ANALYSIS OF BACTERIOPHAGE JLΦ1 INDUCTION IN THE LYSOGENIC HOST BACTERIUM *JANTHINOBACTERIUM LIVIDUM*

THESIS

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Most of all, I would like to thank my fiancé, Lexis Little, my mother, Tashya Schwarzlose, and the rest of my family for their enduring love and scholarly discourse. For nothing exults you or humbles you, like family. Also, I am grateful for the support of my grandfather, Sam Holloman, who helped me financially during my academic years. And last, but not least, I would like to thank *Janthinobacterium lividum*, a rather malodorous, yet extremely intriguing bacteria, that provided me with hours upon hours of frustration, revelation, and jubilation.

My work is dedicated to my father, Rodney E. Schwarzlose, who instilled in me every tool that I would ever need to succeed.

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INTRODUCTION

Studies of the genetics and biology of viruses have led science to the greater understanding of the basic biological processes of life (44). It was through the study of viruses that DNA was discovered to be the carrier molecule of genetic information and that mRNA was an intermediate molecule in the transfer of genetic information to ribosomes (44). Bacteriophage models contributed to the definition and mapping of the first gene, the discovery of the discontinuous nature of DNA replication, and the unraveling of the mechanics of gene regulation (44). Basic research on the biology of bacteriophages has been essential in the establishment of the field of molecular biology and in biotechnology (44). The lytic phages have remarkable antibacterial activities and have been used successfully in therapy (38). Recent studies have shown that bacteriophages interact with planktonic microorganisms in aquatic environments (7, 8, 15, 18, 44). In addition, bacteriophage are an influential factor in population control of bacteria and transduction-mediated genetic exchange (40). VSH-1 virions, for example, package DNA of *Serpulina hyodysenteriae* and are capable of transferring these host genes to neighboring cells (17). Bacteriophage have even been utilized as indicators for the presence of enteric viruses in public groundwater reservoirs (23). It is important for the scientific community to continue research in the relationships between bacteriophages and their hosts in the search for new discoveries and innovations.

Bacteriophage Virulence and Lysogeny

Most bacteriophages are virulent, and multiply vegetatively, thus killing the infected cell at the end of the bacteriophage growth cycle phase (9). Temperate phages, however, can undergo a process known as lysogeny (9). Lysogeny is a process by which viral DNA persists within the host DNA without phage reproduction. This noninfectious form of the virus is known as a prophage (9). The viral DNA replicates along with the host DNA, thus producing daughter cells with an identical prophage (9). Lysogeny is beneficial to the host through the expression of phage genes (41). Phage genes code for toxin production, antibiotic resistance, expanded metabolic capabilities, and homoimmunity (resistance to superinfection by the same or closely related phages) (41). Lysogeny, has been proposed as a means of survival for viral populations that are threatened by poor host cell abundance, a situation in which a lytic infection would deplete the host population (37). Some temperate phages, such as those which integrate into *Escherichia coli* chromosomes, maintain the lysogenic state via a lysis-lysogeny transcriptional regulation system (32). Maintenance of the lysogenic state is regulated by the CI repressor in phage λ, which doubles as a preventative measure against the propagation of a superinfecting phage (32).

The viral DNA in a lysogenic cell will, occasionally, initiate vegetative multiplication under certain conditions (21). Transition of a lysogenic cell to a lytic cell can either be induced by UV light or other DNA-damaging agents (i.e. mitomycin C), or by spontaneous switching, which happens approximately once every 10^4 cell divisions (21). In the case of toxigenic *Vibrio cholerae,* high titers of CTX Φ bacteriophage are produced upon exposure to mitomycin C (11). These cell-free phage particles can then

infect and convert susceptible nontoxigenic *V. cholerae* strains into toxigenic strains (11). Some bacterial cells induce lysis spontaneously, such as *Burkholderia thailandensis* E125 (45). Once a cell lyses, it releases virions which will infect susceptible host cells (9). In known phage-host systems, the susceptible cells lack the prophage (21). The other lysogenic bacterial cells of the culture which harbor the prophage are not susceptible to infection and are said to be immune (9).

A sudden change in the growth rate of a lysogenic bacterium, as a result of improved growth conditions, has also been shown to result in the induction of prophage (44). Studies have shown that the frequency of prophage induction is primarily an effect of the growth conditions of the lysogenic host (25). Moreover, high induction frequencies have been observed under conditions of optimal cell growth (25). In a study performed by Lunde, phage induction rates were found to increase during exponential growth and reach maximum levels during early stationary phase (26). Furthermore, the spontaneous induction of phage was found to be dependent upon the growth phase of the bacterium (26).

Pseudolysogeny

Recent studies have revealed a new type of carrier state of the viral genome known as pseudolysogeny (35). Ripp and Miller describe the pseudolysogenic state as an environmental condition in which starved bacterial cells coexist in an unstable relationship with the infecting viral genomes (35). Under very poor nutrient conditions, host cells do not provide sufficient energy for phage to enter into a true lysogenic or into a lytic cycle (35). When nutrients are replenished, the viral genome establishes either true

lysogeny or becomes lytic (35). Ackermann and Dubow defined pseudolysogeny as a phenonmenon in which phage is constantly being produced in the presence of a high host cell abundance (1). Pseudolysogeny is also ascribed for cultures with high concentrations of plaque forming units with similar turbidities to cultures lacking infectious phage particles (31). The virus is able to coexist with exponential host cell growth and enter either a dormant intracellular phase or become lytic. The phage genome, however, never integrates into the host DNA (1). Conditions that favor phage and host production are inverted (44). Infection at high nutrient concentrations results in lytic growth, whereas, infection at low nutrient concentrations results in pseudolysogeny (44). This type of relationship ensures phage survival when nutrients are low and host growth is poor (44).

Population Density

Observations of lysogenic phage-host systems *in vitro* have led to the conclusion that susceptible lysogenic host cell populations must reach a critical density before phage induction can occur (19). Kasman et al suggest that this threshold density is a requirement of the phage for the host cell to be in a particular metabolic state and that this state is only reached when the cell density is greater than or equal to 10^4 CFU ml^{-1} (19). Some cells monitor their population density through soluble quorum sensing factors or autoinducers (43). At a critical population density, these molecules reach sufficient concentration to alter the expression of genes and thereby regulate the metabolic state of the cells (19). Accumulation of quorum sensing factors, such as acyl-homoserine lactone derivatives, can thus explain the dependence of phage replication on cell density. For

example, proteins that serve as phage receptors may be expressed in response to quorum sensing factors (19).

Quorum Sensing

Quorum sensing is a cell-cell signaling process that enables bacterial populations to collectively control gene expression (46). This process allows for synchronization of activities among the entire bacterial population that are productive only at high population densities (46). A diverse collection of gram-negative bacteria produce acylated homoserine lactone derivatives that function in quorum sensing (13).Violacein production, for example, has been linked to the presence of N-acyl-homoserine lactones (AHLs), which act as quorum sensing signal molecules in *C. violaceum* (28). At low population density, *C. violaceum* colonies are colorless. Following an increase in cell density, quorum sensing target genes are activated and the colonies take on a purple pigmentation (28).

