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The Effect of Iron on the Growth and Luminescence of the Symbiotic Bacterium *Vibrio fischeri*

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Abstract

Light organs of the marine fish *Monocentris japonicus* contain the luminous bacterium *Vibrio fischeri* in a repressed state of growth. Since the luminescence system of the bacteria is inducible, the environment within the light organ must both promote luminescence and repress growth. Several types of nutrient limitation were tested as candidates for a growth repression mechanism by measuring their effects on luminescence of *V. fischeri* in culture. Cultures limited for C, N and P lost their luminescence; in contrast iron limitation had relatively little detrimental effect on luminescence. Luminescence and the effects of iron on growth and luminescence were tested in liquid and on solid media by addition of ferric ammonium citrate to minimal medium and chelation of iron by ethylenediamine di (o-hydroxyphenyl acetic acid), EDDA. Synthesis of luciferase was repressed by iron, thus low iron is optimal for luminescence and iron limitation is well suited as a means of growth repression in *M. japonicus* light organs.

Key words: bioluminescence, luciferase, luminous bacteria, *Vibrio fischeri*, *Monocentris*, symbiosis, iron regulation.

1. Introduction

The Japanese pinecone fish *Monocentris japonicus* possesses external light organs containing luminous bacteria (*Vibrio fischeri*) in a repressed state of growth (Ruby and Nealson, 1976; Haygood et al., 1984). The light organs act as a continuous culture; nutrients are provided by the host, the bacteria luminesce and multiply slowly, and excess bacteria are released into the surrounding seawater (Haygood et al., 1984; Nealson et al., 1984). The mechanism by which growth repression is achieved is not known. If nutrient limitation is the means of growth repression in the light organs it must be compatible with luminescence, since the

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luminescence system is inducible (Nealson, 1977; Rosson and Nealson, 1981) and induction of luminescence may be affected by the growth environment. Thus, as bacteria are luminous in the light organs, a major constraint on a plausible means of growth limitation must be that the proposed form of nutrient have a neutral or positive effect on luminescence.

Many nutrients could limit bacterial growth in the light organs; among the obvious possibilities are the major nutrients carbon, nitrogen and phosphorus and the major trace element, iron. Iron is required by almost all bacteria (except the lactobacilli) at micromolar levels (Weinberg, 1974). To overcome the insolubility of iron under aerobic, neutral pH conditions (Byers and Arceneaux, 1977), microorganisms have developed siderophore systems to solubilize and transport iron (Neilands, 1981). We chose to investigate the effects of iron availability on growth and luminescence in *V. fischeri* because fishes possess an effective mechanism for iron limitation of bacterial growth.

By means of this mechanism, known as "nutritional immunity" animals withhold iron from invading microorganisms (Weinberg, 1974; 1978). Iron in body fluids and secretions of animals, including fishes, is bound by the highly specific iron binding proteins transferrin and lactoferrin (Weinberg, 1978; Hershberger and Pratschner, 1981). These proteins reduce free iron to about 10^{-12} M and are responsible for preventing bacterial growth (Weinberg, 1978; Neilands, 1981). Thus symbiotic luminous fishes may have been preadapted to use iron limitation to repress growth in the light organs.

The effect of iron on growth and luminescence has not been studied in any luminous bacteria known to be light organ symbionts, although iron repression of luminescence was reported in *V. harveyi*, a luminous bacterium not found in light organs (Makemson and Hastings, 1982). The aim of the work described here was to examine growth and light production in *V. fischeri* under conditions of reduced iron availability and to judge whether iron limitation is a good candidate for the growth repression mechanism in the intact symbiosis. Evidence supporting this conclusion would include: 1) iron limitation of growth achieved by transferrin, and 2) low iron having a neutral or positive effect on luminescence.

