Physiological and Morphological Characteristics of Stationary Phase *Vibrio* Cells Able to Support Phage Growth

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(Received 14 November 1979; revised 5 March 1980)

Growth of phage $\alpha 3a$ on stationary phase *Vibrio* cultures requires micro-aerophilic conditions and is inhibited by aeration. Since pre-conditioning of the bacteria by allowing them to stand for 24 h after shaking for 3 d is an important aspect of the stationary phase phage growth system, various physiological and morphological characteristics of the stationary phase cells during the transition from shaking to standing were investigated. Shaken stationary phase cells were less viable and more sensitive to ultraviolet irradiation and heat than standing stationary phase cells. During pre-conditioning the small, non-flagellated cells present in shaken stationary phase cultures underwent morphological changes and became large, flagellated rods which resembled exponential phase cells. The transition of stationary phase cells from shaking to standing was associated with a marked increase in total RNA synthesis but a rapid and large decrease in total protein synthesis. Intracellular concentrations of ATP in shaken stationary phase cells were 53 % lower than those in standing stationary phase cells. Studies on leucine uptake indicated that its transport was inhibited by isoleucine and that the major part (90 %) of the total leucine uptake was due to a shared system for uptake of both amino acids. Shaken stationary phase cells transported less leucine than standing stationary phase cells. Inhibition of phage growth in aerated stationary phase cultures was not due to the prevention of phage adsorption by shaking. It is suggested that the observed differences between shaken and standing stationary phase cells could be due to aeration affecting the template specificity of the *Vibrio* RNA polymerase.

INTRODUCTION

Bacteriophage growth specifically requiring stationary phase bacteria is a unique phenomenon, first reported by Woods (1976) who described a system in which phage $\alpha 3a$ grows on stationary phase *Achromobacter* strain 14 mutant cells but is unable to develop in exponential phase cells of the same strain. The parent strain was isolated from hides and is an aerobic, halotolerant, collagenolytic Gram-negative bacterium which was originally classified as an *Achromobacter* strain (Thomson *et al.*, 1972). This identification was confirmed by the National Collection of Industrial Bacteria, Aberdeen, Scotland, but has since been reinvestigated and the organism is now classified as a *Vibrio* strain.

In addition to the original strain 14 mutant described by Woods (1976), we isolated a rifampicin-resistant mutant which supported phage $\alpha 3a$ growth in both exponential and stationary phase cells (Robb *et al.*, 1977). The rifampicin-resistant mutant had an altered RNA polymerase and it was suggested that the phenomenon of stationary phase phage growth could be due to a change in the template specificity of the bacterial RNA polymerase.

The micro-environment is very critical for stationary phase phage growth and the system requires micro-aerophilic conditions (Woods, 1976; Robb *et al.*, 1977). Stationary phase phage growth occurs in standing cultures but not in either shaken (aerated) or stringent anaerobic cultures (Woods, 1976). Robb *et al.* (1978) investigated the kinetics of phage $\alpha 3a$ growth on stationary phase cells and reported that the system is characterized by a long and variable latent period of 6 to 9 h and an increased burst size of 710 plaque-forming units (p.f.u.) cell⁻¹ as compared with about 150 p.f.u. cell⁻¹ in exponential phase wild-type cells. An important aspect of the stationary phase phage growth system involved pre-conditioning the cells by allowing them to stand for 24 h after shaking for 3 d. Shaken stationary phase cells do not support phage growth and the 6 to 9 h latent period is increased to 18 h when 3 d shaken cultures are not pre-incubated without aeration for 24 h (not pre-conditioned) before the addition of phage. Stationary phase cells which have been allowed to stand retain the ability to be infected and to support phage growth for at least 16 d. Shaken cultures gradually lose the ability to support phage growth (after standing for 24 h) but the phage can persist in the host cell for 10 d until removal from shaking when the lytic cycle can proceed after allowing the cultures to stand.

