasing wealth of evidence supports this correlation (Endo, Ishizawa, Kamiya & Sonoda, 1963; Heinemann & Howard, 1964a; Heinemann, 1971a; Heinemann, 1972). Although not all carcinogens or antineoplastic agents tested have been

shown to be inducers (Price, Buck & Lein, 1964) the correlation is such that induction can be used as a rapid in vitro screening system for potentially harmful carcinogens and useful antineoplastic compounds (Heinemann & Howard, 1964b; Heinemann, 1971b).

Although the mode of action of inducers is not clear they all appear to induce primarily as a result of interference with bacterial DNA synthesis. The selective inhibition of DNA synthesis without effect on RNA or protein synthesis has been observed with antitumor inducers MC (Shiba, Terawaki, Taguchi & Kawamata, 1959; Sekiguchi & Takagi, 1960), streptonigrin (Levine & Borthwick, 1963), phleomycin (Tanaka, Yamaguchi & Umezawa, 1963) and hedamycin (Bradner, Heinemann & Gourevitch, 1966). Selective inhibition of DNA synthesis has also been shown by Heinemann & Howard (1965) for a number of antineoplastic agents which induce phage production in E. coli W1709 (λ). In all cases there was a close relationship between the concentration which inhibited DNA synthesis and that which caused induction.

In studying the correlation between induction and mutagenesis, Terawaki and Greenberg (1965) suggested that only bis-functional alkylating agents (and u.v.) can specifically inhibit DNA synthesis. This inhibition is probably due to the formation of cross-linkages in the DNA. As monofunctional alkylating agents e.g. the mutagen nitrosoguanidine, cannot form such cross-linkages, cross-linkage of DNA is not essential for mutagenesis or carcinogenesis. However, there is increasing evidence that many carcinogens act by specifically binding to DNA (Brookes & Lawley, 1964; Maher, Lesko, Straat & Ts'o, 1971).

Heinemann, Howard & Hollister (1967) developed a procedure whereby paper chromatography of mixtures containing suspected inducers can be used to separate the components. The chromatograms are placed on double-agar-layer plates seeded with E. coli K-12 (λ). The presence of inducers can be detected by clear halos of lysis.

Hydroxylamine is mutagenic (Freese, Bautze-Freeze & Bautze, 1961) and teratogenic (see Heinemann, 1971). Hydroxylamino compounds may be encountered in the environment as hydroxylamine is an intermediate in nitrogen fixation, nitrification and denitrification and derivatives are found in end-products of microbial fermentations (Neilands, 1967). These may represent unsuspected environmental hazards and evidence that aromatic amines and amides can, by N-hydroxylation, be converted into carcinogens in vivo has focussed attention on these compounds (Miller & Miller, 1969). Heinemann (1971a) showed that 18 out of 37 hydroxylamine and 11 out of 45 hydrazine derivatives were effective inducers.

With the demonstration that nitrosamines are carcinogenic in animals (Magee & Barnes, 1967) and their suspected implication in human cancer (Lijinsky & Epstein, 1970) similar studies have been carried out on N-nitroso compounds (Heinemann, 1972). Of the 14 inducers found, 7 have known carcinostatic activity.

N-nitrosodialkylamines are among the most potent carcinogens known (Magee & Schoental, 1964). Sen, Smith & Schwinghamer (1969) demonstrated the in vitro formation of diethylnitrosamine (DEN) when diethylamine and sodium nitrite were incubated with gastric juices from a number of animals and man. The general reaction is as follows:

