Acoustical emissions generated by bacteriophage sk1 and ml3 using *Lactococcus lactis* ssp. *lactis* C2 host

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Abstract

Lactococcus lactis ssp. *lactis* C2 bacteria in M17 medium, at 26C for 8 h were infected with phages sk1 or ml3, and monitored using contact piezoelectric sensors attached to the sides of the growth vessel. The two sensors (5 to 50 kHz range) had individual characteristic and internal amplification mechanisms that were calibrated and adjusted to minimize background noise. After the sensors had been calibrated, the M17 medium was inoculated with *L. lactis* ssp. *lactis* C2 culture (1 x 10^9 cfu/ml), stirred for 1 min, and allowed to grow for approx. 90 min prior to infection (stirred for 1 min) with phages sk1 or ml3. Infection time was set to correspond with the start of the log growth phase. Infection level was 10^5 pfu/ml for both phages sk1 and ml3. Sound intensity from the growth chambers was measured in attojoules (aJ = 10^{-18} Joules) and plotted as the energy rate-per-detected acoustic wave. Acoustic peaks considered significant and beyond internal or external generated noise were those having greater than 3 times the sigma value of the general variation in acoustic intensity over the entire data set of each test. Energy rate data from control tests in which *L. lactis* ssp. *lactis* C2 was grown without phage sk1 or phage ml3 infections contained no acoustic peaks with intensities that exceeded the 3 sigma standard whereas phage sk1 or ml3 infected *L. lactis* ssp. *lactis* C2 culture contained multiple acoustic peaks with intensities that exceeded 3 sigma. The first peaks for phage sk1 appeared at 33.2 4.4 min whereas the first peak for phage ml3 appeared 40 min. Thus, the acoustic data from phage sk1 or phage ml3 infected *L. lactis* ssp. *lactis* C2 were considered to be the result of phage infection. The timings of the acoustic peaks from phage sk1 were sufficiently different from phage ml3, that these two phage could probably be distinguished by acoustic emission monitoring during phage infection of the bacteria.

Introduction

Acoustic emission (AE) measurements have been applied to measure events as diverse as micro-cracking in metals, earthquake tremors, chemical reactions and micro-bubble processes (Boyd and Varley, 2001; Chotard, *et al* 2002; Rzeszotarska, *et al* 1998). Detection of ultrasonic AE, produced during fluid flow through holes in mechanical systems (Wassef, *et al* 1985), has also provided impetus for products that detect and pinpoint the location of tiny gas and liquid leaks from pipes. Hicks et al. (2007a, b) reported that AE measurements could be used to monitor the normal metabolic active of bacteria, beginning just after the bacteria were inoculated into a medium. Some emissions stayed the same throughout the lag, log, and stationary phases, while other AE varied in intensity and frequency as the bacteria moved through these phases. AE correlated with various metabolic and growth periods and emissions became more quiescent as the bacteria entered the stationary phase. Both periodic and vibrational emissions from *L. lactis* ssp. *lactis* and *E. coli* were so varied that differences between genera and closely related strains could distinguished. These acoustic emissions were detected between the frequencies of 50 and 200 kHz, were temperature dependent, and ceased after the addition of a metabolic inhibitor. Research was then conducted to determine if AE methods could be used to determine the infection and lysis of bacteriophage. Lactic c2 phage was first characterized on *L. lactis* ssp. *lactis* C2 host using this AE method. It was determined that the AE equipment could easily follow the infection cycles of c2 phage and that the infection cycle time was approximately 38 minutes at 37℃. This research characterizes the infection cycles of sk1 and ml3 while using *L. lactis* ssp. *lactis* C2 as the host and compares this data with the c2 phage data at the temperature of 26℃.

Materials and Methods

Culture and phage

Lactococcus lactis ssp. *lactis* C2 and c2, sk1, and ml3 bacteriophages were obtained from the University of Kentucky culture Library which is maintained at -80℃.

Medium and chemicals

M17 medium was purchased from Fischer laboratories and all other chemicals were purchase from Aldrich Chemical.

Preparation of media

Media (M17) was prepared by adding 100 ml of 1M $CaCl_2.6H_20$, 5 g of lactose to 1 L of M17 broth. The pH was adjusted to 6.9. The medium was sterilized at 121°C for 15 min. Medium stored at 2℃. Medium was warmed to 26℃ prior to filling the sterilized acoustic growth cells (400 ml).

Enumeration of bacteriophage

Bacteriophage were enumerated using a top/bottom agar assay Petri plate procedure as described by Hicks et al. (2003).