Since pre-conditioning of the stationary phase cells is so important for the novel phage growth system, we have studied various physiological and morphological characteristics of the stationary phase cells during the transition from shaking to standing. As phage growth requires the synthesis of proteins and nucleic acids, the effect of aeration on the synthesis of macromolecules and intracellular ATP concentrations was investigated. The bacterium and related *Vibrio* strains produce extracellular collagenase and proteases in aerated stationary phase cultures (Thomson *et al.*, 1972; Lecroisey *et al.*, 1975). Since enzymes are available in shaken cultures for the degradation and recycling of amino acids from extracellular proteins, the effect of aeration on an amino acid transport system was investigated. Furthermore, the possibility that differences in protein synthesis could be due to variations in the uptake of amino acids was examined. The general viability of standing and shaken stationary phase cultures and their ability to adsorb phage were also determined.

METHODS

Media. The tryptone medium of Thomson & Woods (1973) was used for bacterial growth and for double-layer phage assays. Incubation was at 30 °C.

Bacteria and bacteriophages. The mutant strain previously isolated and classified as an *Achromobacter* strain 14 but recently reclassified as a *Vibrio* strain 14 was used (Woods & Thomson, 1975; Woods, 1976). This strain was used for stationary phase phage growth and for the physiological and morphological studies. The wild-type *Achromobacter* sp. 2 (Thomson *et al.*, 1972) which has been reclassified as the wild-type *Vibrio* sp. 2 was used as the indicator strain for plaque assays. The double-stranded DNA phage $\alpha 3a$ isolated by Thomson & Woods (1974) was grown on the *Vibrio* strains.

Stationary phase cells. Stationary phase *Vibrio* strain 14 cultures were routinely prepared by growing the cells in 600 ml tryptone broth in a 1 l conical flask on a Gallenkamp rotary shaker at 250 rev. min⁻¹ for 3 d (about 2×10^9 cells ml⁻¹) (Robb *et al.*, 1978). The culture was then divided: aeration of one half was continued (shaken culture) while the other half was dispensed in 3 ml volumes in test tubes (12 $\times$ 75 mm) or 10 ml volumes in 28 ml screw-capped containers and incubated aerobically without shaking (standing cultures). The conditions obtained after 24 h standing have been shown to be optimal for stationary phase phage growth (Robb *et al.*, 1978). The viability of shaken and standing stationary phase cells was determined by plating at different times after the initial 3 d aeration.

Ultraviolet and heat inactivation. Standing and shaken stationary phase *Vibrio* strain 14 cells were diluted 100-fold in cold phosphate buffer pH 7.2 (2×10^7 cells ml⁻¹) and heated at 60 °C or irradiated with a Hanovia filtered germicidal lamp which emits > 85 % of its output at 254 nm at an intensity of 0.55 J m⁻² s⁻¹ at a distance of 22 cm. Cell samples (7 ml) were irradiated in open Petri dishes which were continuously agitated. Irradiation was carried out in the dark and post-irradiation manipulations in subdued light and the plates were incubated in the dark. After heating or irradiation for different periods, the cells were diluted and plated.

Phage adsorption. Phage $\alpha 3a$ was added to shaken and standing stationary phase cultures (2×10^9 cells ml⁻¹) at a multiplicity of infection of 10^{-3} and incubated at 30 °C. Samples were removed at different times,

diluted 1000-fold, treated with toluene for 15 min at room temperature and assayed for free phage (Robb *et al.*, 1978).

Nucleic acid synthesis. The amount of DNA and RNA synthesis in shaken and standing stationary phase cells, as well as aerated exponential phase cells, was determined as described previously (Woods, 1976). RNA synthesis was determined by incorporation of [^3H]uracil [$2\ \mu\text{g ml}^{-1}$, $0.4\ \mu\text{Ci ml}^{-1}$ ($15\ \text{kBq ml}^{-1}$)] into trichloroacetic acid (TCA)-precipitable material. As the *Vibrio* strains did not incorporate [^3H]thymidine, DNA synthesis was determined by incorporation of [^3H]adenine [$2\ \mu\text{g ml}^{-1}$, $0.4\ \mu\text{Ci ml}^{-1}$ ($15\ \text{kBq ml}^{-1}$)] into NaOH-hydrolysed TCA-precipitable material. The sampling procedure for assaying nucleic acid synthesis in standing stationary phase cells was the same as that described for protein synthesis determinations (see below).