2. Materials and Methods

2.1 *Media*

Artificial seawater glycerol (ASG) contains per liter, 15.5 g NaCl, 0.75 g KCl, 12.35 g $MgSO_4 \cdot 7H_2O$, 2.9 g $CaCl_2 \cdot 2H_2O$, 1 g NH_4Cl, 0.1 g glycerophosphate, 3 ml glycerol, 10 mM HEPES pH 7.4, 2 mM $NaHCO_3$ and 16 mg ferric ammonium citrate (FAC) unless otherwise specified; FAC (filter-sterilized, 0.2 μm Acrodiscs), HEPES, and $NaHCO_3$ were added as 100x stock solutions after autoclaving the medium. Seawater complete medium (SWC) contains 5 g peptone, 3 g yeast extract, 3 ml glycerol per liter of 75% seawater and is buffered with 20 mM HEPES

pH 7.4. SWC-glucose (SWCG) contains 3 g glucose instead of glycerol. EDDA (Sigma) was deferrated according to Rogers (1973), washed with cold distilled water and dried at 50°C. The stock solution (1.4 mM) was made up in 1 M NaOH and the pH adjusted to ≈9. Solid media for plates contained 15 g agar per liter.

2.2 *Maintenance of bacteria*

The bacterial strain used for this work was *V. fischeri* strain MJ1, isolated from *M. japonicus* (Ruby and Nealson, 1976). Cultures were maintained on ASG, SWC and SWCG plates for preparation of overnight cultures used to inoculate experiments. It was necessary to transfer ASG plate cultures at 1-2 day intervals to ensure good growth when inoculated into liquid ASG; SWC and SWCG plates were transferred weekly. Broth culture experiments were inoculated from liquid overnight cultures in the same medium. SWCG experiments were inoculated from log-phase cultures due to the rapid decrease in light in stationary-phase SWCG cultures.

2.3 *Complete medium plate experiments*

SWC soft agar (2.5 ml, 7.5 g agar per liter) at 45°C with the indicated level of EDDA was mixed with 3 drops of an SWC overnight culture and poured on SWC plates. Sterile concentration discs containing FAC at the indicated concentration were placed on the plates. The plates were examined for growth and luminescence after 12-24 hrs incubation.

2.4 *Iron sources on ASG plates*

ASG plates containing no added iron with 11 mg/l unpurified EDDA were spread with 100 μl of an ASG overnight culture. Concentration discs saturated with filter-sterilized (0.2 μm Gelman Acrodiscs) test substances were placed on the plates and the plates were examined daily for growth stimulation and light production. Test substances were: 320 mg/ml FAC, 500 μM hemin, 100 mg/ml ferritin, 10 mg/ml bovine serum albumin (BSA), BSA plus 16 mg/ml FAC, 65 μM transferrin alone and with 10 and 20 mg/ml FAC.

2.5 *Growth and light measurements in liquid media*

Growth was measured as optical density (OD) at 660 nm on a Bausch and Lomb Spectronic 20 spectrophotometer. Light was measured in an EMI Type 9781A phototube and Pacific Photometrics model 110 amplifier. One light unit equals 5.5×10^5 quanta/sec according to the "J" standard of Hastings and Weber (1963). Cells were grown in 18×150 mm glass test tubes containing 7 ml medium, capped with plastic Morton closures at 25°C on a shaker at 80-100 rpm. For nutrient limitation experiments, ASG was made up with 1 and 10% of standard

levels of NH_4Cl, glycerophosphate and glycerol, and with 0 and 16 μg/ml FAC. The concentration which yielded similar final optical densities ($\approx$0.1) were: no FAC, 1% NH_4Cl (180 μM), 10% glycerophosphate (33 μM) and 1% glycerol (390 μM). These media were chosen for comparison of growth and light production. For iron repression of induction in ASG, new HCl washed tubes were used.

