Salt, pH and Temperature Dependencies of Growth and Bioluminescence of Three Species of Luminous Bacteria Analysed on Gradient Plates

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Two-dimensional diffusion gradients (of NaCl and H⁺ concentrations) were established in solid growth medium containing glycerol and yeast extract as major carbon sources. These were used to investigate conditions favourable for growth and bioluminescence of three species of luminous bacteria during incubation at different temperatures. *Photobacterium leiognathi*, *Photobacterium phosphoreum* and *Vibrio fischeri* all grew over the entire salt range used [0.9–3% (w/v) NaCl] and at pH values <7 at the most favourable temperatures (20 °C, 20 °C and 15 °C respectively); upper and lower temperature limits for growth over a 72 h period were 30 and 10, 25 and 5, and 30 and 5 °C respectively. Bioluminescence was observed at all temperatures that supported growth; in *P. leiognathi* emission at 10 °C was hardly detectable even after 72 h, but at higher temperatures it occurred at all NaCl concentrations. Low pH values and high NaCl concentrations favoured luminescence in the other two organisms; after 48 h light emission decreased from the high pH and low NaCl regions of the gels. These results are discussed with reference to the symbiotic (*P. leiognathi*, *V. fischeri*) or free-living (*P. phosphoreum*) origins of the organisms studied.

INTRODUCTION

The growth of luminous bacteria from marine waters and of those isolated as symbionts of fish light-organs, and the expression of their bioluminescence in the laboratory, may give insights into the environmental conditions favourable for their occurrence (Hastings & Nealson, 1981). Salinity, pH and temperature are of prime importance amongst the factors involved in growth. Thus populations of organisms found in coastal waters correlate with seasonal temperature changes (Ruby & Nealson, 1978; O'Brien & Sizemore, 1979), as well as with local differences in salinity (Shilo & Yetinson, 1979). The temperature range of symbionts correlates well with the temperatures of the habitats of their specific hosts.

The synthesis of the luciferase system and the expression of bioluminescence is dependent on many interacting factors: growth rate, O₂ concentration, availability of iron and arginine, presence of a specific autoinducer, and absence of catabolite repression are some of the more clearly identified ones (Hastings & Nealson, 1977; Hastings *et al.*, 1985). Bacterial light-organ symbionts show some unexpected characteristics; thus Dunlap (1984) has noted that luminescence of *Photobacterium leiognathi* is maximal at the low osmolarity (300 to 400 mosM) and low growth rates which obtain *in situ*. When these bacteria were cultured in a high osmolarity medium [70% (v/v) sea water] at a growth rate of 0.28 h⁻¹, photon emission rates were only one-seventh of those from organisms grown in 30% sea water at half that growth rate (Dunlap, 1985). Early work on the effects of pH and NaCl on several bioluminescent bacteria has been reviewed by Harvey (1952).

Wimpenny *et al.* (1983, 1984) have discussed the usefulness of laboratory models for the investigation of spatial heterogeneity of ecosystems. A potentially powerful method for the study of the effects of two or three environmental variables is that based on the gradient plate technique (Szybalski, 1952; Szybalski & Bryson, 1952). The two-dimensional version of this

method developed by Wimpenny & Waters (1984) has been used in the present work, to examine the effects of pH values and NaCl concentrations on the growth and luminescence of three different luminous bacteria at different temperatures. As well as facilitating determination of optimal culture conditions, the method provides a uniquely convenient approach to a study of multifactorial interactions.

METHODS

Organisms and media. The organisms studied were *Photobacterium leiognathi* LN-1a, isolated from a live *Leiognathus nuchalis* captured off Misaki, Japan, in spring 1983, *Photobacterium phosphoreum* jw1, isolated from a sea water sample from 600 m in the North Atlantic ocean (Ruby *et al.*, 1980), and *Vibrio fischeri* MJ-1 isolated by Ruby & Nealson (1976) from the light organ of a live pinecone fish. Organisms were maintained at 4 °C on Photobacterium Broth (Difco) (66 g per l glass-distilled water) with 15 g Bacto-Agar l⁻¹ (Difco), and for use they were subcultured in 50 ml Photobacterium Broth.

Plate preparation. Two-dimensional gradient plates were prepared by the technique of Wimpenny & Waters (1984), which gives salt and pH gradients at right angles to one another, in Sterilin wettable 100 mm square Petri dishes. Each plate was prepared from four layers of medium which contained (g per l glass-distilled water): Tryptone (Difco), 5; yeast extract (Oxoid), 2.5; NH₄Cl, 0.3; MgSO₄ · 7H₂O, 0.3; FeCl₃, 0.01; KH₂PO₄, 3; Bacto-Agar (Difco), 30; and 20 ml glycerol. This medium was dispensed in volumes of 15 ml. For layers 1 and 2, 0.25 ml 1 M-H₂SO₄ and 0.75 ml 1 M-NaOH respectively were added to 15 ml of the medium. For layer 3, 80 g NaCl was added for every litre of medium. Growth medium was heated to boiling, and autoclaved at 121 °C for 15 min. H₂SO₄ and NaOH were added immediately before pouring, thus preserving the gelling properties of the agar. Media were maintained at 80 °C until poured.