For many years only one quorum sensing system, the LuxI/LuxR circuit, was believed to be involved in cell-cell communication (43). In the LuxI/LuxR signaling system, AHL signal molecules, termed autoinducers, are produced by LuxI proteins (43). These autoinducers then diffuse through the bacterial cell envelope and accumulate at high cell densities (43). Once a sufficient concentration of autoinducers has been produced, the LuxR proteins bind the AHL signal molecules (43). The LuxR-autoinducer complexes then bind target gene promoters and activate transcription (43). Studies have shown that there are many homologous circuits of this LuxI/LuxR system in over 50 species of gram-negative bacteria (43).

Recent studies suggest that some bacterial species possess species-specific signaling processes (i.e. LuxI/LuxR), as well as an additional interspecies signaling system (46). This second type of autoinducer system, termed AI-2, has been shown to be widely distributed in the bacterial domain and to control a variety of traits in different bacteria (46). AI-2 is synthesized by LuxS and detected by two proteins LuxP and LuxQ, which function together as an AI-2 biosensor (16). LuxP is similar to a periplasmic ribose binding protein, and LuxQ is a hybrid two-component protein which contains a sensor kinase and a response regulator domain (16). Unlike the LuxI/LuxR-like autoinducers, which accumulate late in the stationary phase, extracellular AI-2 activity peaks in mid to late exponential phase and declines precipitously in stationary phase (46).

A third type of system, which acts in parallel with a LuxI/LuxR-like (AI-1) system and the AI-2 system, has been discovered in *Vibrio harveyi* (16). *V. harveyi* produces a CqsA-dependent autoinducer CAI-1 and a corresponding sensor CqsS similar to the AI-1 system found in *Vibrio cholera* (16). *V. harveyi* uses this third quorum-sensing system, in parallel to the AI-1 and AI-2 systems, to act as a "three-way coincidence detector" in the regulation of a variety of genes (16).

Janthinobacterium lividum

Similar to *C. violaceum*, *Janthinobacterium lividum* is an aerobic Gram-negative rod which occurs either singly, in pairs or short chains (22). It is motile by means of a single polar flagellum and usually one to four lateral flagella (22). The bacterium produces low, convex, round, violet colonies on solid media, and a violet pellicle in broth (22). *J. lividum* grows at an optimal temperature of 25°C, and a pH of 7-8, however, it

cannot grow in media with more than 6% NaCl (22). This bacterium is common in soil and water in temperate regions (22).

J. lividum is an environmentally significant bacterium. It has been shown to be a metallo-beta-lactamase-producer upon exposure to beta-lactams (36). It has also been reported to naturally degrade phenanthrene, an environmental contaminant (5). The bacterium has even been shown to secrete an extracellular chitinase (14). Chitinase has antifungal properties, giving the bacteria an advantage when competing for resources (30). Furthermore, *J. lividum* has proven successful as a denitrifying microorganism for the removal of nitrate from groundwater (20). This bacterium is an opportunist and has been shown to cause septicemia (34).

Violacein

Isolates of *J. lividum* and *C. violaceum* exhibit acute toxicity to nanoflagellates due to the production of violacein (27). This finding suggests that violacein-producing bacteria, such as *J. lividum* and *C. violaceum,* are an important factor in the population dynamics of bacterivorous protists which, in turn, decrease the grazing pressure on the surrounding bacterial community (27). According to Bergey's Manual of Systematic Bacteriology, violacein production may be suppressed by certain brands of peptone (22). Violacein has been shown to have antibacterial, antitumoral and anti-*Trypanosoma cruzi* activities (3). Violacein is a weak inhibitor of herpes simplex virus type I strain KOS and ATCC/VR-733, and poliovirus type 2 (3).

Present Work

Preliminary studies have shown that *J. lividum* contains a prophage that can spontaneously be induced in growing culture. The aim of this study was to analyze the conditions that lead to the induction of the prophage, designated JLΦ1. Since media composition may affect the growth and life cycle patterns of a bacteriophage-host system, both nutrient-rich and nutrient-poor media were used throughout this study. Here I report on the effect of nutrient limiting conditions and population density of *J. lividum* on the induction of bacteriophage JLΦ1. A pigmentless mutant of *J. lividum* was included in the study to determine if violacein had an effect on phage induction. The production and effect of spent media which contain autoinducers by *J. lividum* on phage induction was also determined.

MATERIALS AND METHODS

Bacterial strains and media. *Janthinobacterium lividum* wild-type strain was isolated from waters of the Edwards Aquifer in Rattlesnake Cave (San Marcos, TX). Sterilized limestone rocks were suspended in the aquifer for 5 days. Bacterial colonies were isolated on R2A medium (Difco, Sparks, MD) following a 48h incubation at 25C. Colonies that produced a purple pigment were purified on R2A agar. The sequence of a 1500bp fragment of the of 16S rRNA gene from the bacterium (MIDI Labs, Newark, DE) was compared to sequence in the NCBI database using Basic Local Alignment Search Tool (BLAST), and showed a 99.57% homology to the RNA of *J. lividum*. A pigmentless strain of *J. lividum* was from laboratory stock following mutagenesis of the wild-type strain by exposure to 1-methyl-3-nitro-nitrosoguanidine (1mg/1ml). A JLΦ1-resistant strain of *J. lividum* (SJS100) was isolated for use as a phage-free source for acyl-homoserine lactones from an agar overlay showing confluent lysis. *Chromobacterium violaceum* (CV026) and *Agrobacterium tumefaciens* A136 AHL biosensor strains, as well as, *C. violaceum* 31532 and *A. tumefaciens* KYC6 AHL overproducers used as positive controls were generously supplied by Robert J.C. McLean (Texas State University, San Marcos, TX) and described previously (29).

Bacterial cultures were grown on nutrient-rich Luria-Bertani (LB) and nutrient-poor R2A (Difco) media. *J. lividum* wild-type strain, *J. lividum* pigmentless strain, *J. lividum* phage-resistant (SJS100) strain, *C. violaceum* (CV026), *C. violaceum* 31532, and *A*

tumefaciens KYC6 were maintained on LB agar. *A. tumefaciens* A136 was maintained on LB with 50 μg/ml spectinomycin and 4.5 μg/ml tetracycline to maintain two plasmids that provide the AHL response system (29).

Isolation of bacteriophage JLΦ1. Phage isolation was performed using methods from Fattouh (12) with the following modifications. Cultures of *J. lividum* were grown in LB and R2A media then assayed for bacteriophage at 18h and 24h using a soft agar overlay method as described by Adams (2). Following phage induction, the cultures were allowed to incubate an additional 48h. After incubation, 50 ml of culture was pipetted into sterile Corning (Corning, NY) centrifuge tubes and centrifuged at 2000g for 20 min at 4C. The supernatant was Millipore filtered (.45 μm) and stored at 4C.

Growth curves and culture conditions. Overnight cultures of *J. lividum* wild-type and pigmentless mutant strains were grown on either LB or R2A agar at 25C. Inoculums of each strain were grown in LB and R2A broth, respectively, and incubated in a water bath shaker (Lab-Line orbital shaker) at 140 rpm and 25C. Bacterial growth was determined by turbidity at 540 nm using a Klett-Summerson photoelectric colorimeter (Klett Mfg Co. Inc., NY) and by viable colony counts. Colony forming units (CFU) were determined on either LB or R2A media using standard dilution techniques.

JLΦ1 bacteriophage release. Cultures of *J. lividum* wild-type and pigmentless strains were grown in either LB or R2A broth with shaking (140 rpm) at 25C. At various

intervals, the turbidity of the culture was determined and assayed for phage using the soft agar overlay method described previously (2).