DIALKYL-AMINE DIALKYL-NITROSAMINE

The nitrosation reaction was also shown in vivo in cats and rabbits. As nitrites and secondary amines occur in human foods (Wick, Underriner & Paneras, 1967) and nitrite is used as a food additive in many countries (Aune, 1972) the amounts could reach levels where nitrosamines are formed in the acid conditions of the stomach. McGlashan and Walters (1969) found dimethylnitrosamine (DMN) and other nitrosamines in African alcoholic drinks and have suggested a correlation between this and the incidence of oesophageal cancer in Africans drinking locally distilled spirits. Molybdenum deficiency in the soil results in the accumulation of nitrate. Should the soil also lack the trace-elements iron, copper and manganese, nitrite cannot be reduced to ammonia. Plants grown on this soil accumulate nitrite and nitrate. In African villages in the Transkei, Burrel, Roach and Shadwell (1966) correlated the presence of such plants with the presence of oesophageal cancer. The plants were used in the preparation of alcoholic beverages and during fermentation nitrate could decompose to nitrite which might react with secondary amines to produce nitrosamines. Nitrosamines have also been found in Transkeian fruits used in the preparation of a major Bantu food source (Du Plessis, Nunn & Roach, 1969) and in a local alcoholic drink in Zambia, called Kachasen (McGlashan, Walters & McLean, 1968). Sen (1972) found traces of DMN in meat products and in bacon (Sen, Donaldson, Iyengar & Panalaks, 1973). In Norway fatal liver poisoning of animals was traced to DMN found in herring meal. The production of DMN was attributed to indiscriminate use of $NaNO_2$ as an additive to raw herring

(Sakshaug, Søgnen, Hansen & Koppang, 1965). Nitrosamines have also been found in cigarette smoke condensate (McCormick, Nicholson, Baylis & Underwood, 1973).

The occurrence of biologically active N-nitroso compounds in the environment may thus constitute a potential hazard to man and animals. The importance of establishing a rapid screening method for the presence of nitrosamines is therefore clear.

Techniques other than phage induction have been developed to screen for carcinogens. As many carcinogens, including nitrosamines, are also mutagens (Bridges, 1972a) tests have been devised which are specific for detecting mutations. Bridges (1972b) used a tryptophan-requiring E. coli WP2 mutant with a deficient repair system to detect mutagens which cause reversion to prototrophy. Parry (1972) used heteroallelic diploid cultures of Saccharomyces cerevisiae to detect prototrophic recombinants caused by mutagens. Cultured mammalian cells which are deficient in hypoxanthine-guanine ribosyl transferase and thus resistant to 8-azoguanine have also been used to detect mutagens (Arlett, 1972).

Ames (1971) used a set of histidine-requiring Salmonella typhimurium strains with a defective excision repair system to screen for mutagens causing base pair substitutions (e.g. alkylating agents), frame-shifts (e.g. nitroso-flourene and many polycyclic hydrocarbon carcinogens) (Ames, Gurney, Miller & Bartsch, 1972; Ames, Sims & Grover, 1972) and large deletions (e.g. X-rays). The S. typhimurium strains have been further improved by introducing a mutation that results in a deficient lipopolysaccharide. This relieves the barrier to penetration by mutagens into the cell (Ames, Lee & Durston, 1973).

As chemical carcinogens appear to act through interaction with the DNA (Brookes & Lawley, 1964) and the structure of DNA is the same in all organisms (Crick, 1967), bacteria can be used for detecting potential human carcinogens, albeit with certain limitations. The most serious of these is that a compound may be metabolised in humans, but not in bacteria, to a true carcinogen. The corollary of this, that mutagens may be detoxified in humans, is also true but it is clear that most compounds mutagenic in bacterial test systems are carcinogenic (Ames, 1971; Miller & Miller, 1971).

It is thought that nitrosamines are metabolised before becoming tumorigenic. This has been shown convincingly for dialkylnitrosamines by Druckrey, Preussmann & Ivankovic (1969) as no sarcomas were found at the site of injections but cancer resulted in remote organs. Although the exact mechanism of this activation is not yet clear, the reaction probably results in the formation of an alkylating agent (Craddock & Magee, 1963; Schoental, 1966). As all carcinogenic nitrosamines are mutagenic (Magee & Barnes, 1967) it is of interest to note that DMN can only cause reversion of *his*$^-$ mutants of *S. typhimurium* after treatment with the liver microsomal fraction responsible for the metabolism of DMN (Malling, 1971). As the main metabolic breakdown of nitrosamines occurs in the liver, this organ is the most commonly affected one by the carcinogens (Magee & Barnes, 1967).

