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Survival and Activity of *lux*-Marked *Aeromonas salmonicida* in Seawater

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The fish pathogen *Aeromonas salmonicida* was chromosomally marked with genes encoding bacterial luciferase, *luxAB*, isolated from *Vibrio fischeri*, resulting in constitutive luciferase production. During exponential growth in liquid batch culture, luminescence was directly proportional to biomass concentration, and luminometry provided a lower detection limit of approximately 10^3 cells ml^{-1}, 1 order of magnitude more sensitive than enzyme-linked immunosorbent assay detection. In sterile seawater at 4°C, *lux*-marked *A. salmonicida* entered a dormant, nonculturable state and population activity decreased rapidly. The activity per viable cell, however, increased by day 4, indicating that a proportion of the population remained active and culturable. Putative dormant cells were not resuscitated after the addition of a range of substrates.

Aeromonas salmonicida was initially characterized as an obligate pathogen (22) that causes furunculosis in salmonids and survives poorly in seawater, as estimated by the ability to form colonies on solid media (13, 25). Indeed, it is difficult to isolate this pathogen from the water surrounding fish farm sites during outbreaks of disease, despite bacteria being shed from infected fish at high rates (24). Recent research (6, 15–17) suggests that *A. salmonicida* cells released from fish enter a dormant state, remaining viable but unable to be cultured by routine laboratory methods. This may prevent the detection of *A. salmonicida* in environmental samples and may explain the occurrence of furunculosis at previously disease-free farm sites, where no infected fish have been introduced (19). The formation of viable but nonculturable cells in several species has been reported (2, 12, 21, 27), and there is evidence for their resuscitation (21), although this may result from the growth of a few remaining culturable cells (26). This paper describes the starvation-survival of *A. salmonicida* in sterile seawater by a luminescence-based technique for measurement of metabolic activity. Luminescence-based marker systems have been used to study the survival and activities of bacteria in soil (1, 7, 14, 23) and plant-pathogen interactions (4, 28, 31) and involve the introduction of *luxAB* genes encoding the luciferase enzyme, with quantification of light emission by luminometry after the addition of the aldehyde substrate. The reactivation of starved, nonculturable *A. salmonicida* cells should result in detectable bioluminescence prior to the growth of any culturable cells (5) without the need for specialized conditions required for cultures of viable but nonculturable cells (30). This paper therefore also describes attempts to resuscitate starved *A. salmonicida* populations after the addition of a range of nutrients.

Bacterial strains and culture conditions. *A. salmonicida* subsp. *salmonicida* MT463 (Scottish Office Agriculture and Fisheries Department Marine Laboratory, Aberdeen, United Kingdom) was isolated in 1988 from Atlantic salmon with furunculosis and is virulent, and its characteristics match those published by Popoff (22). The strain was stored in mist. desiccans at −70°C and routinely cultured on tryptone soya agar (TSA) containing oxolinic acid (5 μg ml^{-1}), producing raised, friable colonies with a characteristic brown diffusible pigment. *Escherichia coli* SM10 (λ pir) was provided by K. Timmis (GBF, Braunschweig, Germany) and routinely cultured on TSA supplemented with tetracycline (12.5 μg ml^{-1}) for the maintenance of plasmid pUT (3, 8).

A. salmonicida subsp. *salmonicida* MT463 was chromosomally marked with luciferase genes, *luxAB*, originally isolated from the naturally bioluminescent marine bacterium *Vibrio fischeri*. This was achieved by introduction of the *luxAB-tet* cassette into *A. salmonicida* MT463 by insertional mutagenesis with the minitransposon Tn*5* (3). The mini-Tn*5* system was harbored on plasmid pUT in *E. coli* SM10 (λ pir), and delivery of the pUT plasmid into *A. salmonicida* MT463 was carried out by filter mating with *E. coli* SM10 (λ pir). Overnight cultures of *E. coli* SM10 (λ pir) and *A. salmonicida* MT463 were prepared in tryptone soya broth (TSB) and incubated at 30 and 22°C, respectively. Samples consisting of 100-μl *E. coli* SM10 (λ pir) and 500-μl *A. salmonicida* MT463 cultures were mixed in 5 ml of sterile 0.85% (wt/vol) NaCl. Bacteria were filtered by spotting the suspension on 0.45-μm-pore-size membrane filters (Whatman). Filters were placed on TSA plates and incubated at 22°C overnight. After incubation, swabs of the bacterial lawn were resuspended in 500 μl of 0.85% NaCl. A 100-μl aliquot was removed and inoculated onto TSA containing tetracycline (12.5 μg ml^{-1}) and oxolinic acid (5 μg ml^{-1}). Plates were incubated at 22°C for 3 days, and brown-pigmented colonies were checked for luminescence. Among the putative *lux*-marked *A. salmonicida* MT463 strains, four were randomly selected for further analysis. Genomic DNAs were prepared from each recombinant strain and wild-type *A. salmonicida* MT463 (29). Plasmid pUT was prepared from *E. coli* SM10 (λ pir) by using Magic Minipreps (Promega). DNA was digested with *Bam*HI and *Hin*dIII, Southern blotted, and probed with

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radiolabelled *luxAB* (32). The restriction patterns of the hybridized bands for these strains were compared.