Statistical analyses. Phage induction and titers were analyzed using Fisher's Exact Test.

Acyl-homoserine lactone assay. The presence of acyl-homoserine lactones was determined using *C. violaceum* (CV026) and *A. tumefaciens* A136 biosensors as described by McLean et al (29).

Effect of spent media which contain acyl-homoserine lactones on phage replication. *J. lividum* SJS100 was grown in R2A and LB for 24h. Cultures were centrifuged at 4000xg for 20 min at 4C. The supernatant was Millipore filtered (.45μm) and used as a bacteriophage-free source of spent media which contain acyl-homoserine lactones. The supernatant was stored at 4°C. Equal volumes of spent media were mixed with fresh medium and inoculated with *J. lividum* wild-type. Following a 10h incubation at 25°C, 10μl of phage stock ($1x10^6$PFU ml^{-1}) was added and at various intervals cultures were assayed for phage as previously described (2). Cultures grown without the addition of supernatant were used as controls.

Effect of spent media which contain acyl-homoserine lactones on phage induction. Equal volumes of spent media were mixed with fresh R2A and LB media and inoculated with *J. lividum* wild-type. The cultures were incubated with shaking (140 rpm) at 25C and assayed for phage at various intervals as previously described (2).

Transmission electron microscopy. TEM preparation protocol was modified from Bettarel (4). A 10 µl sample of filtered virus stock (1 x 10^6 PFU ml^{-1}) was placed on a Formvar-carbon-coated 400-mesh copper grid (SPI Supplies, West Chester, PA) for 1 min and the liquid removed by absorption to Whatman (Maidstone, Kent) filter paper. Grids were air-dried and phage negatively stained by the addition of 10 µl 2% urinyl acetate for 1 min. Grids were examined in a JEOL 1200 EX II electron microscope at an accelerating voltage of 120 kV.

RESULTS

Effect of nutrients on bacterial growth – The growth of *Janthinobacterium lividum* wild-type and a pigmentless mutant strain in LB and R2A is shown in Figures 2A and 2B, respectively. Both the rate of growth and the cell number were greater in R2A as compared to LB (Fig. 2A and B). No significant difference in growth was observed between the wild-type and pigmentless mutant strains in either LB media or R2A media. *J. lividum* wild-type produces low, convex, round, violet colonies on solid media (Fig. 3A and C). The mutant strain produces pigmentless colonies (Fig. 3B and D). Figure 4 shows the presence and absence of pigment production in broth culture by the wild-type and mutant strains, respectively.

When growth of *J. lividum* was analyzed by turbidometric measurements, a higher turbidity was observed in nutrient-rich as compared to nutrient-poor media for both the wild-type and pigmentless mutant strains.

Effect of nutrients on induction of bacteriophage JLΦ1 – The induction of JLΦ1 by wild-type and pigmentless mutant strains in LB and R2A medium is shown in Figures 5A and 5B, respectively. Phage induction patterns were similar on both media during growth of *J. lividum.* Induction was only observed during log growth and early stationary phase. The cell density of *J. lividum* wild-type at the time of phage induction was similar in LB medium (~$9x10^7$ – $9x10^8$ CFU ml^{-1}) and R2A medium (~$8x10^7$ – $4x10^9$CFU ml^{-1})

(Fig. 5A). In addition, mutant strain cell densities at the time of phage induction were similar in LB medium (~$1x10^8$ – $2x10^9$ CFU ml^{-1}) and R2A medium (~$3x10^8$ – $3x10^9$ CFU ml^{-1}) (Fig. 5B). The data indicates that induction of JLΦ1 is cell density dependent (~$8x10^7$ – $4x10^9$ CFU ml^{-1}) under both nutrient-rich and nutrient-poor conditions (Figs. 5A and 5B).

Phage induction during growth of *J. lividum* wild-type and pigmentless mutant strains in LB and R2A medium is shown in Tables 1 and 2. No significant difference in JLΦ1 induction was observed between wild-type and mutant strains in either LB (P = 1.0) or R2A (P = 0.99). The percent of cultures observed to induce phage was 100% for both the wild-type and the mutant in LB, whereas the phage induction was 83.3% for wild-type in R2A, and 78.5% for mutant in R2A (Tables 1 and 2). Although no significant difference was observed in JLΦ1 induction between LB and R2A medias for either wild-type (P = 0.22) or mutant (P =0.22) strain when compared, there was, however, a significant difference observed in phage induction between LB and R2A media when wild-type and mutant samples were combined (P = 0.05). The findings suggest that pigment production has no effect on the induction of JLΦ1, and that phage induction occurs with greater frequency in nutrient-rich as compared to nutrient-poor media.

The percent of cultures which produced phage titers greater than 10^4 PFU ml^{-1} was 69% for wild-type in LB, 86% for mutant in LB, 25% for wild-type in R2A, and 0% for mutant in R2A (Tables 1 and 2). No significant difference was observed for phage induction of titers greater than 10^4 PFU ml^{-1} between the wild-type and mutant strains in LB (P = 0.99), however, a significant difference was observed in R2A medium (P =

0.08). Also significant differences were observed for phage production of greater than 10^4 PFU ml^{-1} between LB and R2A media for both wild-type (P = 0.05) and mutant (P = 0.0001) strains. The data suggest that pigment has no effect on production of large phage titers in LB, but may have some effect in R2A. The results also indicate that phage was produced at significantly higher titers in nutrient-rich as compared to nutrient-poor media.

Acyl-homoserine lactone production – The production of acyl-homoserine lactones (AHLs) by *J. lividum* wild-type, phage-resistant (SJS100), and pigmentless mutant strains is shown in Figures 6A-6D. Acyl-homoserine lactone production was observed by all three strains in the presence of the *Agrobacterium tumefaciens* A136 biosensor (Figs. 6A, C, and D). AHL production was not observed for *J. lividum* wild-type (Fig. 6B), pigmentless mutant, or SJS100 (Data not shown) strains in the presence of the *Chromobacterium violaceum* CV026 biosensor. The data indicates that AHLs produced by *J. lividum* wild-type, SJS100, and pigmentless mutant strains have carbon chains in the range of C6 to C14.

Effect of spent media which contain acyl-homoserine lactones on phage replication – In R2A medium, a ten-fold increase in phage was observed after 4h in two of the three cultures with added spent media as compared to the controls. The third culture was observed to have induced phage and reached a PFU count greater than 10^5 ml^{-1}, before phage was even added (Table 3). In LB medium, the three cultures with added spent media had induced phage and reached PFU counts greater than 10^5 ml^{-1},

before the additional phage was even added. The three controls showed ~100-fold increase in PFU count at 2h (Table 4). The results indicate that spent media did not prevent phage replication, but in fact, may have stimulated the induction and replication of phage.

Effect of spent media which contain acyl-homoserine lactones on phage induction – To determine if spent media prevented the induction of JLΦ1, spent media was added to both R2A and LB mediums and inoculated with *J. lividum* wild-type. The cultures were assayed for phage at various time intervals. Two of three R2A cultures (Table 5) and three of three LB cultures (Table 6) induced high titers of phage in the presence of spent media. The results indicate that spent media did not prevent phage induction, but instead, may have stimulated the induction of phage.