2.6 *Luciferase experiments*

For these experiments 100 ml of culture were placed in 300 ml nephelo flasks. Optical density was measured and 1 ml subsamples were periodically removed and placed in scintillation vials and light measured. These samples were centrifuged 2 min in an Eppendorf microcentrifuge and the pellets were frozen for luciferase determination *in vitro* (Hastings et al., 1978). The ratio of *in vivo* to *in vitro* luminescence was obtained by dividing the luminescence of 1 ml of culture by the luminescence obtained in the luciferase assay corrected to 1 ml of culture.

3. Results

The effect of iron limitation on growth and luminescence was studied with FAC as the iron source. Eight concentrations of iron were added to ASG, and optical density and bioluminescence followed. At FAC concentrations less than 320 ng/ml, growth was limited in this medium (Fig. 1), although because of iron contamination some growth occurred even with no added iron. The iron in FAC is responsible for the growth stimulation; 50 μm citrate (9.6 μg/ml) only slightly enhanced growth (Fig. 1), and the amount of ammonium ion in FAC is negligible relative to the 1 mg/ml NH_4Cl in ASG. More severe iron limitation was achieved by complexing iron in the medium through the addition of human transferrin to tubes containing 160 ng/ml FAC. Transferrin at a concentration of 2.5 μM completely inhibited growth (Fig. 1). Iron limitation of growth enhanced luminescence; cultures with less than 160 ng/ml FAC or with 2.5 μM transferrin had a final ratio of light per OD more than two orders of magnitude higher than cultures with more FAC (Fig. 1).

Growth on various iron sources was tested on low iron ASG plates. Iron in any form stimulated growth except in the presence of high levels of transferrin (data not shown). When examined for light production, all iron sources showed a striking "ring" of light (Fig. 2) suggesting that a low level of iron, just sufficient for growth, is optimal for luminescence. SWC soft agar overlays containing EDDA sufficient to inhibit growth showed a similar response to FAC on a concentration disc (Fig. 2). The light inhibition zone surrounding the disc was not due to acid production since addition of a drop of 1 M HEPES (pH 7.4) did not restore luminescence.

Carbon, nitrogen, phosphate and iron limitation were compared to see if the increased light expression under iron limitation was specific for iron. Cultures

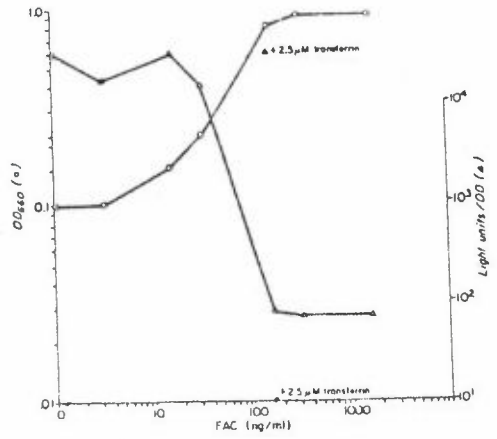

Figure 1. Growth requirement of MJ1 for FAC in ASG and effect of FAC on light emission. (○): Optical density after cessation of growth (3 days) in ASG containing 0 to 1600 ng/ml FAC. Growth at 0 added FAC reflects residual iron in the medium. Addition of 2.5 μM transferrin to a culture with 160 ng/ml FAC further inhibited growth, while 50 μM citrate only slightly enhanced growth. (Δ): Light units per OD after cessation of growth in the same cultures.

Figure 2. See page 44.