Due to the nitrosamine content of plants in the Transkei and the high incidence of oesophageal cancer in this area, the Chemistry Department at Rhodes University under Professor J.R. Nunn has been investigating the chemistry of carcinogenic nitrosamines (Du Plessis & Nunn, 1972). In view of the fact that carcinogenic nitrosamines have been

shown to be unable to induce phage in E. coli W1709 (λ) (Heinemann, 1972), it was decided to attempt to develop an induction system with the ready supply of nitrosamines at Rhodes University using different lysogenic strains. Should such a system be established its sensitivity in detecting the carcinogenic compounds was to be compared with the mutagenic activity of the compounds on his⁻ strains of S. typhimurium. This work was carried out with the kind co-operation of Prof. Nunn, Dr. L.S. du Plessis and F. Kelly.

METHODS

Bacteria. Escherichia coli EMG 21, lysogenic for λ and E. coli EMG 10 sensitive to λ and resistant to streptomycin were obtained from Prof. W. Hayes, Department of Molecular Biology, University of Edinburgh. Salmonella typhimurium his G46 was obtained from Prof. B.N. Ames, Biochemistry Department, University of California, Berkeley, California.

Test materials. Dimethylnitrosamine, di-n-propylnitrosamine, methyl-n-propylnitrosamine, N-nitrosopiperidine, diphenylnitrosamine and diethylamine were supplied by Prof. J.R. Nunn and co-workers of the Chemistry Department, Rhodes University. All compounds were dissolved in sterile deionized water with the exception of diphenylnitrosamine which was dissolved in dichloromethane (CH_2Cl_2).

Induction of λ. A modification of the method of Price et al. (1964) was used. Overnight cultures of EMG 21 in E. coli minimal medium + 0.2% casamino acids (Ruff, Kirby & Goldthwait, 1971) were diluted 1:10 in fresh medium and incubated with aeration at 37 °C for 1 hr. Cells

were centrifuged and resuspended in fresh medium to a concentration of approximately 3×10^5 cells/ml. To 0.8 ml. of this suspension was added 0.2 ml. of inducing agent and the mixture incubated at 37 °C for 60 min. After this induction period, 9 ml. pre-warmed heart infusion broth + 0.1% glucose were added and incubation carried out for 2 hr. in a 37 °C water bath with reciprocal agitation. Samples were diluted in 0.85% NaCl + 10% tryptone broth and plated for plaque-formers. The remainder of each test sample was incubated for a further 8 hr. to permit the development of detectable turbidity. Test samples failing to show growth were retested at lower non-toxic concentrations. Controls of EMG 21 without inducer were carried out for each experiment. If the level of spontaneous induction was greater than 10^2 p.f.u./ml. the experiment was disregarded. Positive controls using MC, a known inducer, were also included in each experiment.

Enumeration of plaque-formers. The indicator strain EMG 10 was grown with aeration at 37 °C for 18 hr. in heart infusion broth + 0.1% glucose. The top agar layer was soft nutrient agar (0.5% agar) supplemented with 0.5% NaCl. The base agar layer was tryptone agar containing 100 μg. streptomycin/ml. In order to obtain an even spread of indicator strain the soft-agar tube was mixed well by rotation between the palms of the hands after the addition of EMG 10 and the induction mixture. Plaques were counted after incubation at 37 °C for 48 hr. Samples which gave an induction index of 10 (ten times the spontaneous phage count) or greater were considered to be active inducers of the lytic cycle in EMG 21 cells (Heinemann & Howard, 1964).