Transmission electron microscopy – A photomicrograph of JLΦ1 is shown in figure 7. The phage head was observed to be 100 nm. The apparently contractile tail was observed to be 120 nm long and 15 nm wide. Morphologically, JLΦ1 belongs to the dsDNA family of viruses, *Myoviridae*.

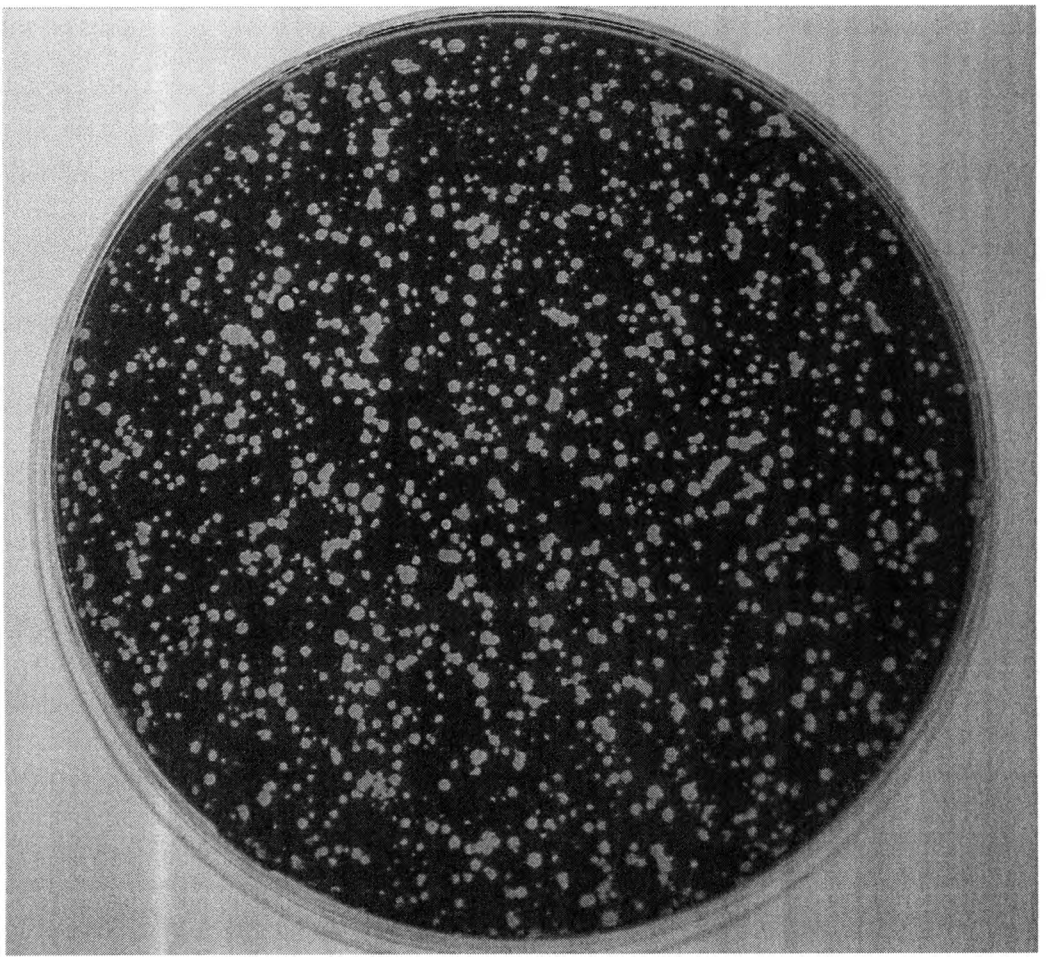

FIG. 1. Plaque assay of J. lividum wild-type strain on R2A agar (Difco) at 25°C.

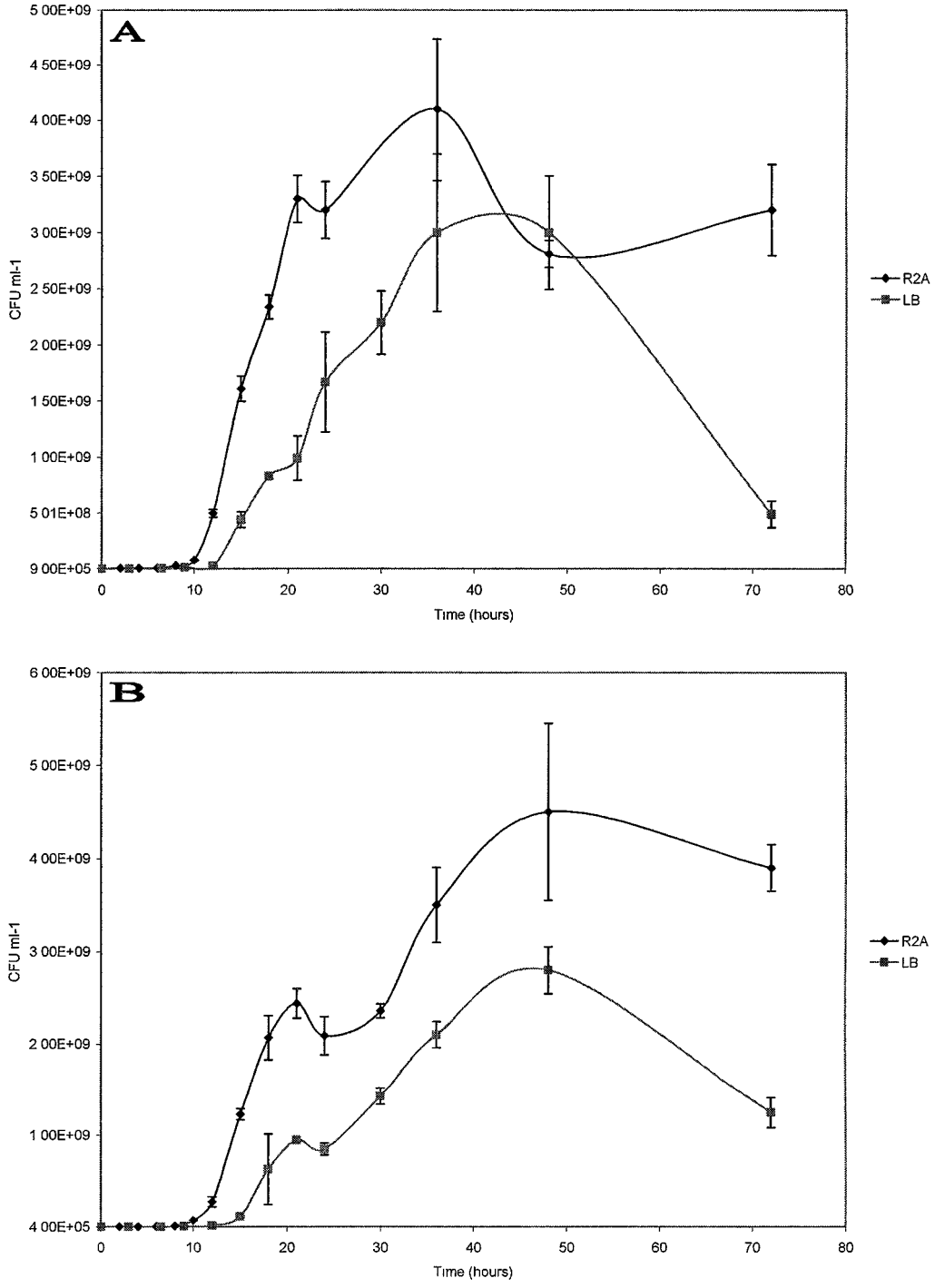

FIG. 2. Effect of nutrients on growth of J. lividum wild-type (A) and pigmentless strain (B). The graph shows the growth curves each *J. lividum* strain in both in nutrient-rich LB (Difco) (■) and nutrient-poor R2A (Difco) (♦) media. Error bars indicate the range of CFU ml^{-1} for triplicate plates.