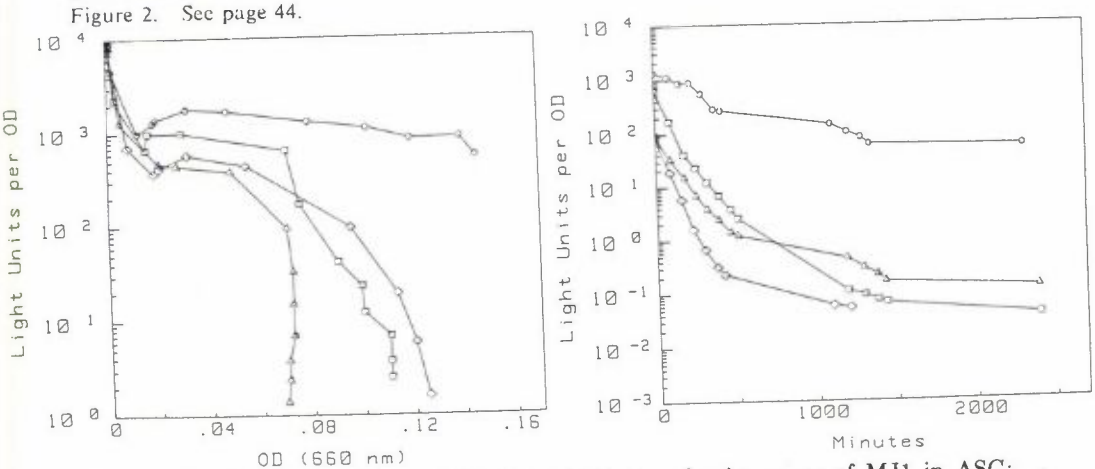

Figure 3. Effects of N, P, C, and Fe limitation on luminescence of MJ1 in ASG: luminescence per OD as a function of growth (OD). (○): Fe limited, (◇): P limited, (□): N limited, (Δ): C limited.

Figure 4. Effect of N, P, C, and Fe limitation on luminescence of MJ1 in ASG: luminescence per OD as a function of time after cessation of growth. (○): Fe limited, (Δ): C limited, (□): N limited, (◇): P limited.