Maximum luminescence during batch culture was measured for each *lux*-marked *A. salmonicida* strain as described below, and the long-term stability of the *luxAB-tet* insert was studied for the two brightest strains during repeated subculture in liquid medium. Light emission was characterized during batch culture of luminescence-marked strains of *A. salmonicida* MT463 by adding a 1% inoculum from an overnight culture to triplicate 250-ml Erlenmeyer flasks containing 100 ml of TSB supplemented with tetracycline (12.5 μg ml^{-1}). Flasks were incubated at 22°C with shaking (100 rpm), and samples were removed for measurements of luminescence and biomass concentration (optical density at 600 nm). Luciferase production in *A. salmonicida* MT463 *luxAB* was constitutive as the regulatory genes *luxIR* were absent, and the absence of *luxCDE* necessitated the exogenous addition of *n*-decyl aldehyde substrate. The optimal conditions for aldehyde addition were determined by incubating 1-ml samples of early-exponential-, mid-exponential-, and stationary-phase cultures of *A. salmonicida* MT463 *luxAB* with 10- or 1-μl volumes of the following concentrations of *n*-decyl aldehyde: 10 μl (100% [vol/vol]) and 1 μl (100, 50, 10, 5, 2 or 1% [vol/vol]). Luminescence was measured repeatedly for 20 min after aldehyde addition with an LKB 1251 luminometer. Thereafter, luminometry was routinely performed after the incubation of 1-ml samples with 1 μl of 2% (vol/vol) *n*-decyl aldehyde for 7 min at room temperature. Sterile TSB, phosphate-buffered saline (PBS), or seawater was used to determine background bioluminescence (as appropriate). To determine the minimum detection limit in liquid batch culture, a 1% inoculum from an overnight culture of *A. salmonicida* MT463 *luxAB* was added to triplicate 250-ml Erlenmeyer flasks containing 100 ml of TSB supplemented with tetracycline (12.5 μg ml^{-1}). Flasks were incubated at 22°C with shaking (100 rpm), and samples were removed for the enumeration of total cells in a counting chamber and for measurements of luminescence and biomass concentration (optical density at 600 nm). The stability of the *luxAB-tet* gene construct was monitored during repeated subculture in nonselective medium. A 1-ml sample of *A. salmonicida* MT463 *luxAB* from an overnight culture in TSB supplemented with tetracycline (12.5 μg ml^{-1}) was centrifuged in a microcentrifuge for 5 min. Cells were washed twice by resuspension in 1 ml of PBS and centrifugation. The cell pellet was resuspended in 1 ml of PBS and added to 9 ml of TSB. Cells were incubated with shaking at 22°C for 2 days. After incubation, cells were subcultured in 9 ml of TSB, and the proportions of tetracycline-resistant cells were determined on TSA in the presence and absence of tetracycline (12.5 μg ml^{-1}) by the drop plate method (9). All plates were incubated at 22°C for 2 days.

Starvation and resuscitation studies. Exponential-phase cultures of wild-type *A. salmonicida* MT463, grown in TSB supplemented with NaCl (3% [wt/vol]), and of the *lux*-marked strain, grown in TSB containing NaCl (3% [wt/vol]) and tetracycline (12.5 μg ml^{-1}), were collected by centrifugation (8,000 × *g*, 15 min). Cells were washed twice by resuspension in an equal volume of sterile seawater and centrifugation. Sterile seawater was prepared by filtration of water from an Aberdeen beach through 0.45- and 0.2-μm-pore-size membrane filters and by autoclaving at 121°C for 30 min. Pellets were resuspended in 10 ml of sterile seawater and diluted 100-fold in the same medium. A 1% inoculum of this suspension was used to inoculate triplicate seawater microcosms, consisting of 250 ml of sterile seawater in 500-ml-capacity Duran flasks. Initial cell concentrations were 10^5 to 10^6 cells ml^{-1}, and samples were incubated without shaking at 4°C. Samples were removed from each flask for determinations of total cell concentration, population activity (as measured by luminometry), and enumeration of CFU on TSA. Total cell enumeration was determined by incubating cells with 2 μg of 4′,6-diamidino-2-phenylindole (DAPI) ml^{-1} for 2 h at 4°C in the presence of 1 mM EDTA (pH 8.0) and by filtering 2-ml samples through 0.2-μm-pore-size black polycarbonate membranes. Cells were observed under UV illumination with an Olympus BH2 fluorescence microscope.