Reversion of his G46. A modification of the method of Ames, Lee & Durston (1973) was used. Crystals or a few μl. of the suspected

mutagens were placed on a lawn of his G46 on Vogel-Bonner minimal agar containing 0.1 μmole histidine and 0.1 μmole biotin per plate (Vogel & Bonner, 1956). For quantitative testing a known amount of mutagen solution was incorporated into the top layer (0.6% agar + 0.5% NaCl) of a double-agar-layer plate. Controls for spontaneous reversion were the tester strain without added mutagen. A sterility check of the mutagen solution was also included in each experiment. Plates were incubated at 37°C and revertants counted after 48 hr.

RESULTS

MC induction

The optimal concentration of MC for λ induction was 5 - 6 μg./ml. (Fig. 7-1a). This concentration was used as a positive control in all induction experiments.

Nitrosamine induction

The concentrations of nitrosamines at which induction was obtained are shown in Table 7-1. No induction was obtained with diphenylnitrosamine (0.02 - 2,000 μg./ml.) or diethylamine (0.2 - 1,900 μg./ml.). There was a rapid rise in induction at low nitrosamine concentrations which levelled off at higher concentrations (Fig. 7-1b).

Reversion of his G46

The nitrosamines, diethylamine, MC and 5BU were all unable to cause reversion of his G46. NG alone of the substances tested was able to do so (Table 7-2).