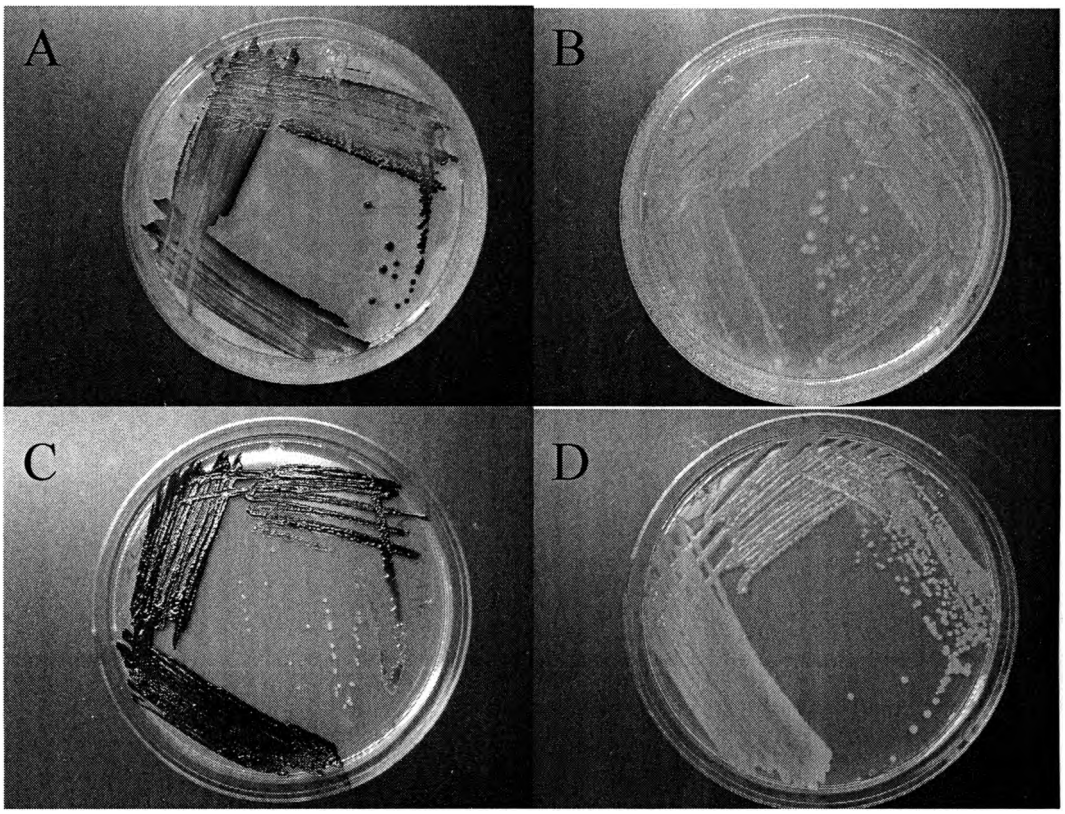

FIG. 3. Streak plates of J. lividum strains grown at 25C: wild-type on R2A agar (Difco) (A), pigmentless mutant on R2A agar (B), wild-type on LB agar (Difco) (C), and pigmentless mutant on LB agar (D).

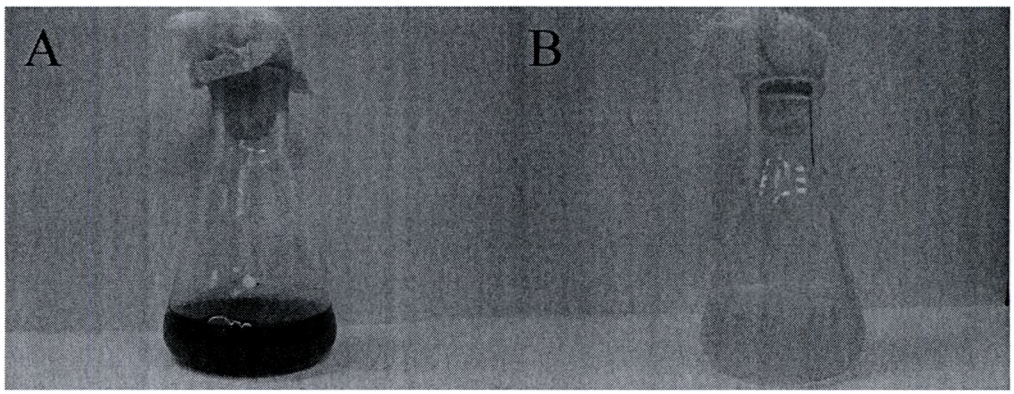

FIG. 4. Broth cultures of J. lividum wild-type (A) and pigmentless strain (B) in R2A broth (Difco) at 25C and shaking (140 rpm).

