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Infection and removal of L-forms of *Listeria monocytogenes* with bred bacteriophage

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Abstract

Phage breeding was employed to produce a bacteriophage (*Listeria monocytogenes* phage ATCC 23074-B1) which was specific for L-forms of *L. monocytogenes*. The bred phage was compared to its unbred parent for lytic activity and specificity. It was also tested for its ability to prevent L-form biofilm formation on stainless steel and compared with an organic acid (lactic) at L-form biofilm inactivation on stainless steel. The bred phage lysed only L-forms of *L. monocytogenes* in broth culture and only plaqued on L-form lawns. Likewise, the unbred phage performed similarly with classical cell-walled culture and lawns. The bred phage successfully inhibited L-form biofilm formation on stainless steel and was as successful as lactic acid (130 ppm) at inactivating pre-formed L-form biofilms. Both reduced viable cell numbers by 3-log cycles over a 6 h period. It appears that phage breeding technology may be an attractive alternative to chemical sanitizers which lack specificity and can be toxic.
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Keywords: Acid sanitizer; Bacteriophage; Biofilm; L-form; *Listeria monocytogenes*; Phage breeding

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1. Introduction

Listeria monocytogenes is an ubiquitous, gram-positive, intracellular parasite that can cause mortality in immunocompromised individuals. It also displays a high mortality rate for other segments of the population. It is principally associated with foods including poultry, red meats, meat products, fish, dairy products, fruits and vegetables (ICMSF, 1994). Many bacteria may develop as L-forms which are those in which the cell wall is deficient or absent in whole or in part. These L-forms differ from parent cell-walled forms in that they are pleomorphic, of variable size and often contain vacuoles and granules (Allan et al., 1993). L-forms may be induced when bacteria are situated in an osmotically stable environment or are exposed to cell wall synthesis-inhibiting compounds such as penicillin and ampicillin (Mattman, 1993). *L. monocytogenes* has been suspected of producing neurological disease such as meningitis and encephalitis while in a cell wall-deficient state (Mattman, 1993) and so a rapid detection system may be needed.

Detection of bacterial L-forms traditionally involves weeks of incubation in a selective medium, with ambigious results. Allan et al. (1992) used a modification of the gram stain to differentiate L-forms of *Enterococcus faecium*, *Bacillus subtilis* and *Pseudomonas syringae* from their parental forms. The use of in vivo bioluminescence for detecting and studying foodborne organisms is gaining acceptance (Baker et al., 1992; Stewart and Williams, 1992). It is clear that assays involving bioluminescence can be sensitive, rapid, and accurate. In vivo bioluminescence has been described as a cellular reporter for research and industry (Jassim et al., 1990, 1993). Denyer et al. (1991) studied the adhesion of *E. coli* K12 to polystyrene and glass coverslips using in vivo bioluminescence and found the technique to be a reliable indicator of the attachment of the organism. Walker et al. (1992) utilized a recombinant bioluminescent *L. monocytogenes* to measure biocide efficacy. More recently, bioluminescence was used to monitor L-form induction of a plant pathogen *Pseudomonas syringae* pv. *phaseolicola* (Waterhouse and Glover, 1994). Hibma et al. (1996a) used bioluminescence to detect the attachment of L-forms of *L. monocytogenes* to stainless steel.

Bacteriophage-based detection systems have been described. Lux^+ (those carrying *lux* genes) phages for *E. coli* and *Salmonella* (Chen and Griffiths, 1996; Stewart et al., 1989; Ulitzer and Kuhn, 1987) and *L. monocytogenes* (Jassim et al., 1992) have been used for the detection of these organisms. Bacteriophage typing of *L. monocytogenes* is well described and is used mostly for epidemiological purposes (Audurier and Martin, 1989; Estela et al., 1992; Loessner and Busse, 1990). Bacteriophage have also been used to remove biofilms in a paper mill but problems with resistant bacteria arose (Araki and Hosomi, 1990). Jassim et al. (1995) have developed phage breeding technologies, and have shown that it is possible to design superphage capable of infecting resistant bacteria. There has been little work published, however, regarding the targeting of bacterial L-forms by bacteriophage. In some respects, phage attack is similar for L-forms and classical bacteria and thus L-forms may be infected by the typical phage with a tail, by rounded phage without an appendage, or by virions (Mattman, 1993).

