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SHORT COMMUNICATION

Pat M. Fidopiastis · Henning Sørum · Edward G. Ruby

Cryptic luminescence in the cold-water fish pathogen *Vibrio salmonicida*

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Abstract The recent discovery that the fish pathogen *Vibrio salmonicida* is closely related to the luminous bacteria *Vibrio fischeri* and *Vibrio logei* suggested that *V. salmonicida* might also be capable of bioluminescence. Interestingly, cells of *V. salmonicida* were found to produce light in culture, but only when exposed to either an aliphatic aldehyde and/or the major *V. fischeri* autoinducer *N*-(3-oxo-hexanoyl)-L-homoserine lactone, a transcriptional activator of the luminescence (*lux*) genes. An extract of spent medium of *V. salmonicida* that should contain any *V. salmonicida* acyl-homoserine lactone autoinducer, when added to *V. fischeri* cells, led to an induction of their luminescence. These results show that *V. salmonicida* is a newly recognized luminous bacterial species that apparently both produces an autoinducer activity and responds to exogenous *V. fischeri* autoinducer.

Key words *Vibrio salmonicida* · Luminescence · Luciferase · Autoinducer · Quorum sensing

Abbreviations *Acyl-HSL* Acyl-homoserine lactone · *$FMNH_2$* Reduced flavin mononucleotide · *VAI-1 Vibrio fischeri* autoinducer *N*-(3-oxo-hexanoyl)-L-homoserine lactone

Introduction

Vibrio salmonicida is a psychrophilic bacterium that is the causative agent of cold-water vibriosis in Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), and cod (*Gadus morhua*) (Egidius 1986; Wiik et al. 1989; Sørum et al. 1990). Analyses of 16S rRNA gene sequences have revealed a close phylogenetic relationship between *V. salmonicida* and the luminous marine bacteria *Vibrio fischeri* and *Vibrio logei* (Wiik et al. 1995; Fidopiastis et al. 1998). However, phenotypic characterization has shown that *V. salmonicida* is metabolically quite distinct from both of those species (Farmer and Hickman-Brenner 1991). Since several other marine luminous bacterial species cluster phylogenetically (Nealson and Hastings 1991), we examined isolates of *V. salmonicida*, a species not previously known to be luminous, for visible luminescence in culture. Interestingly, we discovered that cultures of this species did produce detectable luminescence if they were supplemented with an aliphatic aldehyde.

Bacterial luminescence results from the activity of luciferase, a mixed function oxidase that couples the oxidation of reduced flavin mononucteotide ($FMNH_2$) and an aliphatic aldehyde by O_2, yielding light as a product. The genes encoding the enzymes required for luminescence have been examined in at least five species and are typically organized in a single operon (Nealson and Hastings 1991). In each case, the operon contains the luciferase genes (*luxA* and *B*) and those encoding the synthesis of aldehyde substrate (*luxC*, *D*, and *E*), while in *V. fischeri* the locus also contains two regulatory genes, *luxI* [the VAI-1 autoinducer synthase gene; VAI-1 is the *V. fischeri* autoinducer *N*-(3-oxo-hexanoyl)-L-homoserine lactone] and *luxR* (the VAI-1 receptor gene). The luciferase and aldehyde synthesis genes have been shown to be cotranscribed, and the regulation of this operon in several species is influenced by the concentration of an acyl-homoserine lactone (acyl-HSL) autoinducer (Nealson and Hastings 1991).

The ability to produce light indicated that *V. salmonicida* expresses luciferase genes; however, the apparent requirement for exogenous aldehyde suggested that *V. salmonicida* either may lack the genes for aliphatic aldehyde synthesis or, alternatively, may uncouple the regulation of *luxA* and *B* from *lux C*, *D*, and *E* (Nealson 1977; Fuqua et al. 1996). In the latter case, *V. salmonicida* might sepa-

P. M. Fidopiastis · E. G. Ruby (✉)
Pacific Biomedical Research Center, University of Hawai'i,
41 Ahui Street, Honolulu, HI 96813, USA
e-mail: eruby@hawaii.edu
Tel. +1-808-539-7309; Fax +1-808-599-4817

H. Sørum
Norwegian College of Veterinary Medicine, Oslo, Norway

rately regulate aldehyde synthesis (and, thus, luminescence) only in response to the presence of an acyl-HSL autoinducer produced by other luminous bacteria (Visick and Ruby 1998). In this study, we describe some aspects of the physiology of luminescence of *V. salmonicida*, and discuss our results in the light of two important questions first posed by Nealson and Hastings (1991):

1. Are there groups of bacteria that possess genes for luciferase (or aldehyde synthesis) but do not normally express them?
2. Is luminescence the primary role for luciferase in luminous bacteria?