Protein synthesis. Protein synthesis was measured by the incorporation of [^{14}C]leucine into TCA-precipitable material (Eichenlaub & Winkler, 1974). Unlabelled leucine ($10\ \mu\text{g ml}^{-1}$) and [^{14}C]leucine ($1\ \mu\text{Ci ml}^{-1}$, $37\ \text{kBq ml}^{-1}$) were added to 3 ml shaken and standing stationary phase cultures. For assaying leucine incorporation in the standing cultures, leucine was added to a number of identical 3 ml cultures at time 0. At each sampling time, samples were taken from a different culture which was then discarded. This was to ensure that the aeration conditions of the standing cultures were not altered during sampling as Robb *et al.* (1978) showed that standing cultures which were repeatedly opened and moved during sampling did not support phage growth.

Intracellular ATP concentrations. Intracellular ATP was measured by the luciferin-luciferase assay by counting the flashes of light emitted by mixtures of cell extracts and firefly lantern extracts (Sigma) by the method of Foulds (1971).

Leucine transport. Exponential and stationary (shaken and standing) phase cells were washed twice by centrifugation and resuspension in phosphate buffer pH 6.9 at room temperature. The cell density was adjusted turbidimetrically to 1×10^9 cells ml^{-1} . The leucine-specific and LIV (leucine, isoleucine and valine) systems (Wood, 1975) were assayed essentially as described by Rahmanian *et al.* (1973). Cells ($50\ \mu\text{l}$) were added to $50\ \mu\text{l}$ of double-strength transport buffer and held for 60 s before starting the reaction by adding $50\ \mu\text{l}$ [^{14}C]leucine (final concentration $10\ \mu\text{M}$). All the assay mixtures contained chloramphenicol ($50\ \mu\text{g ml}^{-1}$), glucose (0.5 %, w/v) as the energy source and 0.4 M-NaCl. Transport was measured at 20°C and the reactions were stopped after 15, 30, 45, 60 and 120 s by filtration of $100\ \mu\text{l}$ samples and washing with buffer on $0.45\ \mu\text{m}$ (pore size) membrane filters (Gelman Instrument Co.). Each nmol leucine was represented by 209000 c.p.m. The leucine and LIV systems were assayed together in the absence of isoleucine and the leucine-specific system was measured by leucine uptake in the presence of $200\ \mu\text{M}$ -isoleucine which completely inhibits the LIV system in *Escherichia coli* (Rahmanian *et al.*, 1973). The LIV system was determined by difference. Protein was determined by the Folin method of Lowry.

Cell morphology. The morphologies of exponential and stationary (shaken and standing) phase cells were investigated. Wet mounted specimens were observed with a Zeiss photomicroscope fitted with phase contrast optics. Glutaraldehyde-fixed and metal-shadowed specimens were examined with a Jeol JSN U3 scanning electron microscope. Metal-shadowed bacterial suspensions were observed with a Hitachi HU 11B transmission electron microscope.

RESULTS

Viability of stationary phase cells. The viability of stationary phase cells was affected by aeration (Fig. 1). Shaken stationary phase cells showed an immediate and almost exponential decrease in viable count from 2.7×10^9 to 1.5×10^7 cells ml^{-1} after 18 d (0.55 % survival). The survival of standing stationary cultures was characterized by a shoulder from 3 to 7 d and after 18 d the viable count was 2×10^8 cells ml^{-1} (8.33 % survival). The total microscopic cell count remained constant in both the shaken and standing cultures and lysis was not observed.

Ultraviolet and heat inactivation. Standing stationary phase cells were more resistant to u.v. irradiation than shaken stationary phase cells (Fig. 2). The u.v. survival curve of standing cells was characterized by a pronounced shoulder and 66 % of the bacteria survived after 60 s irradiation compared with a survival of 0.1 % in the shaken culture. Standing stationary phase cells were also more resistant to inactivation by heat. After 30 min at 60°C the viable counts had decreased from 1.7×10^7 to 6.3×10^3 and $< 1 \times 10^2$ cells ml^{-1} for standing and shaken cultures, respectively.

Phage adsorption. Phage $\alpha 3a$ adsorption to shaken stationary phase cells was greater than to standing stationary phase cells. The percentage of phage adsorbed after 30 min with

Fig. 1

Fig. 2

Fig. 1. Viability of stationary phase aerated and non-aerated *Vibrio* strain 14 cultures. A culture was grown with shaking for 3 d before being divided: aeration of one half was continued (○) while the other half was dispensed and allowed to stand (●).