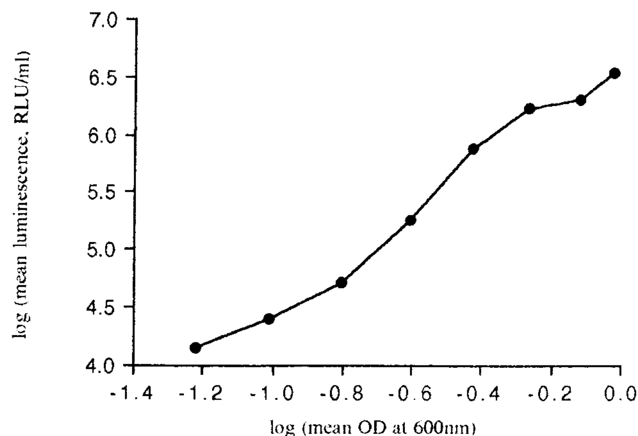

FIG. 1. Relationship between luminescence and biomass concentration during batch culture of *A. salmonicida* MT463 *luxAB*. Data are the means of triplicate cultures. OD, optical density.

For resuscitation studies, cell supernatants were harvested from mid-exponential-phase and late-exponential-phase batch cultures of *A. salmonicida* MT463 *luxAB* grown in TSB supplemented with tetracycline (12.5 μg ml^{-1}). Supernatants were sterilized by filtration (0.45-μm-pore-size filters followed by 0.2-μm-pore-size filters [Millipore]). Atlantic salmon parr were sacrificed, and the gill arches were aseptically removed. Patches of skin and mucus (approximately 1 cm^2) were also removed and added aseptically to sterile Universal bottles. Fish serum was donated by T. Bowden (SOAFD Marine Laboratory). The resuscitation of starved cells was carried out by measuring the changes in luminescence and growth after the addition of the following media to triplicate flasks of starved cells prepared as described above: TSB at 0.1 and 5% (wt/vol), TSB (5% [wt/vol]) supplemented with sucrose (10% [wt/vol]), yeast extract (0.025% [wt/vol]), yeast extract (0.025% [wt/vol]) supplemented with sucrose (10% [wt/vol]), TSB supplemented with 1 ml of sterile supernatant, fish gill tissue, fish skin and mucus, and fish serum. Growth was assessed by turbidity and dilution plate counting on TSA (3% [wt/vol]), TSA (5% [wt/vol]), TSA (5%) supplemented with sucrose (10%), nutrient agar, and nutrient agar supplemented with sucrose (10%). All plates were incubated at 22°C until discrete colonies were distinguished.

Characterization of *lux*-marked *A. salmonicida* MT463. The DNA restriction pattern of the brightest *lux*-marked *A. salmonicida* MT463 strain revealed that chromosomal integration of the entire pUT plasmid had occurred, and the resultant construct was found to be unstable (data not shown). Chromosomal insertion was established for the second-brightest strain (data not shown), and this strain, *A. salmonicida* MT463 *luxAB*, was used for all subsequent studies. During batch growth of this strain, light emission and the biomass concentration increased at the same specific rate (Fig. 1) and luciferase synthesis was constitutive. During logarithmic growth, the light

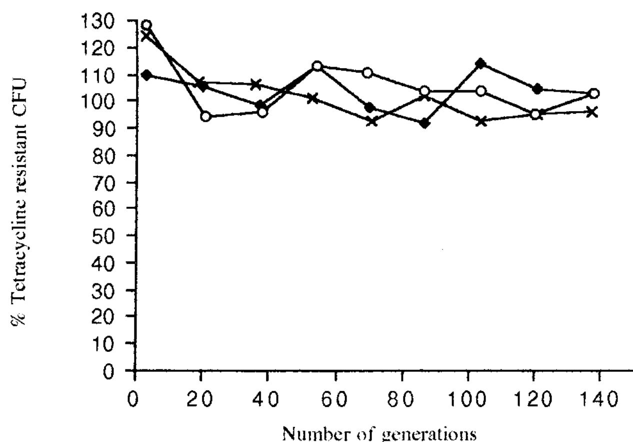

FIG. 2. Stability of the *luxAB-tet* gene construct in triplicate cultures of *A. salmonicida* MT463 *luxAB*, showing the percentages of tetracycline-resistant cells during repeated subculture in nonselective medium. Results are presented for three replicates (○, ×, and ◆).