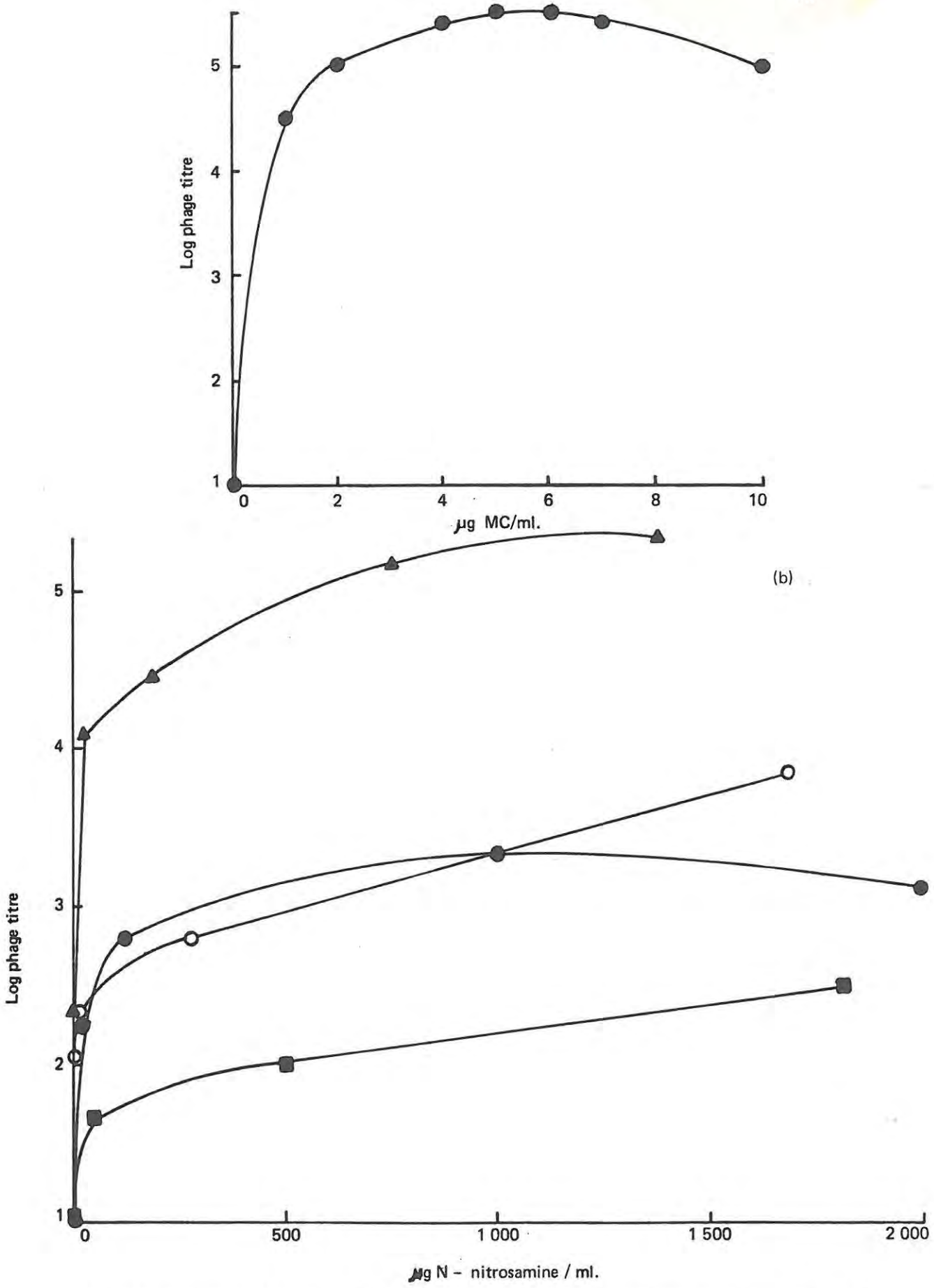


Fig. 7–1. Induction of E. coli EMG 21 by (a) MC and (b) carcinogenic N-nitrosamines: (●—●) dimethylnitrosamine, (■—■) di-n-propylnitrosamine, (▲—▲) methyl-n-propylnitrosamine, (○—○) N-nitrosopiperidine.

Table 7-1. E. coli (λ) induction by nitrosamines

Inducing agent	Concentrations causing induction (μg./ml.)		Concentrations not causing induction (μg./ml.)	
Dimethylnitrosamine	2,000	20		0.02
	800	2		
	200	0.2		
Di-n-propylnitrosamine	1,800	50		1
	500	10		0.1
Methyl-n-propyl-nitrosamine	1,400	14		0.01
	750	1.4		
	140	0.14		
Nitrosopiperidine	1,700	1.7		0.01
	170	0.17		
Diphenylnitrosamine			2,000	2
			200	0.2
			20	0.02
Diethylamine			1,900	1.9
			190	0.2
			19	

Table 7-2. Reversion of Salmonella typhimurium his G46

Test substance	Concentration (μg./plate)	No. tests	Revertants/plate
Control	-	4	0 - 7
MC	10	3	2 - 6
	5	1	4
	0.1	1	1
Di-n-propylnitrosamine	800	2	5
	8	2	0 - 4
Dimethylnitrosamine	1,000	2	2
	10	3	2
	0.1	1	0
Methyl-n-propyl-nitrosamine	702	2	2 - 3
	7	3	1 - 4
	0.07	1	0
N-nitrosopiperidine	856	2	3
	8.56	2	3 - 4
Diethylamine	967	2	5 - 6
	9.67	2	1 - 3
5BU	100	1	1
	25	1	3
	20	1	0
	2	1	2
	0.02	1	2
NG	650	1	3×10^4
	65	1	1×10^4
	10	2	9.3×10^2
	1	1	1×10^2
	0.1	1	2.4×10^1
	0.01	1	2
	0.001	1	0

DISCUSSION

The ability of the carcinogenic N-nitrosamines tested to induce λ in a lysogenic strain of E. coli provides an excellent screening system for potentially harmful nitrosamines. Previous attempts to use nitrosamines as inducers of E. coli W1709 (λ) have been unsuccessful (Heinemann, 1972). In the system outlined above the minimal concentrations of inducers are similar to those for N-nitrosamidines and N-nitrosamides (Heinemann, 1972) which range from 0.1 to 25 μg./ml. It is of interest to note that the non-carcinogenic diphenylnitrosamine (Boyland, Carter, Gorrod & Roe, 1968) did not induce. However, as this compound was dissolved in CH_2Cl_2 there may have been insufficient contact between bacteria and nitrosamine for induction to occur. This aspect will be studied further as a system which detects only carcinogenic nitrosamines would be very useful. It is thought that nitrosamine carcinogenicity may depend on at least one disposable H atom in the alpha position (Druckrey et al., 1969).