In this paper we describe the targeting of in vivo bioluminescent L-forms of *L. monocytogenes* with bred bacteriophage and use the bred phage to prevent attachment and to remove attached L-forms from stainless steel.

2. Materials and methods

2.1. Bacterial strain and growth media

L. monocytogenes ATCC 23074 (serotype 4b), containing a *lux* AB gene expression plasmid, [pSB331], was supplied by Dr Gordon Stewart, Nottingham University, UK. Cultures were maintained on tryptic soy agar (TSA, Difco, Detroit, MI) and grown in tryptic soy broth (TSB, Difco) when needed. Ampicillin was included at 5.0 μg/ml to maintain selection for plasmid pSB331. A mutation of classical *L. monocytogenes*, which eliminated the requirement for ampicillin selection for the plasmid, was isolated after the addition of the L-form bred phage to the classical culture. Ampicillin was then only used to maintain L-form cultures (i.e. keep them from reverting back to the classical form). L-form cultures were maintained on TSA supplemented with 0.5 μg/ml ampicillin and grown in TSB supplemented with 0.5 μg/ml ampillin as needed. λ-Buffer (2.5 g $MgSO_4 \cdot 7H_2O$, 0.05g gelatin, 6 ml 1 M Tris, pH 7.2 per liter) was used for all rinsing.

2.2. L-form induction

One hundred microliters of classical (cell-walled) culture (10^8 cfu) was inoculated into 10 ml of TSB containing 50 μg/ml ampicillin and cultured for 18 h at 30°C. The culture (10^7 cfu/ml) was then plated onto TSA (containing 0.5 μg/ml ampicillin) and incubated at 30°C for up to 3 weeks. Colony morphologies were compared to those described by Brem and Eveland (1968), and cell wall deficient forms were confirmed by transmission electron microscopy (TEM) as previously described (Hibma et al., 1996b). Viable counts were carried out according to the procedure of Miles et al. (1938).

2.3. Bacteriophage

L. monoctyogenes phage ATCC 23074-B1 was obtained from the ATCC and maintained in λ-buffer. The phage was bred (a chemical treatment) according to the method of Jassim et al. (1995) to be specific for the L-form of *L. monocytogenes*. The phage stocks were developed to high titre on an appropriate host strain by the plate lysis procedure, essentially equivalent to growing bacteriophage λ-derived vectors (Ausubel et al., 1991). The original phage for the classical form was serially diluted (10-fold) seven times in λ-buffer. An 18 h classical culture (100 μl) was added to 100 μl of each phage dilution and incubated for 10 min at 30°C to facilitate attachment of the phage to the bacteria. After incubation, 3 ml of molten 0.4% agar was added to each phage-bacteria mixture and these were poured onto

and spread around the surface of TSA plates and allowed to harden. Plates were incubated for 18 h at 30°C and examined for plaque formation and phage titre. Typical phage titres of 10^{10} cfu/ml were obtained. Stocks of bred phage were prepared according to the above methods. Phage stocks were maintained in λ-buffer at 4°C for several months. Bred and unbred phage were prepared for TEM as previously described (Hibma et al., 1996b) and observed for morphological differences.

2.4. Bacteriophage specificity and lytic activities

The unbred and bred phages were tested with both classical and L-form cultures. Unbred (classical) and bred (L-form) phage were added to the classical and L-form