Fig. 2. Survival of u.v.-irradiated *Vibrio* strain 14 stationary phase cells. Shaken (○) and standing (●) stationary phase cells were diluted 100-fold in phosphate buffer (2×10^7 cells ml⁻¹) and u.v.-irradiated at 254 nm at an intensity of $0.55 \text{ J m}^{-2} \text{ s}^{-1}$.

shaken cells was 54 % (average of five experiments) compared with 44 % (average of five experiments) with standing cells.

Nucleic acid synthesis. Woods (1976) investigated nucleic acid synthesis in stationary phase cells and reported that DNA synthesis was not observed in shaken or standing stationary phase cultures. No RNA synthesis was detected in shaken stationary phase cultures but RNA synthesis occurred in standing stationary phase cultures. The incorporation of [³H]uracil into standing stationary phase cells over 60 min was 50 % of that into exponential phase cells. As Robb *et al.* (1978) had shown that the sampling technique for detecting stationary phase phage growth markedly affected phage growth, it was considered important to re-examine nucleic acid synthesis using their technique. Although different sampling techniques were utilized the results obtained by Woods (1976) were essentially confirmed except that very low levels of DNA synthesis (0.88 % of that in exponential cultures after 60 min) were detected in shaken stationary phase cultures.

Protein synthesis. Studies on the incorporation of [¹⁴C]leucine into protein indicated that protein synthesis was markedly reduced in standing stationary phase cultures compared with that in shaken stationary phase cultures (Fig. 3). In seven different experiments protein synthesis in standing stationary phase cultures never exceeded 4 % of that in shaken stationary phase cultures. Protein synthesis in shaken stationary phase cultures was 38.5 % of that in exponential phase cultures after 30 min incorporation of [¹⁴C]leucine. In standing stationary phase cultures protein synthesis was 0.5 % of that in exponential phase cultures. An experiment was carried out to determine whether the low [¹⁴C]leucine incorporation into proteins in standing stationary phase cells was attained gradually during the 24 h standing period. A 3 d old shaken culture was dispensed into standard containers (10 ml) and into 12 × 75 mm test tubes (3 ml) and placed at 30 °C. Incorporation of [¹⁴C]leucine was measured over 30 min in samples which had been standing for 0 to 24 h. Incorporation of [¹⁴C]leucine immediately after dispensing and standing was 2 % of that in the parent shaken culture. From 0 to 24 h there was no variation in the level of protein synthesis in the standing cultures. There was therefore an immediate and almost total reduction in protein synthesis in changing from shaking to standing conditions.

Intracellular ATP concentrations. Intracellular ATP concentrations of shaken exponential phase, standing stationary phase and shaken stationary phase cells were, respectively, 54.4,

Fig. 3. Protein synthesis in shaken and standing stationary phase *Vibrio* strain 14 cultures. Incorporation of [^{14}C]leucine in shaken ($\circ$) and standing ($\bullet$) cultures.

Fig. 4

Fig. 5

Fig. 4. Leucine uptake by exponential phase wild-type *Vibrio* sp. 2 cells in the presence of different concentrations of isoleucine. Uptake of [^{14}C]leucine by cells grown in the absence ($\circ$) or presence ($\bullet$) of leucine (5 mg ml^{-1}).

Fig. 5. Leucine uptake by stationary phase *Vibrio* strain 14 cells. Uptake of [^{14}C]leucine in shaken ($\bullet$) and standing ($\blacksquare$) stationary phase cells without isoleucine and in shaken ($\circ$) and standing ($\square$) stationary phase cells in the presence of $200 \mu\text{M}$ -isoleucine.

43.9 and $23.3 \text{ pmol (mg protein)}^{-1}$. Thus, ATP concentrations in standing stationary phase cells were 80 % of those in exponential phase cells, and concentrations were 53 % lower in shaken stationary phase cells than in standing stationary phase cells.

Leucine transport. As the kinetics of leucine uptake in *Vibrio* cells had not been investigated previously it was initially studied in exponential phase *Vibrio* wild-type cells which had been grown in minimal medium (Woods & Thomson, 1975). The uptake of leucine over 30 s was measured with increasing concentrations of isoleucine in the assay mixture (Fig. 4). Isoleucine inhibited 90 % of the leucine uptake (major transport system) but did not affect the remaining 10 % of leucine transport activity.