Acoustic monitoring device

A chamber having two identical temperature controlled compartments (Fig. 1) were sound insulated and vibration isolated to minimize interference from acoustic sources external to them. The AE sensors and software used were manufactured by Physical Acoustics Corporation (PAC, Princeton Junction, NJ). The sensors (model number R6) had a sensitivity between 20 – 100 kHz. Acoustic signals were pre-amplified and then analyzed using PAC AEWin and AEPost software. A growth cell containing the sterilized medium was place in each chamber (26℃) with two sensors attached to the cell (Fig. 2). An additional guard sensor was attached to the cabinet to monitor acoustic noise from sources external to the growth cells. A thin layer of silicon grease was applied to the face of each sensor to enhance the acoustic signal transfer. The apparatus holding the sensors to the cell applied a constant reproducible pressure to the sensors (Fig. 3). The compartments were closed and background AE data were collected to establish threshold levels for each sensor. These levels were used as an input parameter to the software; if acoustic wave intensities above the sensor's threshold were detected the software enabled storage. *L. lactis* ssp. *lactis* C2 was used as the host to inoculated (1% or 4 mls of culture containing 10^9 cfu/ml) the medium. The medium was stirred for 1 min prior to data collection (up to 8 h). Bacteriophages ml3 or sk1 were added to the medium at an infection level of 10^5 pfu/ml (final concentration in growth cell) approx. 90 min after inoculation of the host.

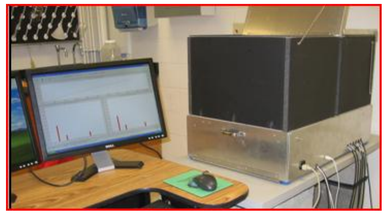

Fig. 1. View of sound chamber with equipment attached to the computer..

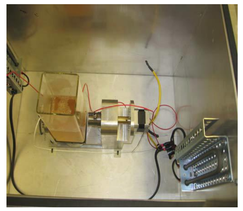

Fig. 2. View looking down at growth cell, sensors, and device to hold sensors in place

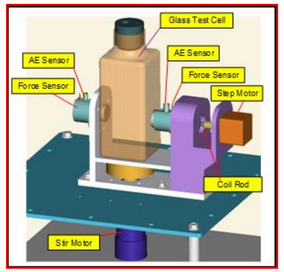

Fig. 3. Schematic showing the arrangement of the sensors and test cell.

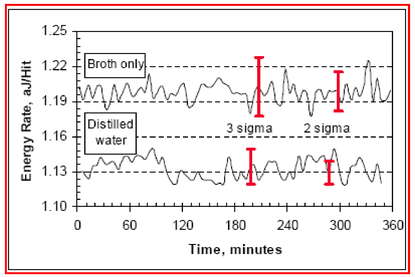

Fig. 4. Acoustic output when only water or growth medium was in the growth cells. Note that no data exceeded the 3 ☞ level.

Monitoring of cell growth

A sterile pipette and collection tube was used to collect a sample (3 ml) at 30 min intervals. AE measurements were stopped, the cell was stirred for 1 min, the sample was collected, and the AE measurements were resumed when sampling was complete. The OD of the sample was determined at 600 nm, using a UNIC 2100 spectrophotometer, to estimate cell growth. The sample was serially diluted, plated on aerobic Petri-film, and incubated at 30℃to enumerate cell numbers .

Monitoring of M17 and water

As a control both M17 medium and water (Fig. 4) were monitored to determine if the noise level produce by these substances would exceed the 3☞ level. Some noise signals exceeded 2 ☞, but none exceeded 3 ☞, thus 3 ☞ was chosen as the point where AE could be attributed only to the treatment.

Statistical analysis

Analysis of AE was conducted using statistical software, where only the peaks that were greater than 3 ☞ were considered significant. All experimental treatments were replicated 3 times. Data shown are an average of the analyzed data.

Results and Discussion

Initially, lyses curves were determined for ml3 and sk1 phage at infection levels of 10^4, 10^5 10^6 and 10^7 pfu/ml (Fig. 5 & 6). To amplify the AE affect the maximum cell count was desired, therefore, the lyses of the host should take place just prior to the host entering the stationary phase (control). At 10^4 pfu/ml the phage concentration was not sufficient in some cases, that the host could enter into the stationary phase without total lyses occurring, thus an infection level of 10^5 pfu/ml was selected as the desired infection concentration to be used. This infection level allowed for a near maximum AE output to be recorded.

At the base line setting used the AE from the host did not exceed the 3☞ level (shown by the black bar, Fig. 7.), so all AE being derived from the host, *L. lactis* ssp. *lactis* C2 was considered part of the background noise pickup by the sensor. Fig. 7 shows the AE recorded from sensor 1 as plotted on Channel 1 (Ch1). When ml3 was added at 90 min (Fig. 8, purple dash infect line) to infect the host both sensors 1 (Ch1) and 2 (Ch2) pick up AE that exceeded the 3 ☞ level (level noted by red and blue

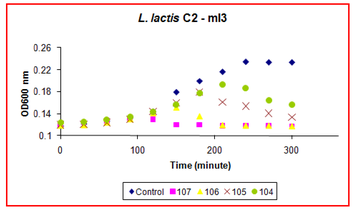

Fig. 5. Lyses curves for *L. lactis* ssp. *lactis* when infected with ml3 phage at 10^4, 10^5, 10^6, and 10^7 pfu/ml.