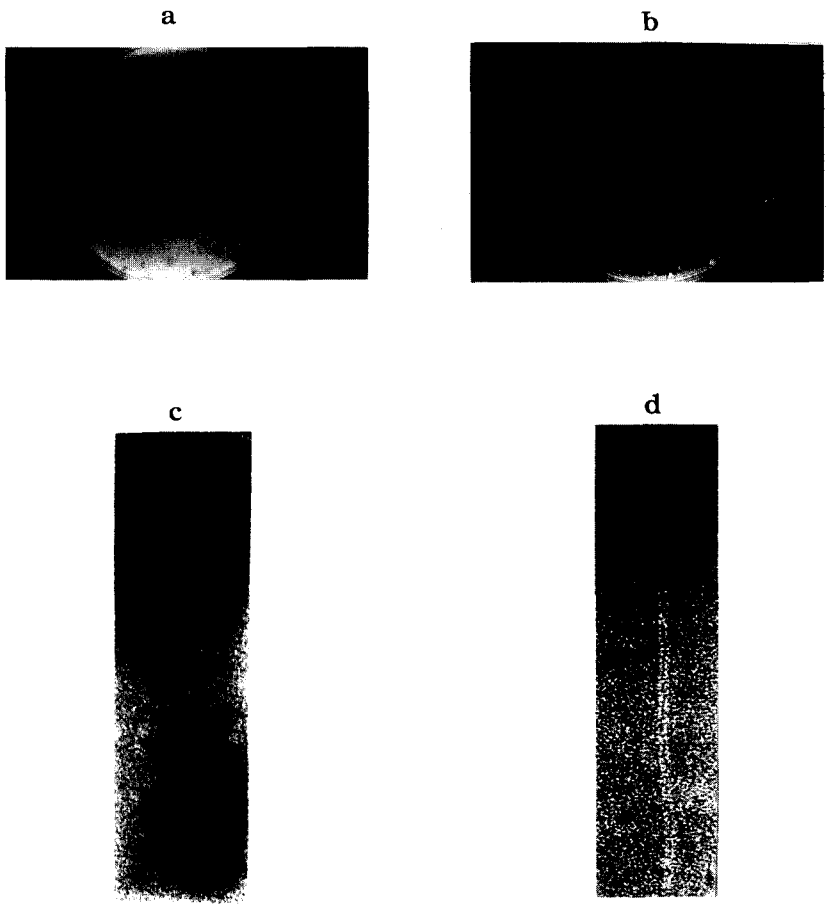

Fig. 1. Photographs of lysate plates of (a) unbred phage on lawn of classical *L. monocytogenes* and (b) bred phage on lawn of L-form *L. monocytogenes*. Transmission electron photomicrographs of (c) unbred *L. monocytogenes* phage ATCC 23074-B1, and (d) bred L-form phage. (Mag. × 288 000).

cultures. In each case 1 ml of phage (10^{10} pfu) was added to 10 ml of fresh culture (10^7 cfu) and incubated for 6 h at 30°C. At 1 h intervals, 1 ml of the phage-bacteria mixture was withdrawn and optical density (660 nm), viable counts, and bioluminescence measured. Specificity was also examined by lysis plates, as described above.

2.5. Bioluminescence assay

Nonyl decanal (Sigma, MO, 1% v/v in ethanol) was added to the culture (50 μl/ml) as a substrate for the bioluminescence reaction. Bioluminescence of broth cultures was measured using a Biotrace multilite luminometer (Biotrace, Bridgend, UK). The bioluminescence of colonies was confirmed using a BIQ bioview biomedical image quantifier (Cambridge Imaging, Cambridge, UK) after 100 μl of nonyl aldehyde was spotted onto the lid of the colony-containing agar plates.

2.6. Prevention of L-form biofilm formation on stainless steel

Bred phage was tested for its ability to prevent or hinder the attachment of L-forms to stainless steel. Six sterile stainless steel squares (No. 304, 1/2 in. × 1/2 in. × 1/8 in.) were immersed in 30 ml of L-form culture (10^6 cfu/ml) and 10 ml of bred L-form phage (10^{10} pfu/ml) in a sterile Petri plate. Likewise, squares were immersed in 30 ml of L-form culture and 10 ml λ-buffer as a control. Plates were incubated for 6 h at 30°C and rotated slowly at 25 rpm. At 1 h intervals, a square was removed and rinsed twice in λ-buffer. It was placed in a 50 ml centrifuge tube containing 1 ml TSB supplemented with 2% Tween 80, and vigorously vortexed for 30 s to remove adhered bacteria. In triplicate experiments, attachment was assessed by bioluminescence and viable count.

2.7. Organic acid versus bacteriophage on L-form biofilms

An organic acid was compared to the bred phage at removing L-form biofilms on stainless steel. L-forms were allowed to form biofilms for 18 h on stainless steel squares as described above. Squares with biofilms were treated for 6 h in either 10 ml lactic acid solution (130 ppm, pH 3.2), or 10 ml bred phage (10^{10} pfu/ml), or 10 ml λ-buffer as a control. After the given treatment, squares were removed, and rinsed twice in λ-buffer, and vigorously vortexed for 30 s in 1 ml TSB supplemented with 2% Tween 80. Viable counts and bioluminescence measurements were performed and compared to those from squares receiving no treatment with phage or lactic acid.