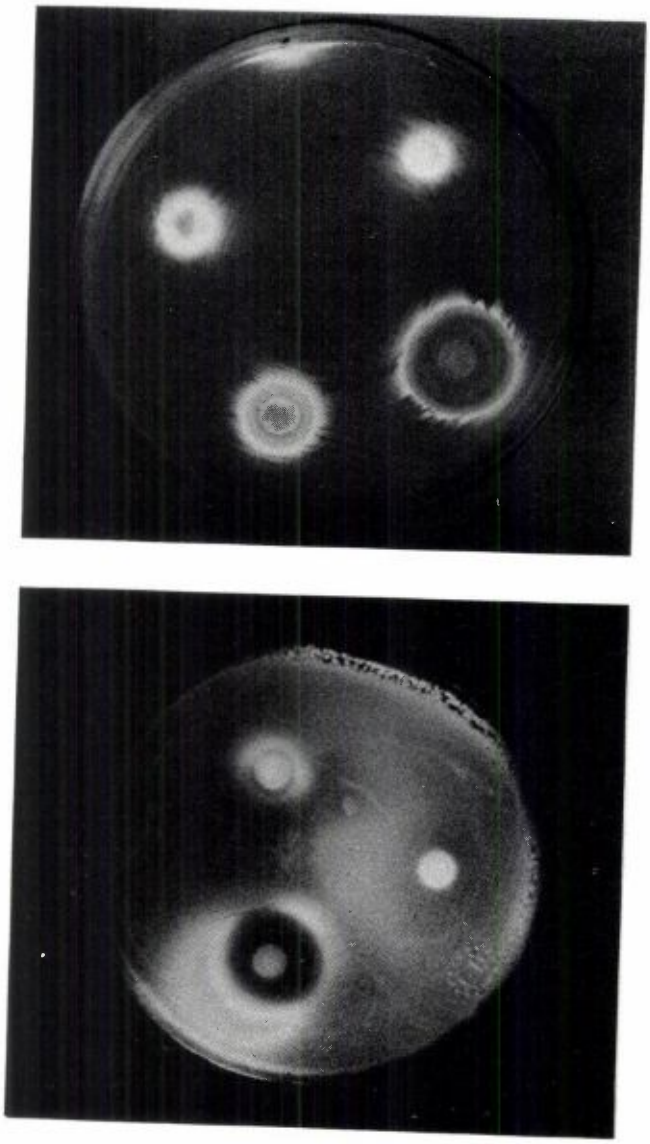

Figure 2. Effect of FAC on luminescence of MJ1 on plates. Above: Low iron minimal medium plate (ASG without added iron, with 11 μg/ml unpurified EDDA) spread with a culture of MJ1. Concentration discs contain clockwise from lower right, 3, 0.3, 0.03, and 0.003 mg/ml FAC. The plate was photographed under a red light; whitish rings are bacterial luminescence. Below: SWC plate containing 30 μM EDDA in the overlay. Concentration discs contain, clockwise from lower left, 3, 0.3, and 0.03 mg/ml FAC. Photographed by its own luminescence.

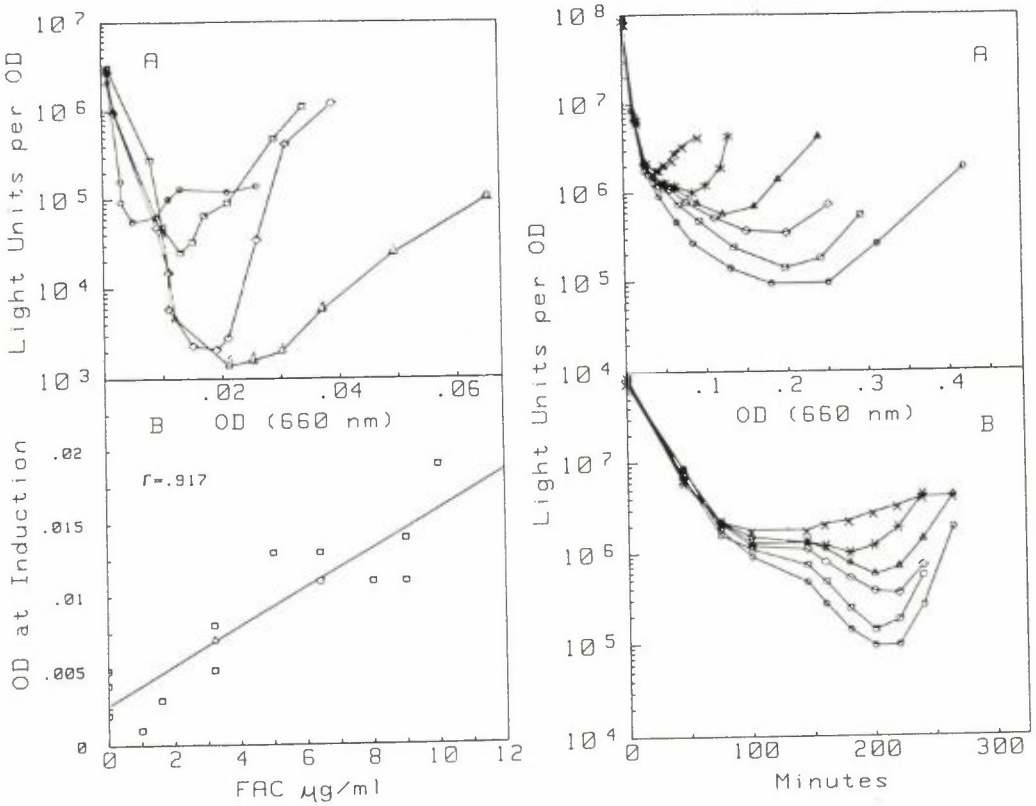

Figure 5. Effect of FAC on induction of luminescence of MJ1 in ASG. A: 0(○), 5(□), 10(◇), and 15(Δ) µg/ml FAC were added to ASG cultures of MJ1 as indicated. Luminescence per OD as a function of growth shows that cultures with more FAC induced at a higher cell density. B: Optical density at induction of luminescence from six experiments shows a linear relationship between FAC in the medium and OD at induction. Citrate ((Δ) 50 µM) had no effect on induction.