Although all carcinogenic nitrosamines are mutagenic (Magee & Barnes, 1967) the nitrosamines tested were unable to revert his G46. This is in agreement with the theory (Craddock & Magee, 1963) that nitrosamines are metabolised before becoming carcinogenic. Malling (1971) was able to use metabolic products of DMN to revert his^- mutants of S. typhimurium. It is therefore intended to do similar experiments with the nitrosamines at our disposal.

The λ induction system will be used to test for the in vivo formation of nitrosamines by anaerobic bacteria supplied with nitrite and secondary amines. Discharges from patients suffering from cancer

of the uterine cervix contain many bacteria which might synthesize nitrosamines (Harington, Nunn & Irwig, 1973). Non-enzymic *in vitro* formation of nitrosamines by bacteria has been reported by *Streptococcus* (Collins-Thompson, Sen, Aris & Schwinghamer, 1972) and by aerobic soil bacteria (Ayanaba & Alexander, 1973). The *in situ* formation of nitrosamines may well present a potential hazard.

Although the induction test is useful for preliminary investigations and rapid screening, this should in all cases be accompanied by more stringent chemical analyses for the detection of nitrosamines.

APPENDIX II

MEDIA AND SOURCES OF REAGENTS

All percentage compositions were w/v unless otherwise stated.

Achromobacter minimal medium

Salts solution:	K_2HPO_4	10.5 g.
	KH_2PO_4	4.5 g.
	Sodium-citrate.$2H_2O$	0.47 g.
	$(NH_4)_2SO_4$	1 g.

Above salts dissolved in 100 ml. distilled water. To the solution is added 0.1 g. $MgSO_4.7H_2O$. The salts solution is sterilized by filtration.

Glucose: 20% (w/v) is sterilized by filtration.

Water agar:	Oxoid Ion-agar No. 2	12 g.
	NaCl	23.4 g.
	Distilled water	1 ℓ.

Sterilize by autoclaving.

To make up:	Water agar	70 ml.
	Salts solution	8 ml.
	Glucose	1 ml.

For slightly enriched minimal medium add 2.5 ml. nutrient broth per 100 ml. medium.

Acid Orcinol Reagent

	$FeCl_3.6H_2O$	0.05 g.
	Concentrated HCl	100 ml.

Alcohol-Orcinol Reagent

Orcinol	6 g.
95% Ethanol	100 ml.

Alcaligenes minimal medium

NH_4NO_3	0.25 g.
NaCl	8.0 g.
K_2HPO_4	1.5 g.
$MgSO_4$	0.5 g.
Asparagine	2.0 g.
Cysteine	0.02 g.
Distilled water	1 ℓ.

Adjust pH to 7.8.

Bacillus subtilis minimal medium

Minimal salts:	$(NH_4)_2SO_4$	10 g.
	K_2HPO_4	70 g.
	KH_2PO_4	30 g.
	Sodium citrate	5 g.
	$MgSO_4.7H_2O$	1 g.
	Distilled water to	1 ℓ.

Dissolve each salt in turn and store over chloroform at 4 °C.

To make up:	Minimal salts	20.00 ml.
	Distilled water	76.50 ml.
	20% glucose	2.50 ml.
	2% casein hydrolysate	0.50 ml.
	10^{-4} M $MnSO_4$	0.10 ml.

The salts, water and $MnSO_4$ may be autoclaved together, the remaining components must be added asceptically after separate sterilization.

Borate buffer pH 8.6

Borate solution A:	H_3BO_3	62 g.	
	NaOH	20 g.	
	Distilled water to	5 ℓ.	
Borate buffer:	Borate solution A	350 ml.	Borate solution B
	0.1 N HCl	150 ml.	
	NaCl (0.85% w/v)	500 ml.	
	Distilled water	1000 ml.	
Borate sugar:	Sucrose	300 g.	
	Borate solution B	300 ml.	
	Distilled water to	600 ml.	