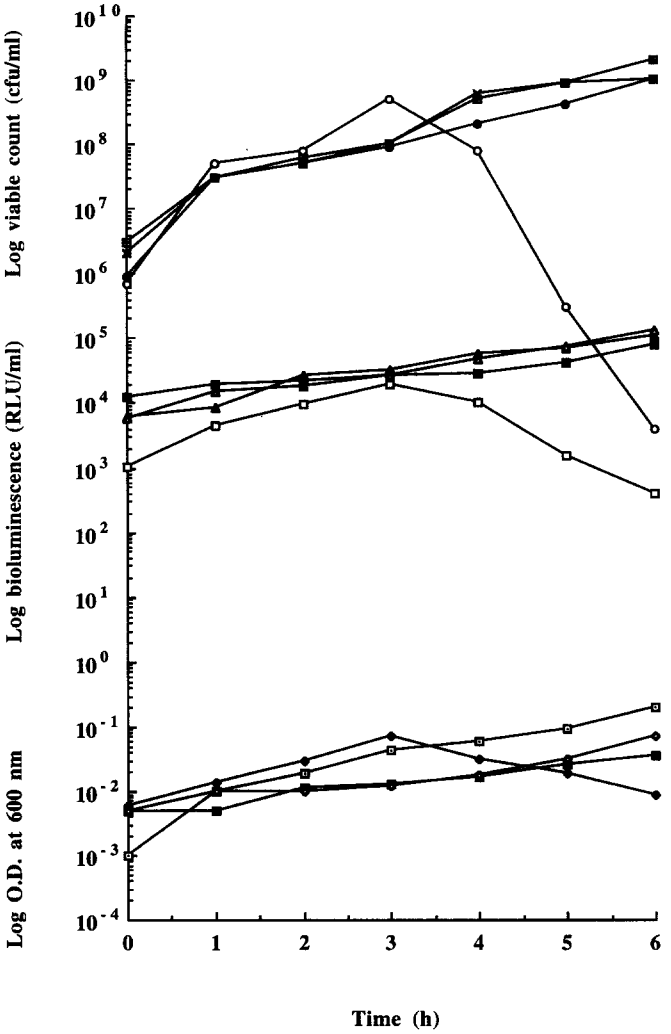

Fig. 2. Growth curves of classical and L-forms of *L. monocytogenes* in the presence of unbred phage. (&z.rvbull;) O.D. classical cells only, (◆) O.D. classical cell and unbred phage, (□) O.D. L-form cells only, (◇) O.D. L-form cells and unbred phage, (■) bioluminescence classical cells only, (□) bioluminescence classical cells and unbred phage, (▲) bioluminescence L-form cells only, (△) bioluminescence L-form cells and unbred phage, (●) viable count classical cells only, (○) viable count classical cells and unbred phage, (⊠) viable count L-form cells only, (×) viable count L-form cells and unbred phage.

3. Results and discussion

3.1. Confirmation of the L-form

L-form colonies required at least 72 h and up to 3 weeks to be visible on agar plates. Initially, they could be detected by bioluminescence after 8 h of incubation.

These colonies were compared to *L. monocytogenes* L-forms shown by Brem and Eveland (1968) and were very similar. TEM comparision of cell types (Hibma et al., 1996a) revealed that L-forms were smaller and oval with no cell walls while classical cells were longer, rod shaped and displayed cell walls.

3.2. Bacteriophage breeding and comparison

The morphological properties of unbred phage on the lawns of classical *L. monocytogenes* produced large round plaques up to 8 mm in diameter, some of