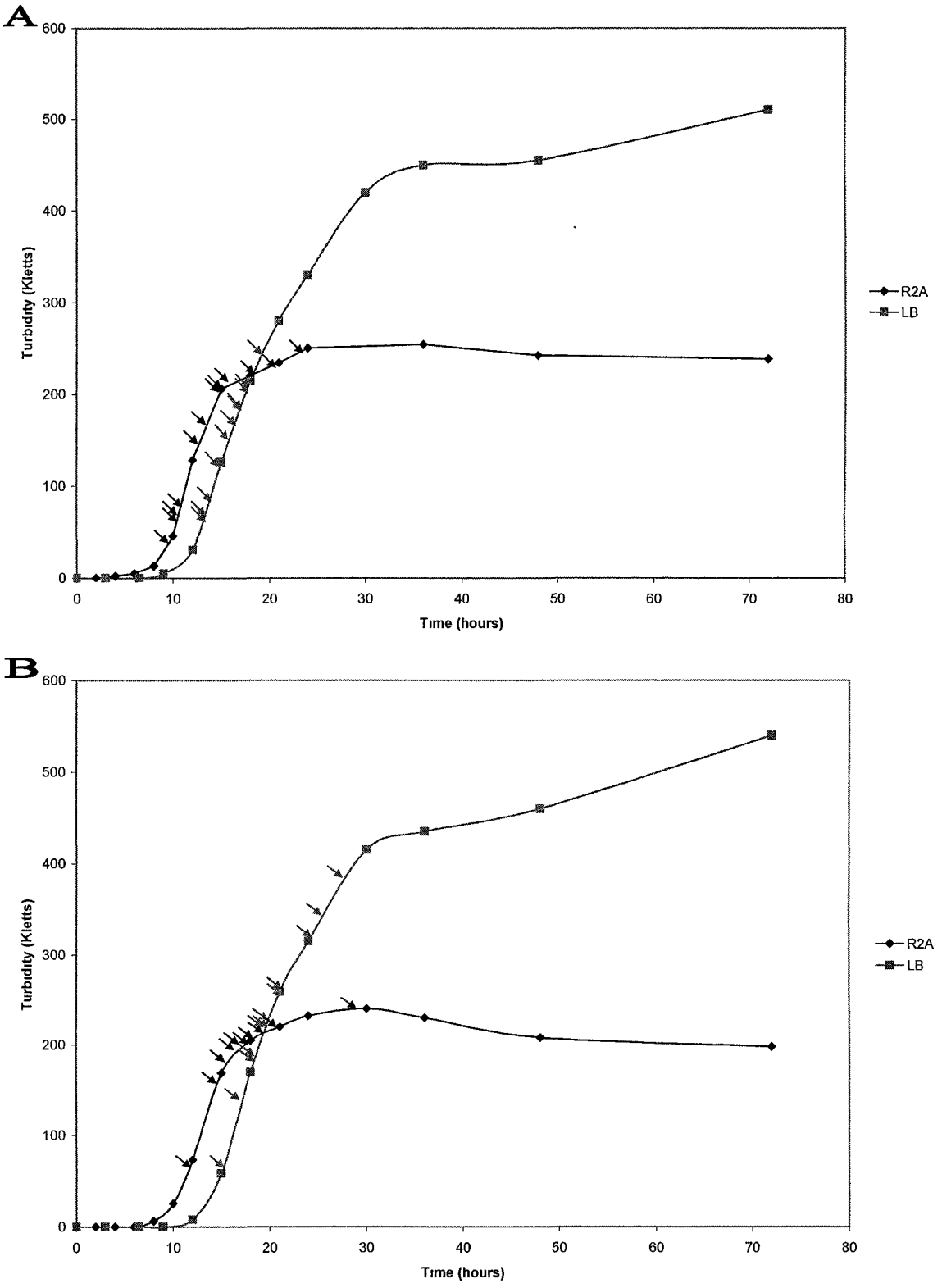

FIG. 5. Effect of nutrients and population density on induction of JLΦ1 in J. lividum (A) wild-type and (B) pigmentless mutant strains. The graph shows phage induction (↓) of each *J. lividum* strain in both nutrient-rich LB (■) and nutrient-poor R2A (♦) media.

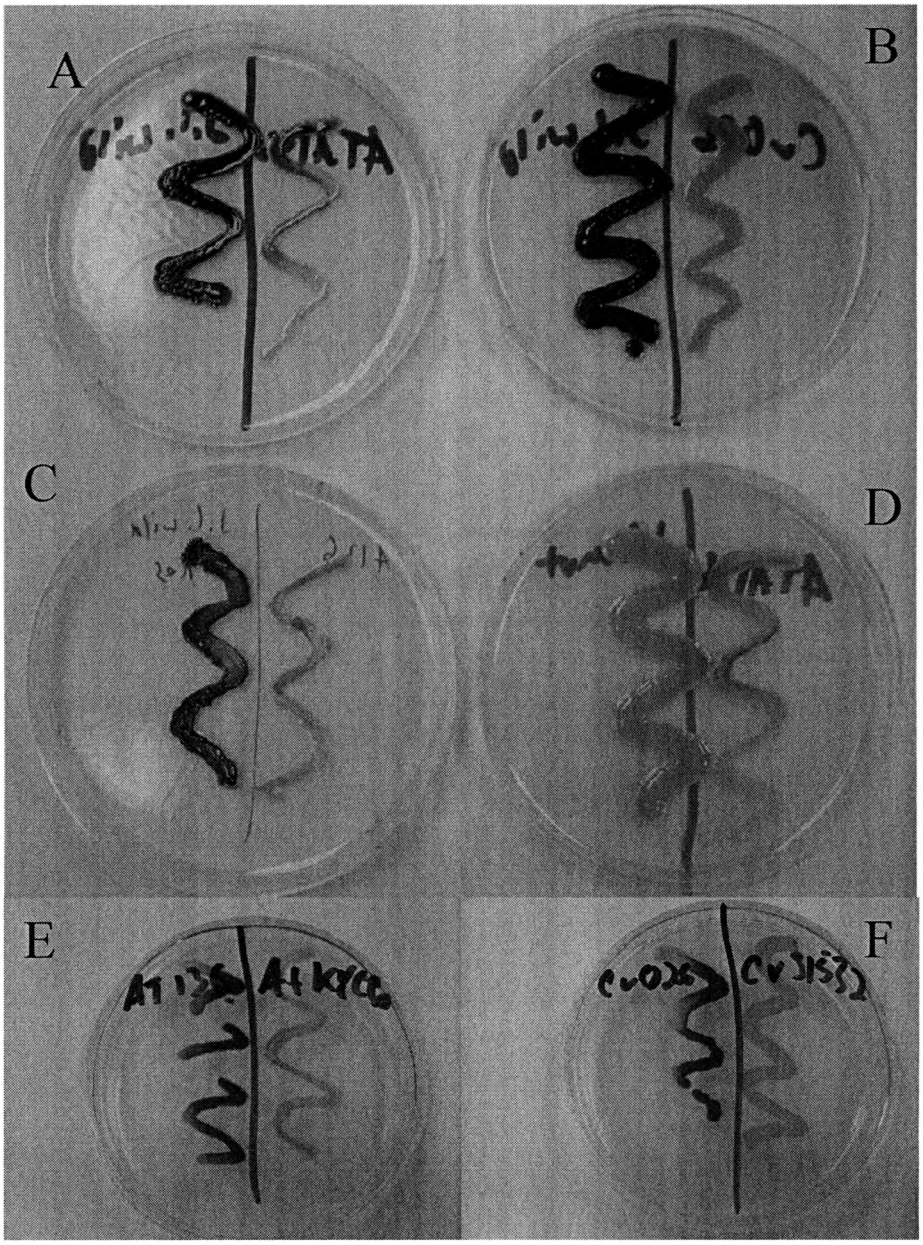

FIG. 6. Acyl-homoserine lactone production. *J. lividum* wild-type in the presence of *A. tumefaciens* A136 biosensor shows AHL production (A). No AHL production is shown for wild-type in the presence of *C. violaceum* CV026 biosensor (B). AHL production is shown for *J. lividum* SJS100 in the presence of *A. tumefaciens* A136 (C) and pigmentless mutant in the presence of *A. tumefaciens* A136 (D). Positive controls *A. tumefaciens* KYC6 in the presence of *A. tumefaciens* A136 (E) and *C. violaceum* CV 31532 in the presence of *C. violaceum* CV026(F) show AHL production.