Pouring the plates. The method used was as described by Wimpenny & Waters (1984). Additionally, all plates were left at room temperature for 24 h to equilibrate. For use, the plates were dried at 37 °C with the lids removed and then stored at 30 °C.

Inoculation. The inoculum was incubated at 20 °C overnight in a shaking water bath, then 0.5 ml of this culture was removed and spread over the surface, using an alcohol-flamed glass spreader: incubation was for up to 72 h.

Mapping the results. Both growth and bioluminescence were recorded using the x and y co-ordinates from a grid etched in both dimensions at 5 mm intervals.

pH and NaCl measurements. These were made as described by Wimpenny & Waters (1984). However, the pH electrode used was a microcombination probe (MJ-410-PYE; Microelectrodes Inc.).

Stability of NaCl and pH gradients over periods of up to 96 h has been demonstrated (Wimpenny & Waters, 1984). All experiments were done in duplicate: patterns of growth and bioluminescence were almost identical on both sets of plates.

RESULTS

P. leiognathi. Growth of this organism occurred over the range 10–30 °C, but it was poor at 10 °C, and at this temperature luminescence was not detectable until after 72 h incubation (Fig. 1). The organism grew over the entire range of NaCl concentrations at temperatures below 30 °C; at 30 °C luminescence was not observed at 1.2% NaCl. At 15–25 °C luminescence spread from the lowest NaCl concentrations progressively, but at the highest NaCl concentrations was markedly pH dependent, with an optimum between pH 5.8 and 6.5. Along the pH axis, the growth almost always (except at 30 °C) occurred beyond the region of detectable luminescence. An intermediate growth front, i.e. a secondary boundary, observed between the regions of heavy and light growth may have arisen as a result of local modifications of gradients in the medium due to metabolic activity. This phenomenon was most pronounced at the sub-optimal temperature of 15 °C. Luminescence was most persistent at low NaCl concentrations at temperatures < 25 °C, but at 30 °C was longer lived at high NaCl concentrations.

P. phosphoreum. This organism was able to grow and luminesce over the range 5–25 °C, but not at higher temperatures (Fig. 2). Growth occurred over the entire NaCl range, but not at pH values higher than 7.0. No luminescence was observed at NaCl concentrations < 1.0% or pH values > 6.8. The upper limit for the pH dependence of luminescence was noticeably temperature dependent (pH 5.8 at 5 °C but 6.8 at 25 °C). Luminescence was less stable at the higher temperatures, and was lost progressively over the second and third day of incubation from the regions of lower NaCl. That acidity plays a role in this decline is evident after 72 h at 25 °C from the skewed trailing edge of luminescence. A front of heavy growth was noted at 15 and 20 °C after 72 h.

Fig. 1. Patterns of growth and bioluminescence of *Photobacterium leiognathi* LN-1a on two-dimensional diffusion plates incubated for up to 72 h at different temperatures. Areas of perceptible growth are indicated by solid lines; limits of heavy growth (---) and secondary boundaries (---) are also indicated. Hatched areas indicate bioluminescence. The dots indicate 10 mm grid intersections.

Fig. 2. Patterns of growth and bioluminescence of *Photobacterium phosphoreum* jw1. See Fig. 1 for other details.

Fig. 3. Patterns of growth and bioluminescence of *Vibrio fischeri* MJ-1. See Fig. 1 for other details.

V. fischeri. This organism grew and showed luminescence at all temperatures between 5 and 30 °C (Fig. 3). Growth occurred at all NaCl concentrations, but bioluminescence over the entire NaCl range was observed only at temperatures < 20 °C. At higher temperatures luminescence required high NaCl, and its persistence showed a similar NaCl dependency. At all temperatures the pH requirements for growth and luminescence were identical and temperature independent; pH 6.8 was the upper limit. A zone of heavy growth was established after 24 h at all temperatures > 10 °C and did not change in position over the next 48 h of incubation.

DISCUSSION

These results confirm the usefulness of the two-dimensional gradient plate method for investigation of the optimal conditions for growth and light emission of bioluminescent bacteria. The observed patterns give a rapid indication of the temperature, pH and salinity limits for growth and bioluminescence. That these are not identical for the three species examined may be related to their differing ecological habitats, e.g. the fact that lower NaCl concentrations support luminescence of the two symbionts than in the case of *P. phosphoreum*, isolated as a free-living species, is in accord with the recent observations of Prosser (1973) that the ambient NaCl concentration within fish light-organs is much lower than that of sea-water.

As well as enabling distinctions to be drawn between species (Wimpenny & Waters, 1984), indications of unexpected interactions between temperature, pH and NaCl dependencies suggest promising avenues for further physiological and biochemical investigations. Other determinants of bacterial luminescence that could be investigated by this technique include the concentrations of glucose (Nealson & Hastings, 1979), iron (Makemson & Hastings, 1982) and arginine (Waters & Hastings, 1977).

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