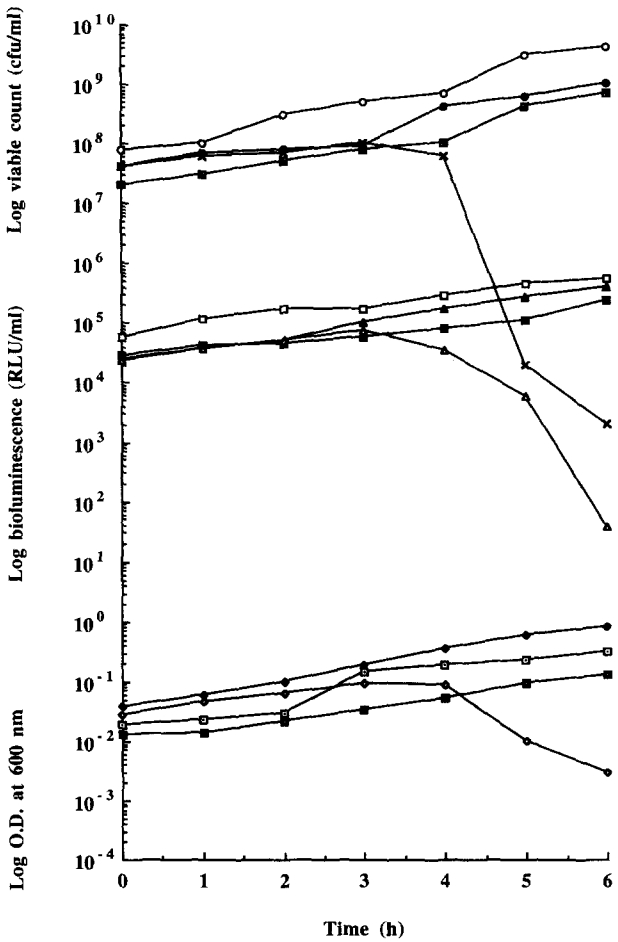

Fig. 3. Growth curves of classical and L-forms of *L. monocytogenes* in the presence of bred L-form phage. (&z.rvbull;) O.D. classical cells only, (◆) O.D. classical cells and bred phage, (□) O.D. L-form cells only, (◇) O.D. L-form cells and bred phage, (■) bioluminescence classical cells only, (□) bioluminescence classical cells and bred phage, (▲) bioluminescence L-form cells only, (△) bioluminescence L-form cells and bred phage, (●) viable count classical cells only, (○) viable count classical cells and bred phage, (⊠) viable count L-form cells only, (×) viable count L-form cells and bred phage.

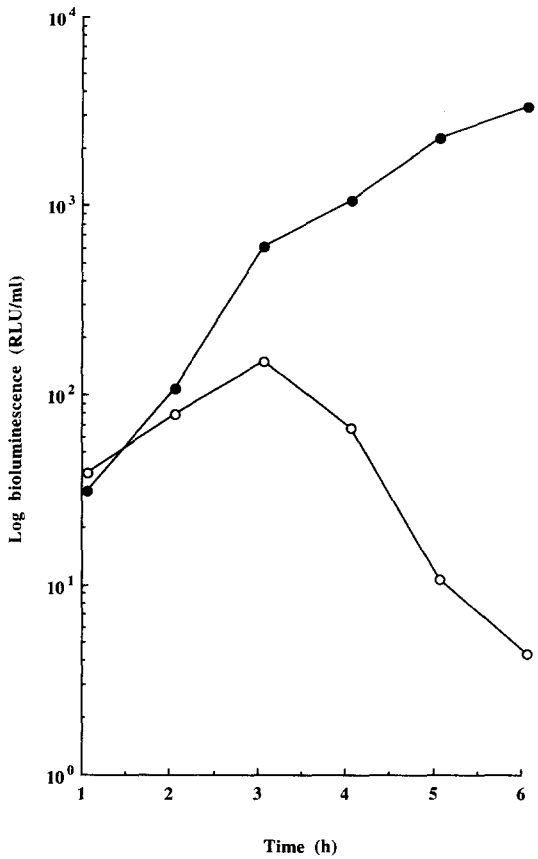

Fig. 4. Attachment of L-forms of *L. monocytogenes* to stainless steel in the presence (○) and absence (●) of bred L-form phage as measured by bioluminescence.

which were turbid and sharp cut (Fig. 1(a)). The L-form bred phage (ATCC 23074-B1) produced different sized plaques, some large and some pinpoint in size on lawns of *L. monocytogenes* L-forms (Fig. 1(b)). Plaques were detectable by bioluminescence within 3 and 7 h, respectively. In comparison, visible colony and plaque detection required between 5 h and 3 days for the classical and L-form, respectively. Classical phage did not plaque on L-form lawns, and L-form bred phage did not plaque on classical cell lawns.

The lytic activity of both phages were tested on both culture types over a 6 h period. Typical growth (optical density, viable count, bioluminescence) inhibition curves for classical cells of *L. monocytogenes* were associated with the rapid amplification and lysis of unbred phage (Fig. 2). However, the growth was unaffected by the presence of L-form bred phage. Likewise, similar results were obtained with the bred phage with both cell types (Fig. 3) indicating that only the L-form culture was infected and lysed by the bred phage.