Figure 6. Effect of EDDA on induction of luminescence in MJ1 in SWC (0(○), 3(□), 5(◇), 7.5(Δ), 10(*), and 20(X) µM EDDA. A: Luminescence per OD as a function of growth. B: Luminescence as a function of time.

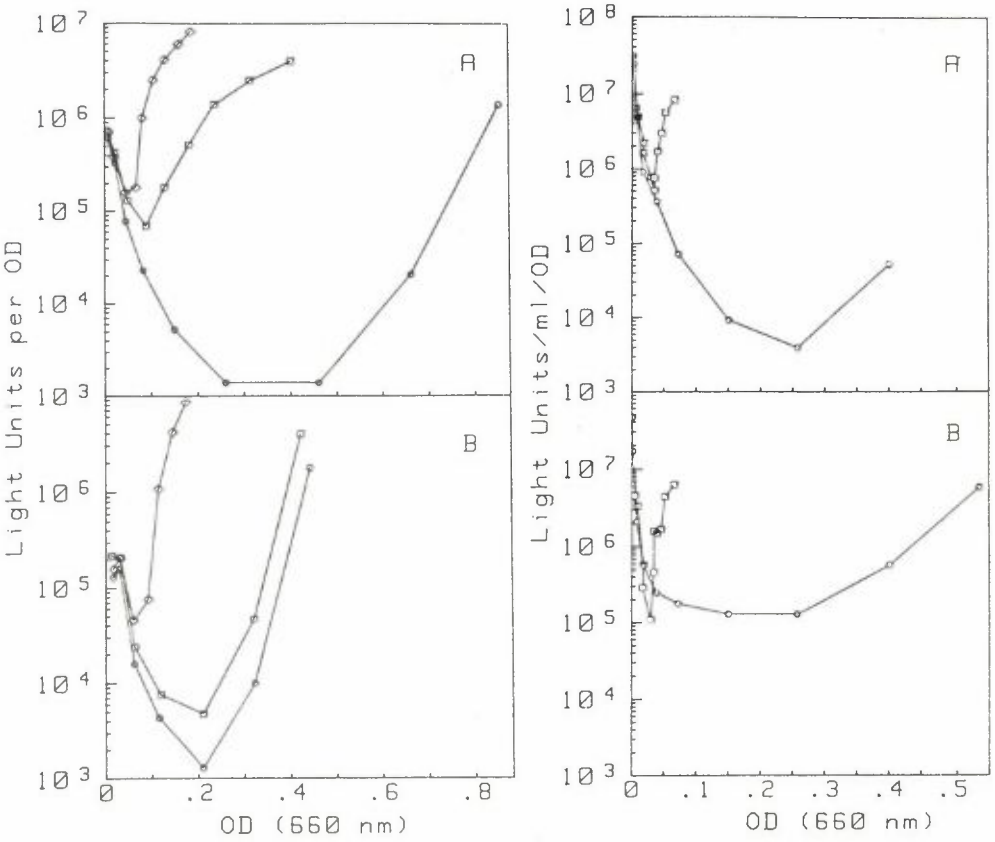

Figure 7. Comparison of EDDA effect in MJ1 in SWC and SWCG. 0(○), 2(□) and 5(◇) μM EDDA added. A: Luminescence per OD as a function of growth in SWCG. B: Lunimescence per OD as a function of growth in SWC.

Figure 8. Comparison of the effect of EDDA on luminescence (*in vivo*) and luciferase activity (*in vitro*) in the same culture. Zero (0) and 10(□) μM EDDA added. A: *in vivo* luminescence/ml/OD as a function of growth (optical density). B: *in vitro* luciferase activity/ml/OD as a function of growth. EDDA causes induction of luciferase synthesis as well as luminescence at a lower cell density.