Adjust to pH 8.6 with about 13 ml. 1 N NaOH.

Carnoy's fluid

Glacial acetic acid	10 ml.
Chloroform	30 ml.
Ethanol	60 ml.

Diphenylamine reagent

Diphenylamine	1 g.
Glacial acetic acid	100 ml.
H_2SO_4	2.7 ml.

The reagent is stored in the dark at 4°C. Before use it is warmed to room temperature.

Escherichia coli minimal medium

Minimal salts:	NH_4Cl	20 g.
	NH_4NO_3	4 g.
	Na_2SO_4 (anhydrous)	8 g.
	K_2HPO_4	12 g.
	KH_2PO_4	4 g.
	$MgSO_4.7H_2O$	0.4 g.
	Distilled water	500 ml.

Dissolve each salt in cold water in this order and wait for complete solution before adding the next one. Store over chloroform at 4 °C.

To make up:	Oxoid Ion-agar No. 2	5 g.
	Minimal salts	31.25 ml.
	10% glucose	5 ml.
	Distilled water	213.75 ml.

Autoclave at 20 lbs. for 20 min.

Glucose broth

Glucose	5 g.
Difco tryptone	13 g.
NaCl	23.4 g.
Distilled water	1 ℓ.

Heart Infusion broth

Difco Bacto-Beef heart for infusion	50 g.
Distilled water	1000 ml.

Infuse at 50 °C for one hr. Heat to 80 °C for 5 min. to coagulate proteins. Filter and add any other ingredients. Boil, filter and autoclave.

Lysozyme spheroplasting medium

Sucrose	171 g.
1 M tris pH 8.0	30 ml.
Distilled water	1 ℓ.

Modified McIlvaine's buffer

Citric acid 0.1 M	6 ml.
Na_2HPO_4 0.15 M	4 ml.

Molybdic acid solution

MoO_3	1 g.

Boil for 1 - 2 min. in 100 ml. distilled water. Cool and decant supernatant from precipitate. Dilute 9:1 before use.

NaCl-EDTA buffer

NaCl	0.15 M
EDTA	0.01 M

Adjust pH to 8.0.

Nutrient broth

Difco nutrient broth	8 g.
NaCl	23.4 g.
Distilled water	1 ℓ.

Pasteurella minimal medium

L-arginine	0.4 g.
Asparagine	0.4 g.
Histidine	0.2 g.
Methionine	0.4 g.
Spermidine	0.04 g.
NaCl	15.8 g.
$MnSO_4$	0.75 g.
$MgSO_4$	6.0 g.
$CaCl_2$	2.94 g.
Tris	6.05 g.
Distilled water to	1 ℓ.

Adjust pH to 6.8.

Phage broth

Difco tryptone	13 g.
NaCl	23.4 g.
Glucose	1.5 g.
Distilled water	1 ℓ.

Phage buffer

Solution A.	KH_2PO_4	7.5 g.
	NaCl	23.4 g.
	Anhydrous Na_2HPO_4	15.0 g.
	K_2SO_4	25.0 g.
	$CaCl_2$	0.055 g.
	Deionised water to	1 ℓ.

Store over chloroform at 4°C.

Solution B.	$MgSO_4.7H_2O$	4.8 g.
	Deionised water	100 ml.
Solution C.	Gelatin	0.2 g.
	Deionised water	100 ml.
To make up:	Solution A	40 ml.
	Deionised water	158 ml.
	Solution B	1 ml.
	Solution C	1 ml.

Phosphate acetate buffer

Na_2HPO_4 0.15 M

Bring to pH 5.5 with 10% (w/v) acetic acid. Mg-acetate added to a concentration of 0.003 M to improve the efficiency of the DNAse.