Materials and methods

Determination of luminescence on agar plates

V. salmonicida strains NCMB 2262 (Egidius et al. 1986; Table 1), VS1, VS201, VS224, and VS420 (Sørum et al. 1988), each isolated from diseased Atlantic salmon, were streaked onto a seawater-tryptone-yeast extract (SWT) agar medium (Boettcher and Ruby 1990) and incubated for up to 8 days at either 2, 8, or 16 °C. From the time they first appeared and throughout their subsequent growth, individual colonies were removed and their luminescence was measured in a sensitive photometer (TD-20/20 Luminometer; Turner Designs, Sunnyvale, Calif., USA) either with or without exposure to fumes of decyl aldehyde (Sigma-Aldrich, St. Louis, Mo., USA).

Luciferase enzyme kinetics

Luciferase activity was assayed in vitro as described previously (Nealson 1978), with the exception that reactions were performed at 24 °C rather than at 30 °C. Briefly, several colonies from overnight cultures grown on SWT agar plates were placed into separate Eppendorf tubes containing 1.0 ml cold lysis buffer (10 mM Na EDTA, pH 7.5; 1 mM dithiothreitol); the cell suspension was vortexed and then incubated on ice for 20 min. Twenty-microliter aliquots of each of the resulting cell lysates were then added to separate 5-ml glass vials containing 10 µl 0.01% dodecyl aldehyde (Sigma-Aldrich) suspension in 10 mM potassium phosphate buffer (pH 7.1). The vials were placed into a light-tight photometer chamber, and 1 ml of a 50 µM solution of $FMNH_2$ was injected into each. The resulting level of light emission was continuously recorded on a strip chart recorder, and the values at intervals of 1 s were plotted to calculate the enzyme turnover kinetics from the rate of decay of luminescence.

Table 1 Luciferase enzyme decay kinetics of some bacterial strains used in this study

Species	Source, reference	Luciferase in vitro turnover kinetics ($T_{1/2}$, in s)
Vibrio harveyi		
B392	Seawater[a]	17
Vibrio logei		
SA6	*Sepiola affinis* light organ[b]	2.2
SR6	*Sepiola robusta* light organ[b]	2.1
Vibrio fischeri		
SA1	*Sepiola affinis* light organ[b]	1.7
SR5	*Sepiola robusta* light organ[b]	2.0
ES114	*Euprymna scolopes* light organ[c]	0.7
Vibrio salmonicida		
NCMB 2262	Diseased Atlantic salmon[d]	2.0

[a] Reichelt and Baumann (1973)
[b] Fidopiastis et al. (1998)
[c] Boettcher and Ruby (1990)
[d] Egidius et al. (1986)

Luminescence induction in the presence of added aldehyde

Five strains of *V. salmonicida* were inoculated separately into each of two sets of flasks containing either SWT broth or a *V. harveyi*-conditioned SWT broth (VHCM). VHCM was prepared as described previously (Makemson 1973) to remove an inhibitor of luminescence induction. The flasks were shaken at either 8 or 16 °C, and aliquots were taken throughout growth of the culture and measured for both optical density (OD) at 600 nm and luminescence. The luminescence measurements were made both with and without exposure to a final decyl aldehyde concentration of 50 ng/ml which was added at the time of measurement.

Effect of the addition of VAI-1 on luminescence induction

Cells of either *V. fischeri* strain ES114, *V. logei* strain SR6, or *V. salmonicida* strain NCMB 2262 were grown to an OD_{600} of 0.2–0.4, and were then diluted to a final OD_{600} of 0.01–0.04 in flasks containing 15 ml SWT broth. This procedure reduces carryover of any intrinsic autoinducer activity that the cells may have secreted into the growth medium. The cultures were grown with shaking at 16° C (the upper temperature limit at which these strains might be expected to coexist in nature), and the OD_{600} and luminescence (either with or without added decyl aldehyde) of culture aliquots were measured periodically. In some cases, the medium was supplemented with VAI-1 (Sigma-Aldrich) to a final concentration of 2.4–240 ng/ml. Cells of *V. salmonicida* were also exposed to VAI-1 at a final concentration of 2 µg/ml because lower concentrations of VAI-1 were not able to induce them to luminesce without the addition of aldehyde. These cells were grown to an OD_{600} of 0.8 and were then diluted to a final OD_{600} of 0.01–0.04 in 15 ml SWT broth, after which they remained luminous when exposed to aldehyde.