The growth of *Vibrio* cells in a medium containing $5 \text{ mg leucine ml}^{-1}$ enhanced the major

transport system (Fig. 4) which has a K_m of approximately $2 \mu\text{M}$ -leucine (unpublished results). Uptake rates for cells grown in tryptone and minimal medium were 0.06815 and $0.05532 \text{ nmol leucine } (A \text{ unit})^{-1} \text{ min}^{-1}$, respectively. The levels of active transport of leucine into stationary phase *Vibrio* strain 14 cells were low in shaken cultures compared with standing cultures (Fig. 5). After 120 s cells from the standing culture had transported about four times more [^{14}C]leucine across the membrane than cells from the shaken culture. As in exponential phase cells the major leucine transport system was inhibited by isoleucine and reduced to about 10 % of that measured without isoleucine in cells from both standing and shaken cultures.

Cell morphology. Exponential phase cells were large, flagellated (peritrichous), motile rods. Many dividing cells were observed in exponential phase cultures. In contrast, shaken stationary phase cells were small (about $1.3 \times 0.7 \mu\text{m}$), non-flagellated and non-motile. After standing for 24 h the cells increased in size (about $3 \times 1.25 \mu\text{m}$), resynthesized an abundance of peritrichous flagella and resembled exponential phase cells. Although cells from standing cultures were motile they tended to form filaments of cells linked end to end. By 12 h after the addition of phage $\alpha 3a$ the cells from standing cultures had increased slightly in width, become highly motile and the filaments had disappeared.

DISCUSSION

Studies on the transition from shaking to standing in stationary phase *Vibrio* cells were undertaken with a view to understanding how vigorous aeration inhibited $\alpha 3a$ phage development during the stationary growth phase. Adsorption of phage was better in aerated than in standing stationary phase cultures. Therefore, the inhibition of phage development in shaken cultures is not due to inhibition of phage adsorption.

Interesting changes in RNA and protein synthesis occur when shaken stationary phase cells are allowed to stand. RNA synthesis is not detected in shaken stationary phase cells. These cells are not able to support phage growth in contrast to standing stationary phase cells. A possible explanation is that phage growth is prevented in shaken stationary phase cells because transcription is completely or partially inhibited by aeration. Complete inhibition of RNA synthesis is unlikely as the high rates of protein synthesis over long periods of aeration indicate that mRNA synthesis is occurring. The synthesis of unstable mRNA can be difficult to detect in stationary phase cells (Nierlich, 1978). Alterations in template specificity of the *Vibrio* RNA polymerase may be affected by aeration and as a result the phage genome is not transcribed. In a similar way, variations in template specificity of the *Vibrio* RNA polymerase were suggested by Robb *et al.* (1977) to account for the isolation of a rifampicin-resistant mutant with an altered RNA polymerase which supported $\alpha 3a$ phage growth in stationary phase cells. The ability of the RNA polymerase from shaken and standing stationary phase cells to synthesize RNA from a purified $\alpha 3a$ DNA template *in vitro* is being investigated to determine whether aeration affects template specificity. Stationary phase cells are capable of adapting to the environment by changes at the transcriptional level (Mandelstam, 1957) and bacterial endospore formation is a morphological example of this type of differentiation (Pine, 1972).

It is not known whether the stimulation of RNA synthesis in standing stationary phase cells represents proportionate increases in mRNA, rRNA and tRNA, whether one class is favoured or what role degradation of RNA plays in this apparent stimulation. It is likely that the increase observed in RNA synthesis is due to changes in the regulation of stable RNA since mRNA usually constitutes a minor fraction of total bacterial RNA and is independent of growth rate in *E. coli* (Forchhammer & Lindahl, 1971; Norris & Koch, 1972). The increase in total RNA synthesis on standing indicates the presence of a reserve transcriptional capacity but the control of this synthesis is not known and could involve the concentration of RNA polymerase, ATP, GTP and ppGpp (Nierlich, 1978). Studies are

being undertaken to determine the levels of RNA polymerase and ppGpp in shaken and standing stationary phase cells.