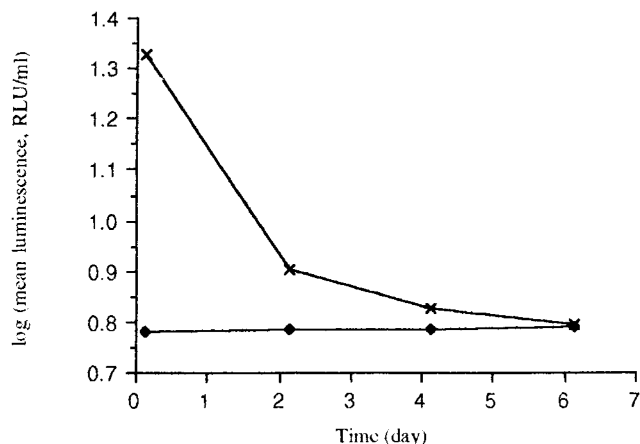

FIG. 4. Changes in the luminescence of *A. salmonicida* MT463 *luxAB* (×) and background luminescence (◆) during nutrient stress in sterile seawater at 4°C. Data are the means of triplicate flasks.

output was directly proportional to biomass concentration. This relationship does not hold when cells are not fully active. The specific growth rates of the wild-type and *lux*-marked strains were 0.567 and 0.458 h^{-1}, respectively (significantly different at the 95% significance level). Log-log plots of light output against cell concentration during logarithmic growth in liquid batch culture gave a mean value for light output of 10^{-3} relative light units (RLU) $cell^{-1}$. The lower detection limit was calculated to be approximately 10^3 cells ml^{-1} by estimating the number of cells resulting in a value of 1 RLU above the background level. The long-term stability of the *luxAB-tet* cassette in *A. salmonicida* MT463 *luxAB* was monitored by repeated subculture in nonselective liquid medium (Fig. 2). There was no significant loss of tetracycline resistance after 140 generations, and high levels of luminescence in nonselective liquid media were maintained throughout this period.

Starvation-survival and resuscitation. During starvation in sterile seawater at 4°C, the viabilities of both wild-type and *lux*-marked *A. salmonicida* strains decreased rapidly within 4 days (Fig. 3); after approximately 9 weeks, the number of cells capable of forming colonies on TSA fell to the limit of detection (0.1 cells ml^{-1} [data not shown]). The total counts for both strains, however, fell to approximately 12% of the original numbers after starvation for 23 days (Fig. 3). Microscopic examination of DAPI-stained cells revealed that they had maintained their characteristic coccobacillus morphology, but cell size was reduced. No significant differences in the responses of these two *A. salmonicida* MT463 strains to starvation stress were found (total count, $P = 0.272$; viable count, $P = 0.333$). The population activity of *A. salmonicida* MT463 *luxAB* (in RLU ml^{-1}) decreased exponentially to background levels within 6 days of inoculation into sterile seawater (Fig. 4). However, the average light output per viable cell increased from 5.6×10^{-5} RLU (day 0) and 2.5×10^{-5} RLU (day 2) to 3.6×10^{-3} RLU (day 4) (similar to the activity of exponentially growing cells) before luminescence fell to background levels on day 6. The response of *A. salmonicida* MT463 *luxAB* cells, starved for approximately 9 weeks, to the addition of a range of substrates was studied by monitoring changes in cell activity and culturability on solid media. No CFU of *A. salmonicida* MT463 *luxAB* were recovered either with or without the prior addition of substrate to cells suspended in seawater. Further, no CFU were recovered on solid medium containing sucrose as an osmotic support, and there was no observed increase in the metabolic activity of starved cells under any of the conditions tested.

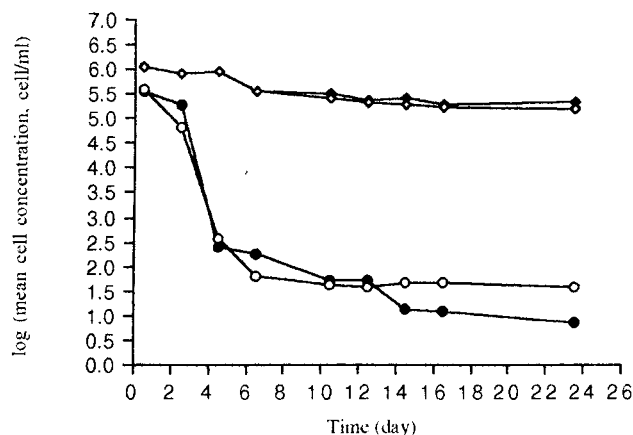

FIG. 3. Changes in the viable cell concentrations (circles) and total cell concentrations (diamonds) of wild-type (open symbols) and *lux*-marked (closed symbols) cells of *A. salmonicida* MT463 during starvation at 4°C in sterile seawater. Data are the means of triplicate flasks.