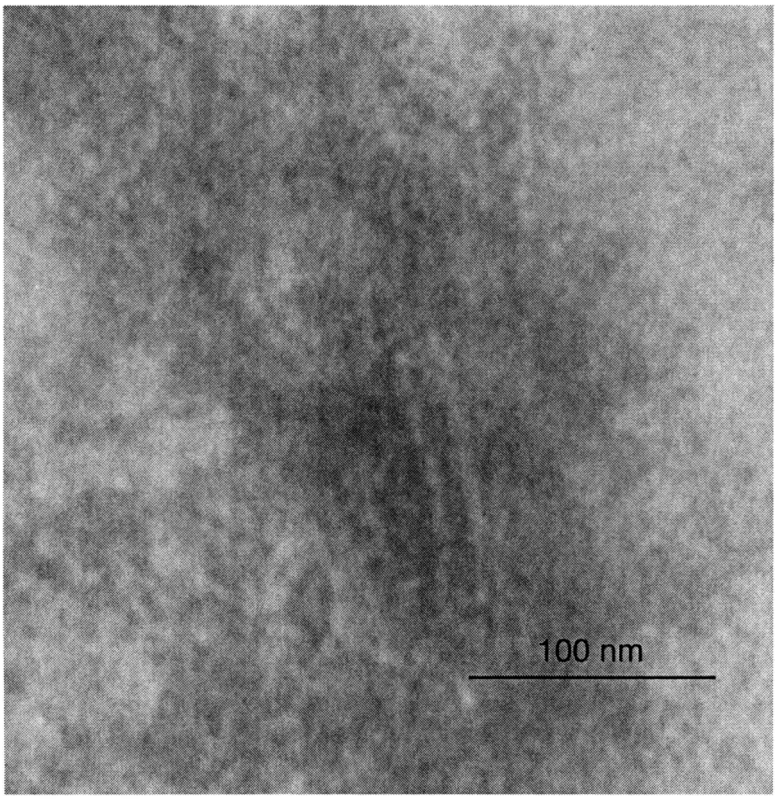

FIG 7. Transmission electron micrograph of JLΦ1

TABLE 1. Induction frequency and viral yield in *Janthinobacterium lividum* wild-type cultures under both nutrient-rich (LB) and nutrient-poor (R2A) conditions

Culture[a]	LB		R2A	
	Induction[b]	PFU $>10^4$ ml^{-1}	Induction[b]	PFU $>10^4$ ml^{-1}
1	+	+	+	-
2	+	+	+	-
3	+	-	+	+
4	+	+	+	-
5	+	+	+	-
6	+	+	+	+
7	+	-	+	-
8	+	+	-	-
9	+	-	-	-
10	+	+	+	-
11	+	+	+	-
12	+	+	+	-
13	+	-	N/A[c]	N/A[c]

[a] Cultures incubated at 25°C and assayed for phage at various times
[b] Prophage induction detected by plaque assay
[c] Samples not taken

TABLE 2. Induction frequency and viral yield in *Janthinobacterium lividum* pigmentless mutant cultures under both nutrient-rich (LB) and nutrient-poor (R2A) conditions

Culture[a]	LB		R2A	
	Induction[b]	PFU $>10^4$ ml^{-1}	Induction[b]	PFU $>10^4$ ml^{-1}
1	+	+	+	-
2	+	+	-	-
3	+	+	+	-
4	+	+	+	-
5	+	-	-	-
6	+	+	+	-
7	+	+	+	-
8	+	+	-	-
9	+	-	+	-
10	+	+	+	-
11	+	+	+	-
12	+	-	+	-
13	N/A[c]	N/A[c]	+	-
14	N/A[c]	N/A[c]	+	-

[a] Cultures incubated at 25°C and assayed for phage at various times
[b] Prophage induction detected by plaque assay
[c] Samples not taken

TABLE 3. Effect of increased concentration of spent media on phage replication in R2A

Time (min)[b]	Cultures (PFU ml^{-1})[a]				
	Spent media [c]			Control[d]	
	1	2	3	1	2
0	26	36	$>10^5$	38	18
30	22	50	$>10^5$	34	32
60	34	46	$>10^5$	46	38
90	26	54	$>10^5$	36	22
120	38	82	$>10^5$	36	50
180	78	120	$>10^5$	64	26
240	934	758	$>10^5$	110	64
300	$1.0x10^3$	978	$>10^5$	94	92

[a] Incubated at 25°C for 10h before the addition of phage
[b] Infectious phage determined by plaque assay at indicated times following addition of phage at time zero
[c] Cultures grown in equal volumes of spent media and fresh media
[d] Cultures grown in fresh media

TABLE 4. Effect of increased concentration spent media on phage replication in LB

Time (min)[b]	Cultures (PFU ml^{-1})[a]				
	Spent media[c]			Control[d]	
	1	2	3	1	2
0	$>10^5$	$>10^5$	$>10^5$	22	28
30	$>10^5$	$>10^5$	$>10^5$	40	50
60	$>10^5$	$>10^5$	$>10^5$	24	52
90	$>10^5$	$>10^5$	$>10^5$	32	26
120	$>10^5$	$>10^5$	$>10^5$	$8X10^2$	$1.1X10^3$
180	$>10^5$	$>10^5$	$>10^5$	$>10^4$	$>10^4$
240	$>10^5$	$>10^5$	$>10^5$	$>10^5$	$>10^5$
300	$>10^5$	$>10^5$	$>10^5$	$>10^5$	$>10^5$

[a] Incubated at 25°C for 10h before addition of phage
[b] Infectious phage determined by plaque assay at indicated times following addition of phage at time zero
[c] Cultures grown in equal volumes of spent media and fresh media
[d] Cultures grown in fresh media

TABLE 5. Effect of increased concentration of spent media on phage induction in R2A

Time (h)[b]	Cultures (PFU ml^{-1})[a]					
	Spent media[c]			Control[d]		
	1	2	3	1	2	3
18	0	$>10^4$	$8x10^3$	0	0	0
24	0	$>10^4$	$8x10^3$	0	0	0
36	0	$>10^4$	$4x10^3$	0	0	0

[a] Incubated at 25°C
[b] Infectious phage determined by plaque assay at indicated time intervals
[c] Cultures grown in equal volumes of spent media and fresh media
[d] Cultures grown in fresh media

TABLE 6. Effect of increased concentration of spent media on phage induction in LB

Time (h)[b]	Cultures (PFU ml^{-1})[a]					
	Spent media[c]			Control[d]		
	1	2	3	1	2	3
18	$>10^5$	$>10^5$	$>10^5$	680	120	0
24	$>10^5$	$>10^5$	$>10^5$	$\sim10^4$	$1.6x10^3$	0
36	$>10^5$	$>10^5$	$>10^5$	$>10^4$	$\sim6x10^3$	100

[a] Incubated at 25°C
[b] Infectious phage determined by plaque assay at indicated time intervals
[c] Cultures grown in equal volumes of spent media and fresh media
[d] Cultures grown in fresh media

DISCUSSION

Recently, numerous studies have reported on the lysogenic and lytic interactions between various bacteriophages and their hosts (8, 18, 31, 33, 42, 44). To my knowledge, there has been only one report which describes a lysogenic relationship which results in the infection of the host bacterium by the homologous phage (42). The lysogens, however, were lost upon storage at -80°C and it was necessary to reinfect the host in order to regain the lysogenic relationship (42). This study reports on a naturally occurring lysogenic bacterium, *Janthinobacterium lividum,* susceptible to infection by the homologous phage, designated JLΦ1. The host, *J. lividum,* a purple-pigmented, lysogenic bacteria was isolated from the oligotrophic environment of the Edwards aquifer. JLΦ1 is characterized as a member of the dsDNA virus family *Myoviridae*, based on a 100nm x 90nm icosahedral head and 120nm long x 15nm wide contractile tail (39).

J. lividum has been observed to produce phage at high cell density (Fig. 5), characteristic of pseudolysogeny (1). In addition, *J. lividum* cultures which contain high plaque forming units have been observed to maintain similar turbidities as cultures which lack infectious phage particles (data not shown), also characteristic of a pseudolysogeny (31). The data suggest that the *J. lividum* phage-host system is maintained in a lysogenic state which resembles pseudolysogeny. Pseudolysogeny may provide bacteriophage an opportunity to survive in times of low host cell abundance (35).