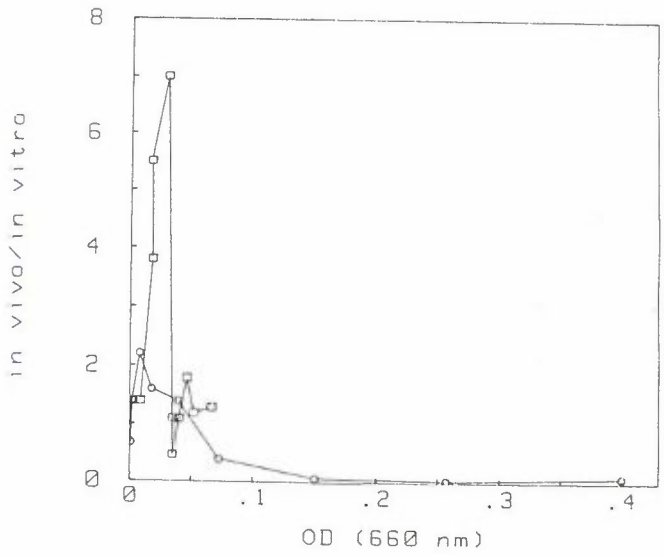

Figure 9. Effect of EDDA on luciferase expression (*in vivo* luminescence/ml divided by *in vitro* luciferase activity/ml) in the experiment shown in Fig. 8. Zero(○) and 10(□) μM EDDA added. *In vivo/in vitro* luminescence as a function of growth.

limited by glycerol, nitrogen and phosphate lost virtually all their luminescence, while cultures limited by iron retained about a tenth of their original luminescence (Figs. 3, 4).

In minimal medium (ASG) addition of increasing amounts of FAC caused induction at higher cell densities (Fig. 5). There was a linear relationship between FAC concentration and the cell density (OD) at which induction occurred up to 10 μg/ml (Fig. 5). Citrate (9.6 μg/ml, 50 μM) did not significantly affect induction (Fig. 5).

The effect of low iron on luminescence was also tested in complete medium by adding EDDA to SWC in order to chelate iron. As shown in Fig. 6, increasing levels of EDDA (corresponding to decreasing available iron) caused induction at lower cell densities. A similar effect was seen with glucose as the major carbon source (Fig. 7). *In vitro* luciferase assays showed that the effect could also be seen at the level of luciferase synthesis (Fig. 8). FAC (32 μg/ml) had no effect on the *in vitro* assay itself.

The patterns of expression of luciferase with and without EDDA were quite different (Fig. 9). Without EDDA luciferase expression (the ratio of *in vivo* to *in vitro* light) decreased 2 orders of magnitude prior to induction, followed by a recovery to initial levels. With EDDA, however, an increase in expression was seen prior to induction followed by a decline to initial levels. Just prior to induction the cells produced about eight-fold more light *in vivo* than *in vitro*.

4. Discussion

Nealson (1979) has proposed that oxygen limitation controls the *V. fischeri*-*M. japonicus* symbiosis. He hypothesized that the fish supplies glucose to the bacteria as the carbon source, a large portion of which is excreted by the bacteria as pyruvate (Ruby and Nealson, 1977); pyruvate is then aerobically metabolized by the light organ tissue, thereby lowering the oxygen concentration in the tubules and slowing growth. This model was particularly attractive because low oxygen increases luciferase production in *Photobacterium phosphoreum* (Nealson and Hastings, 1977) and possibly also in *V. fischeri* (Ruby, E.G., Ph.D. Thesis, University of California, San Diego, 1977).