The use of luminometry to assess *A. salmonicida* activity during starvation in sterile seawater is a novel approach and facilitates rapid in situ measurement. This system may be extended to nonsterile aquatic systems since luminescence in the *lux*-marked strain requires the addition of a fatty aldehyde substrate, enabling a distinction from naturally luminescent microorganisms. The lower detection limit for marked cells in liquid culture (10^3 cells ml^{-1}) is higher than some reported values, for example, 1.7×10^2 cells ml^{-1} for a plasmid-bearing construct of *E. coli* in liquid culture (23). Plasmid markers give higher levels of luminescence but are less stable in the absence of selective pressure (1), and chromosomally marked strains provide a more direct relationship between luminescence and biomass concentration. The high levels of light output from chromosomally marked *A. salmonicida* MT463 *luxAB* enabled sensitive quantification of population survival and activity, but marked strains grew more slowly than the parent strain did. High-level expression of the *luxAB-tet* construct was dependent on integration at a site downstream of a strong promoter, and

the gene disrupted by insertion may have been important for cell growth. The reduction in growth may also have been due to the increased metabolic burden of replicating this foreign DNA.

The limited survival of this fish pathogen in seawater observed here agrees with other studies (6, 13, 25), but our data indicate an increase in the activity of cells on day 4 of starvation. This may have been due to the synthesis of novel proteins in response to starvation stress (18), but such an increase is more likely to occur immediately after starvation. An alternative explanation is the increased activity of culturable cells surviving by cryptic growth.

Luminometry was used to detect regrowth or reactivation of starved *lux*-marked cells after the addition of nutrients without the necessity of cultivation on laboratory media or complications associated with the growth of a small number of culturable cells. However, there was no detectable increase in either luminescence or culturable cells after the addition of a range of nutrients to starved wild-type or *lux*-marked cells. Nutrients were chosen to test hypotheses regarding reactivation suggested by previous studies. For example, Effendi and Austin (6) suggested the formation of specialized cell wall-defective variants, but we found no evidence of resuscitation of nonculturable strains on sucrose-amended medium designed to support the growth of L forms. Effendi and Austin (6) also reported the formation of viable but nonculturable forms of *A. salmonicida*, detecting viability by the direct viable count assay of Kogure et al. (11) and involving activation by yeast extract. We found no evidence of activation or resuscitation after the addition of yeast extract (0.025% [wt/vol]) to starved cells with or without the addition of sucrose. Reactivation and regrowth were not observed in complex or diluted complex media, the latter reducing the possibility of substrate-accelerated death. Reactivation after contact with susceptible fish was tested by amending media with fish gill tissue, skin, mucus, and serum, but no increase in luminescence or cell concentration was detected.

Therefore, the addition of nutrients in itself was not sufficient to reactivate nonculturable *A. salmonicida* after long-term starvation in sterile seawater. Other evidence indicates that this pathogen becomes dormant. For example, cell integrity, RNA, and plasmid DNA levels are maintained (16, 17), and more subtle mechanisms may control the exit of cells from dormancy. Recent research with *Micrococcus luteus* (30) suggests that recovery is due to the excretion of a pheromone by viable cells, but we found no activation in the presence of supernatants harvested from cells at various stages during batch culture.

Despite extensive research, our studies did not identify factor(s) or conditions capable of reestablishing culturable *A. salmonicida* after prolonged starvation. This suggests that the transmission of *A. salmonicida* does not involve dormant organisms, and the mechanism of transmission after shedding from diseased fish remains unclear. In some cases, infection is not associated with direct contact with infected fish and has been attributed to a waterborne source (19), perhaps carried by a planktonic vector. King and Shotts (10) describe the proliferation of *A. salmonicida* through ingestion by the freshwater protozoan *Tetrahymena pyriformis*, and this pathogen has been isolated from marine zooplankton, including the salmon louse *Lepeoptheirus salmonis* (20). Further research on the relationship between plankton and *A. salmonicida* is being carried out to test these alternative mechanisms of transmission.

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