The finding that protein synthesis showed an opposite response to that of RNA synthesis when stationary phase cells were removed from aeration was unexpected. During growth, bacteria respond to changes in the environment by increasing or decreasing RNA and protein proportionately over the same period of time (Mandelstam, 1960). The stringent response caused by the starvation of an amino acid results in the inhibition of protein synthesis and a reduction in RNA synthesis. It was suggested previously that aeration could affect the control of transcription in stationary phase *Vibrio* cells. The inability to control the template specificity of RNA polymerase in aerated stationary phase cells could also account for the high levels of protein synthesis in the small unhealthy cells from shaken stationary phase cultures. Non-specific and erroneous transcription in aerated stationary phase cells could result in a 'sick' uncontrolled protein synthesis similar to that in relaxed mutants (Hall & Gallant, 1972; Travers, 1976). Micro-aerophilic conditions could enhance the regulation of transcription such that only appropriate proteins are made resulting in the transition from small unhealthy non-flagellated cells to large healthy flagellated rods which support phage growth. The ability of standing stationary phase cells to carry out essential activities more efficiently is emphasized by the greater viability of the cells, increased resistance to u.v. irradiation and heat, higher ATP concentrations and increased leucine transport. The regulation of all these physiological responses could be controlled by the effect of aeration on transcription in stationary phase cells. It is more likely that aeration will affect a single essential core process rather than a component of each individual activity.

As the different levels of protein synthesis in shaken and standing stationary phase cells could have been due to variations in the ability of the cells to transport [^{14}C]leucine, it was important to determine the effect of aeration on leucine uptake. The reduced incorporation of [^{14}C]leucine into protein in standing stationary phase cells is not due to the inability of the cells to transport leucine. Standing cells were actually shown to have a far greater capacity for leucine uptake than shaken cells.

The *Vibrio* strain is highly proteolytic and produces proteases in aerated stationary phase cultures which could be utilized for the degradation of pre-formed proteins and the recycling of amino acids. The inability to grow phage in aerated stationary cells could be due to amino acid starvation as a result of the inhibition of the transport of the amino acids released by the proteases. Leucine uptake was studied as an example of an amino acid transport system. Aeration of stationary phase cells markedly reduced leucine transport but it was not completely inhibited. As high concentrations of [^{14}C]leucine are incorporated into proteins in shaken stationary phase cells, it is suggested that the levels of amino acids are not limiting and that the low level of leucine uptake is sufficient.

Since these are the first studies on leucine transport in a *Vibrio* strain, it is interesting to compare them with similar studies in *E. coli*. The kinetics of inhibition of leucine transport by isoleucine were similar to those of *E. coli*. The major component of the transport system represented 90 % of the total leucine uptake and is a shared system for leucine and isoleucine uptake, analogous to the *E. coli* LIV system (Wood, 1975). The residual 10 % of transport activity represented the minor leucine-specific system which probably has a high affinity for leucine. Growth of the *Vibrio* cells in a medium containing leucine increased the major transport system whereas in *E. coli* it is repressed by growth in leucine (Wood, 1975). The K_m for the shared transport system is similar in both *Vibrio* and *E. coli*.

Nierlich (1978) suggested that the controls of slowly growing *E. coli* cells have evolved to provide rapid adaptability. The ability of stationary phase *Vibrio* cells to react immediately to aeration conditions is demonstrated by the rapid decrease in protein synthesis on removal from shaking. Although an improvement in the environmental conditions is suggested by the increase in cell size, production of flagella and more resistant cells, there is a decrease in protein synthesis which differs from slowly growing *E. coli* cells where an immediate

increase in the rate of protein synthesis occurs after enrichment (Koch & Deppe, 1971). The substantial physiological and morphological changes represent a stationary phase differentiation or regulation system which is controlled by aeration. Few studies have been carried out on the regulation of non-sporulating stationary phase cells and the control mechanisms in stationary phase cells may be different from those operating in growing cells (Koch & Deppe, 1971; Nierlich, 1978). The stationary phase phage growth system provides a unique experimental system for studying differentiation and possible changes in template specificity in a non-sporulating Gram-negative bacterium.

D. R. W. acknowledges research grants from the South African Council for Scientific and Industrial Research.

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