Saline citrate buffer

Standard saline citrate

NaCl	0.15 M	pH 7.0
Sodium citrate	0.015 M	

Dilute saline citrate

NaCl	0.015 M	pH 7.0
Sodium citrate	0.0015 M	

Concentrated saline citrate

NaCl	1.5 M	pH 7.0
Sodium citrate	0.15 M	

Salt agar

Difco tryptone	13 g.
NaCl	23.4 g.
Glucose	1.5 g.
Oxoid agar No. 3	15 g.
Distilled water	1 ℓ.

Salt agar toplayer

Difco tryptone	10 g.
NaCl	23.4 g.
Glucose	3 g.
Oxoid agar No. 3	6 g.
Distilled water	1 ℓ.

Spheroplast medium

Difco tryptone	13.00 g.
NaCl	23.40 g.
Glucose	1.50 g.
Sucrose	171.15 g.
$MgSO_4.7H_2O$	2.00 g.
Distilled water	1 ℓ.

Transfection medium

Difco nutrient broth	1 g.
Difco yeast extract	0.1 g.
Glucose	0.05 g.
K_2HPO_4	0.2 g.
KH_2PO_4	0.1 g.
NaCl	2.34 g.
Sucrose	17.1 g.
Distilled water	100 ml.

Tryptone agar

Tryptone broth	1 ℓ.
Oxoid agar No. 3	15 g.

Tryptone broth

Difco tryptone	13 g.
NaCl	8.5 g.
Glucose	1.5 g.
Distilled water	1 ℓ.

Vogel-Bonner minimal medium

$MgSO_4.7H_2O$	10.0 g.
Citric acid . H_2O	100.0 g.
K_2HPO_4.anhydrous	500.0 g.
$NaNH_4HPO_4.4H_2O$	175.0 g.
Distilled water	670 ml.

Dilute 50-fold with distilled water and autoclave. Supplement with 5 g./ℓ. glucose autoclaved separately.

Vogel-Bonner minimal agar

Vogel-Bonner minimal medium	1 ℓ.
Oxoid Ion-agar No. 2	15 g.

Water-saturated phenol

Phenol	500 g.
Distilled water	200 ml.
8-hydroxyquinolene	0.7 g.

Leave overnight at 37 °C. Shake well and store at 4 °C in a dark container.

INCUBATION TEMPERATURE

This was 30 °C unless otherwise specified.

SOURCES OF REAGENTS AND CHEMICALS

Amino acids	British Drug Houses Ltd., Poole, England (BDH)
Ammonium acetate	E. Merck A.G., Darmstadt, Germany (Merck)
5BU	Koch-Light Laboratories, Colnbrook, England
Casamino acids	Difco Laboratories, Detroit, Michigan, U.S.A.
Collagenase	Koch-Light
Cytochrome c	BDH
Diphenylamine	BDH
DNAse	Miles-Seravac, Cape Town, South Africa
EDTA	Merck
Folin-Ciolcateau reagent	BDH
Glycine	Merck
Iso-amyl alcohol	BDH
Iso-propanol	Merck
Lysozyme	BDH
Methyl cellulose	Merck
Mitomycin C	Calbiochem, San Diego, California, U.S.A.
Nalidixic acid	Winthrop Laboratories, Mobeni, Durban, S.A.
Nitrosoguanidine	Koch-Light
Orcinol	BDH
Palladium-gold (40%)	Johnson Matthey (Pty.) Ltd., Wadeville, Transvaal
Phenol red	BDH
Potassium phosphotungstate	Hopkin & Williams, Ltd., Essex, England
Pronase	Miles-Seravac
RNAse	Miles-Seravac
Sodium benzyl-penicillin	Glaxo-Allenbury (S.A.), Wadeville, Transvaal, S.A.
Sodium dodecyl sulphate	BDH
Sodium perchlorate	Merck
Spermidine	Koch-Light
Streptomycin sulphate	Hoechst Pharmaceuticals (Pty.) Ltd., Johannesburg
Trypsin	BDH

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