In vitro, *J. lividum,* was observed to grow more efficiently in nutrient-poor medium as compared to nutrient-rich medium (Fig. 2). This is expected for organisms isolated from nutrient depleted environments (6, 10, 24). However, turbidity readings were much higher for cultures grown in nutrient-rich as compared to nutrient-poor media. This may be due to a larger cell size for cultures grown in LB. The spontaneous induction of JLΦ1 was observed during logarithmic growth phase in both nutrient-poor and nutrient-rich media, which demonstrates a dependency of JLΦ1 induction on the bacterial growth phase (Fig 5). However, phage induction was also observed to occur at a lower frequency under nutrient-poor as compared to nutrient-rich conditions (Tables 1 and 2). Some studies have suggested that prophage induction frequency is mainly an effect of the growth conditions rather than the growth behavior of the lysogenic host (25). It is possible, however, that the lower induction frequency observed for cultures in nutrient-poor media may be attributed to a shorter log phase growth under nutrient-poor conditions (10h) as compared to nutrient-rich conditions (18h) for *J. lividum* (Fig. 5).

It is unclear what proportion of phage production is due to phage induction versus phage replication. Cultures lacking infectious particles showed an increase in phage production upon infection with a known concentration of JLΦ1 (Tables 3 and 4). The observed increase in phage titer, as compared to the absence of phage in control uninfected cultures, indicates phage production is, in part, due to replication of JLΦ1. The data indicate that infectious virus is produced as a result of phage induction (Fig 5), thus phage undergo replication in culture (Tables 3 and 4). The term phage production, therefore, will be used to indicate the virus produced as a result of both induction and replication.

Production of JLΦ1 occurred during exponential cell growth (Fig. 5), corresponding with nearly all of the thousands of bacteriophages previously described that apparently infect and replicate only in rapidly growing hosts (44). In addition, viral production was observed to be significantly higher for *J. lividum* wild-type ($P = 0.05$) and pigmentless mutant ($P = 0.0001$) cultures in nutrient-rich as compared to nutrient-poor conditions, even though host density was shown to be higher in nutrient-poor medium (Tables 1 and 2). This is in agreement with the observations that bacterial populations produce a greater number of virus with higher infection rates under nutrient-rich conditions (44). The results suggest that production of JLΦ1 is dependent upon both a minimum host cell density and optimal nutrient conditions. This is in accordance with other studies on pseudolysogeny which have shown that infection of bacteria at high nutrient concentrations results in lytic growth, and infection at low nutrient concentrations results in pseudolysogeny (44). This may account for the observation that *J. lividum* exhibited low bacterial host populations (Fig 2), with high phage concentrations under nutrient-rich conditions (Tables 1 and 2).

A pigmentless mutant strain of *J. lividum* was isolated to determine if violacein production had an effect on bacterial growth and phage production. No difference between the growth patterns of the wild-type bacterium and the pigmentless mutant was observed (Fig. 2). In addition, no significant difference in the frequency of induction or the number of phage produced were found to occur between the wild-type and pigmentless mutant (Tables 1 and 2). Violacein production has been linked to the presence of N-acyl-homoserine lactones (AHLs), which act as quorum sensing signal molecules, in *C. violaceum* (28). *J. lividum* produces AHLs with carbon chains ranging

between C6 to C14 as detected by the biosensor *Agrobacterium tumefaciens* A136 (Fig. 6), as well as, the pigment violacein. The production of AHLs and violacein (Fig. 3) by *J. lividum*, thus, suggests that cultures of *J. lividum* may exhibit quorum sensing. The data indicate that violacein production had no effect upon JLΦ1 induction or replication, and violacein production is not a factor in the growth of *J. lividum* or the production of JLΦ1 in culture.

It was initially hypothesized that *J. lividum* may acquire resistance to infection at high population densities due to mechanisms controlled by AHLs, such as violacein production. Neither spent media containing AHLs nor violacein production had an effect on resistance of the culture to phage infection or induction (Tables 3-6). It may be speculated that immunity to infection may be due either to lysogeny which provides homoimmunity (44) or the emergence of phage-resistant host mutants (42).

Studies of viruses in nature have shown that viral abundance exceeds bacterial abundance, even in oligotrophic environments (44). In contrast, *J. lividum* cultures were observed to exhibit much lower viral concentrations (10^6 PFU ml^{-1}) as compared to bacterial concentrations (10^9 CFU ml^{-1}). Since host cells acquire immunity to further infection through lysogeny (44), this pattern may be attributed to the presence of a greater percentage of homoimmune lysogenic host cells in cultures of *J. lividum* as compared to other phage-host systems.

It has been proposed that temperate bacteriophages have evolved to maximize their survival using unknown mechanisms to sense unfavorable bacterial growth conditions (25). A rationale proposed for high induction frequency under nutrient-rich growth conditions is the ability of the phages to sense that the cell population is growing

well (25). It is possible that the presence of acyl-homoserine lactones in spent media, the same molecules used by some bacterial species in quorum sensing (46), may be a mechanism by which phage sense cell population density. A ten-fold increase in phage production in two cultures with the addition of spent media was observed in nutrient-poor media, as well as, a high titer of induced phage in three other cultures (Tables 3 and 5). All cultures grown in nutrient-rich media induced high titers of phage upon the addition of spent media (Tables 4 and 6). JLΦ1 may be exploiting *J. lividum's* quorum sensing system to determine when the population density has reached a critical threshold for phage induction. This increase in phage replication in the presence of additional spent media suggests an ability of the virus to detect an increase in AHL concentration, which may regulate infection and replication conducive to phage survival.

In conclusion, bacteriophage JLΦ1 and *J. lividum*, were found to interact in a pseudolysogenic relationship dependent upon host cell density, growth phase, and nutrient availability. Additionally, *J. lividum* uses quorum sensing signals, which may be exploited by JLΦ1 to sense host cell populations and regulate phage production.

SUMMARY

Janthinobacterium lividum is a lysogenic bacterium susceptible to infection by the homologous phage. The prophage, designated JLΦ1, is spontaneously induced from a pseudolysogenic state in growing culture and produces clear plaques on a lawn of the host bacterium. The bacterium produces a purple pigment, violacien, as well as, acyl-homoserine lactones (AHLs) with carbon chains in the range of C6-C14. Phage induction occurred during exponential growth phase in both nutrient-rich and nutrient-poor growth media. Although bacterial growth was more efficient in nutrient-poor growth conditions, both the frequency of phage induction and viral yield was greater under nutrient-rich growth conditions. In general, bacteriophage production was dependent upon a minimal host cell density and nutrients present during growth of the lysogenic host. Although violacein had no effect on either bacterial growth or phage production, the addition of spent media containing AHLs to cultures of *J. lividum* under both nutrient-rich and nutrient-poor growth conditions stimulated phage induction. The results suggest JLΦ1 may exploit bacterial quorum sensing signals to sense cell density and bacterial growth for efficient virus replication. JLΦ1 consists of a head 100 nm in diameter with a flexible tail 120 nm in length and 15 nm in diameter. JLΦ1 is morphologically similar to viruses in the family

Myoviridae. This is the first report of a naturally occurring lysogenic bacterium susceptible to infection by the homologous phage.

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