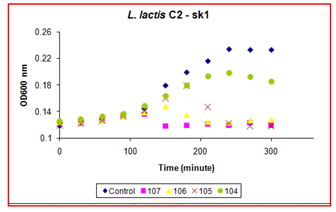

Fig. 6. Lyses curves for *L. lactis* ssp. *lactis* when infected with sk1 phage at 10^4, 10^5, 10^6, and 10^7pfu/ml.

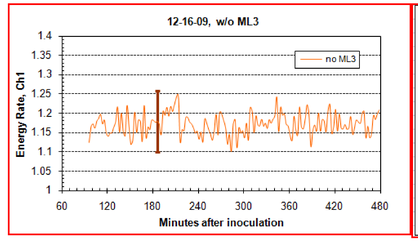

Fig. 7. AE signal coming from host growth without being infected with ml3 phage. Note there are no significant peaks.

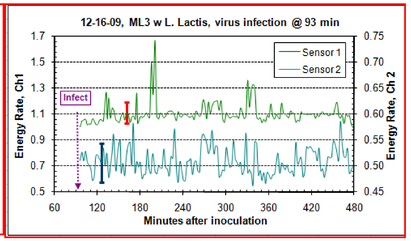

Fig. 8. AE signal coming in from sensors 1 & 2 when host was infected with ml3 phage.

Results and Discussion (continued)

bars as recorded on Ch1 and 2, respectively, Fig. 8). From this data it is presumed that the initial infection occurred immediately after the phage was added to the test cell, prior to the data collection system being turned on. Thus, two initial peaks at130 and 135 min (Ch1) and two larger peaks at 185 and 190 min suggest that lyses occurred prior to the peaks and infection was occurring at these intervals. Since ml3 is noted for its larger burst sizes, the second series of peaks would be expected to be larger than the first series of peaks, which they were. The initial peaks were 40 and 45 min from time of infection and the second series of peaks were exactly 55 min after the first series of peaks, suggesting that the sustained infection cycle of ml3 phage was 55 min. After 190 min the culture would have been moving toward total lyses (Fig. 5) so no addition peaks would be expected, which was the case. Sensor 2 (Ch2, Fig. 8) picked up a lot more background noise than sensor 1 and only a few emissions exceeded the 3☞ level. The difference of sensor 1 from sensor 2 shows the current state of the ceramic building art. Sensor 1 always out performed sensor 2, thus the following data will only show the information collected from sensor 1 and 3 which preformed similarly. When experiments were run with sk1 phage no AE output exceeded the 3☞ level when the phage was not present (Ch3, Fig. 9). However, when the host, *L. lactis* ssp. *lactis* C2, was infected with sk1 phage at 93 min after host inoculation into the medium, a significant AE peaks occurred at 126 min, which averaged 33 .2 min after infection. All other peaks were less than the 3☞ level. When these data were compared to earlier work with c2 phage which had an infection cycle of 38 min it was apparent that all three phage had different infection cycle times. These data suggest that AE might be a good technique to follow phage infections and that the type of phage might be distinguished by this method.

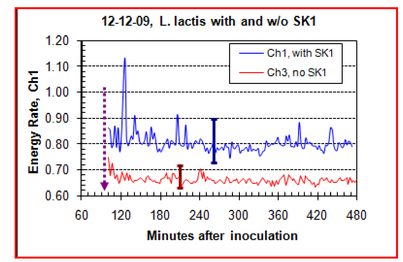

Fig. 9. AE signal of host growth without sk1 phage infection (Ch3) and with infection (Ch1).

Conclusions

AE output from c2, sk1, and ml3 phage could be distinguished from background and external noise. The AE observed appeared to be quite specific for the infection of the host organism, *L. lactis* ssp. *lactis* C2. Earlier data showed that the infection cycle for c2 phage was approximately 38 minutes at 37℃. Sk1 phage produced a >3☞ peak at 33.2 4.4 min whereas the first peak for phage ml3 appeared 40 min. after infection and that the second infection cycle was 55 min after the first. Thus, this acoustic data collected from phage sk1 and ml3 were considered to be the sound of phage infection the host. AE peak height for second cycle infections compared to first cycle infections were as expected. The timings of the acoustic peaks for phage sk1 and ml3, were sufficiently different that these two phage could probably be distinguished by their acoustic emissions.

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