The electron photomicrographs at 288 000 × magnification of the two phages (Fig. 1(c, d)) revealed some morphological differences. The unbred phage (Fig. 1(c)) displayed a capsid full head and long (364 nm in length, noncontractile state) noncontractile tail with cross-striations and a thick basal structure with spike-like terminal structures. The bred phage (Fig. 1(d)) displayed a full head and a long (but shorter than the unbred phage) thin noncontractile tail (304 nm in length, extended state) with cross-striations and a rounded appendage-less end. Differences between the two may be further confirmed at the molecular level. The fact that bred phage infects only the cell wall-deficient form is supported by the generalization that phage receptors are on the cytoplasmic membranes of some gram-positive bacteria, but are lacking on gram-negative organisms (Mattman, 1993). The breeding may have altered the specificity of the phage from a cell wall receptor to a cell membrane receptor.

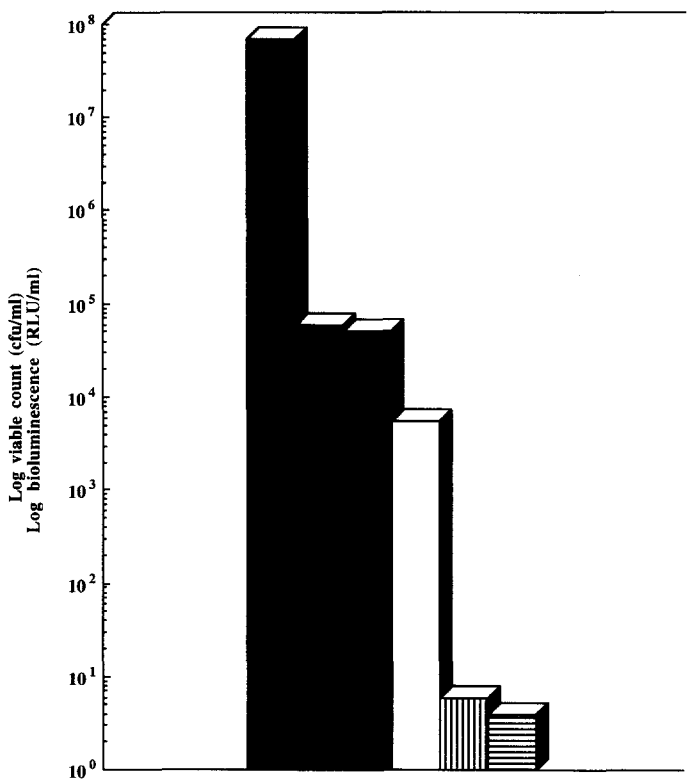

Fig. 5. Comparison of 6 h treatments with 130 ppm lactic acid and bred L-form phage for inactivating L-form biofilms of *L. monocytogenes* on stainless steel. (■) Viable count of biofilm receiving no treatment, (■) viable count of biofilm treated with bred phage, (■) viable count of biofilm treated with acid, (□) bioluminescence of biofilm receiving no treatment, (□) bioluminescence of biofilm treated with bred phage, (□) bioluminescence of biofilm treated with acid.

3.3. Inhibition of L-form biofilm formation on stainless steel

Over a 6 h period, a biofilm formed on stainless steel submersed in L-form culture in the absence of bred phage, as demonstrated by bioluminescence measurements (Fig. 4). However, the presence of the bred phage in the L-form culture prevented the formation of L-form biofilms, as was demonstrated by a decrease in in vivo bioluminescence.

It appears that in vivo bioluminescence for classical or L-form types can be a useful tool for such studies, in that it can demonstrate biofilm formation control, in situ.

3.4. Organic acid versus bacteriophage at inactivating biofilms

Biofilms of *L. monocytogenes* L-forms formed on stainless steel were treated for 6 h with bred phage and lactic acid (130 ppm). The bred phage was as effective as the organic acid at inactivating the L-form biofilm (Fig. 5). Both treatments reduced attached L-form bacteria by 3-log cycles when determined by conventional viable count and real-time bioluminescence measurement.

This paper has demonstrated the breeding of a bacteriophage that is specific for L-forms of *L. monocytogenes*. Exact applications of this phage remain to be elucidated, but it has been shown to be able to inhibit L-form biofilm formation on stainless steel. In addition, the phage has been shown to be as adept as an organic acid (lactic) at inactivating L-form biofilms. This could make phage breeding an attractive alternative to chemical sanitizers as a bio-control agent.

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