The oxygen limitation model poses some problems. When facultatively anaerobic bacteria are grown on a fermentable substrate, such as glucose, growth can continue in the complete absence of oxygen; thus under these conditions oxygen cannot be a classic limiting nutrient. When the bacteria shift from aerobic to anaerobic growth a nutrient other than oxygen will become limiting. It could be argued that if the maximum growth rate (μ_{max}) is much less under anaerobic than aerobic conditions, oxygen may effectively limit growth. However, the difference in growth rate of *V. fischeri* with and without oxygen on a complete glucose medium is negligible (Eberhard et al., 1979), and thus, although low oxygen may be maintained in the light organ by the pyruvate excretion mechanism and thereby enhance light production, low oxygen alone is probably not responsible for growth repression in the light organs if the bacteria are growing on a fermentable substrate. If bacteria are grown on a non-fermentable substrate (to which Nealson's model does not apply), oxygen can limit growth, but glucose has generally been perceived as the most likely *in vivo* substrate due to its availability in blood. Strains of *V. fischeri* can grow on non-fermentable substrates including glycerol, pyruvate, glycerate, succinate, fumarate and citrate (Reichelt and Baumann, 1973).

The results of our work show that iron is a good candidate for the symbiotic growth-limiting nutrient. Iron limitation is relatively readily achieved in culture, and transferrin, a probable means of iron limitation in the symbiosis, is extremely effective in limiting growth. Further, iron was shown to repress luminescence by all means tested: on minimal and complete solid media, as well as minimal and complete liquid media. In addition, low iron is not only favorable for expression of luminescence, in contrast to nitrogen, phosphate and carbon limitation, but iron regulates synthesis of luciferase. Iron represses induction of luciferase, and therefore low iron is optimal for both synthesis and expression of luciferase. Thus iron limitation is particularly well-suited as a mechanism of growth repression in light organs. Iron repression of luminescence also occurs in the light organ symbionts *P. leiognathi* and *P. phosphoreum* (Haygood and Nealson, 1984). Considering the universal occurrence of iron-based nutritional immunity in animals, iron limitation may be important, not only in luminous symbioses, but in other symbioses between bacteria and higher organisms.

The increased luciferase expression under low-iron conditions (Fig. 9) is not surprising, since cytochrome levels typically decrease when iron is limiting (Light and Clegg, 1974), and reduced cytochromes would, in turn be expected to channel electron flow via luciferase. As shown by the luciferase assay experiment, luciferase is fully expressed only under low iron stress (EDDA) just prior to induction, while enzyme levels are relatively low (Fig. 9), a previously unreported pattern of expression. In the control culture, on the other hand, luciferase is markedly underexpressed prior to induction. This underexpression is due to lack of substrate, which is diluted out as the cells grow (Nealson, 1970; Ulitzur et al., 1981). The difference in expression with and without EDDA is apparently not due to quenching of luminescence by iron, since FAC added to the luciferase assay did not decrease light production. In the EDDA containing culture, the emission of more light *in vivo* than *in vitro* (all measurements were normalized to 1 ml of culture) implies that the *in vivo* reaction can be considerably more efficient than the *in vitro* reaction. There are several possible reasons for this: 1) the *in vivo* aldehyde substrate may be more effective than the dodecanal used *in vitro* (Hastings et al., 1966); 2) there may be a physical arrangement of the components of the system that increases efficiency *in vivo*; 3) there may be a secondary emitter present *in vivo* (Gast and Lee, 1978).

When *M. japonicus* specimens are available, one approach to test the hypothesis of iron limitation of *V. fischeri* in light organs would be to use the iron overload techniques that have demonstrated the role of iron availability in bacterial pathogenesis in rodents (Sawatski et al., 1983). Specifically, fish could be injected with iron, and monitored for an increase in release of bacteria as described in Haygood et al. (1984). Electron microscopy of light organs after administration of iron might show an increase in division figures or invasion of fish tissue by the bacteria. Another approach would be to determine specific biochemical constituents affected by iron, such as cytochrome or outer membrane proteins specifically induced under low iron stress (Klebba et al., 1982) and compare levels of these constituents in cells cultured under various forms of nutrient limitation with those levels in cells from light organs.

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