Effect of the addition of *V. salmonicida* spent-culture extract on the luminescence induction of *V. fischeri* ES114

V. salmonicida spent-culture extract was prepared as described by Nealson (1977). Briefly, strain NCMB 2262 was grown to a density of 2.9×10^8 cells/ml, and 1 l cell-free spent medium was extracted with ethyl acetate and concentrated to a volume of 3.0 ml. The extract should contain any VAI-1-like acyl-HSL autoinducer produced by *V. salmonicida*. *V. fischeri* cells were grown to an OD_{600} of 0.4 and were then diluted to an OD_{600} of 0.05 in 15 ml VHCM that contained an ethyl acetate extract from an equivalent of 15 ml of a *V. salmonicida* culture at an OD_{600} of 4.8. Prior to adding the medium, the ethyl acetate solvent was allowed to evaporate completely, leaving any acyl-HSL produced by the *V. salmonicida* cells. *V. fischeri* cells were then grown with shaking at their temperature optimum (28 °C), and the OD_{600} and luminescence (both with and without added decyl aldehyde) of culture aliquots were measured periodically.

Results and discussion

V. salmonicida has not been previously described as being a luminescent species; however, because of recent evi-

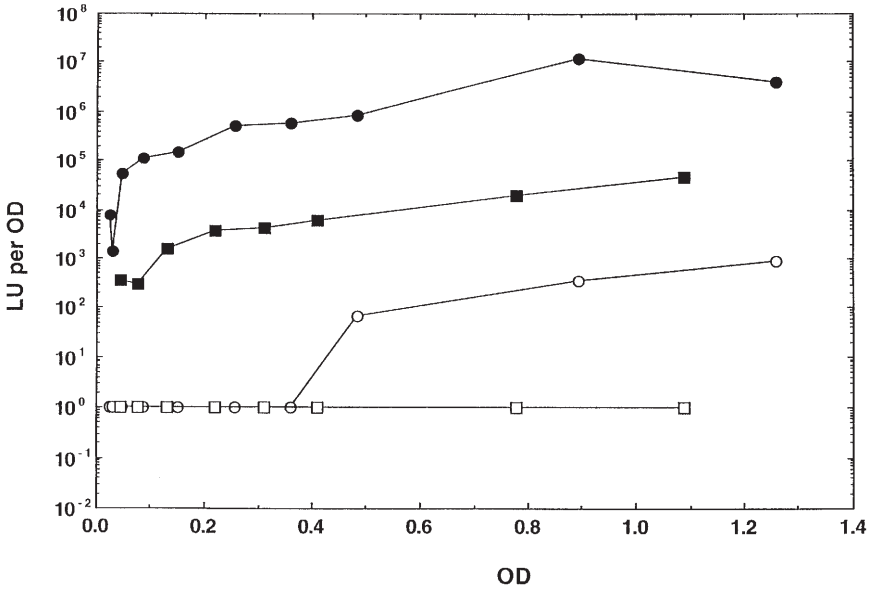

Fig. 1 Luminescence of *Vibrio salmonicida* strain NCMB 2262 grown in the presence (●, ○) or absence (■, □) of *Vibrio fischeri* autoinducer *N*-(3-oxo-hexanoyl)-L-homoserine lactone. Light emission of culture aliquots was measured at the indicated culture densities (*OD values*) either in the presence (●, ■) or in the absence (○, □) of added decyl aldehyde. Essentially the same results were obtained when all five *V. salmonicida* strains were tested (1 luminescence unit [LU] = 1.7×10^3 quanta/s; the detection limit was 10^0 LU/OD)

dence that the level of luminescence of *V. logei* is inhibited at elevated growth temperatures (Fidopiastis et al. 1998), colonies of *V. salmonicida* were assayed for luminescence during growth within its psychrophilic temperature range (2–16 °C). Colonies of *V. salmonicida* were not detectably luminous over this entire range, either visibly or by sensitive photometry. However, if the colonies were exposed to decyl aldehyde, they immediately became visibly light-emitting, indicating that *V. salmonicida* is a luminous bacterium that expresses a luciferase but not all the cofactors normally required to produce light. Using an in vitro enzyme assay on cell lysates, we confirmed that the light was produced by a typical bacterial luciferase that required $FMNH_2$, O_2, and an aliphatic aldehyde. Further, analysis of luciferase enzyme decay kinetics (Table 1) revealed that the *V. salmonicida* activity grouped with the "fast-kinetics" enzymes of *V. fischeri* and *V. logei*, whose turnover rates are distinct from those of the slower luciferases of *V. harveyi* and related species (Nealson 1978).

All five *V. salmonicida* strains that we examined induced luminescence early in the exponential phase of growth regardless of either the medium (SWT or VHCM broth) or growth temperature (8 or 16 °C) used, but in all cases exogenous aldehyde was necessary to detect luminescence. Because *V. salmonicida* cells grow faster in SWT ($t_{gen} \cong 3$ h at 16 °C) than in VHCM ($t_{gen} \cong 5$ h at 16 °C) medium, and luminescence induction was similar in all strains tested, cells of strain NCMB 2262 grown in SWT broth were used in subsequent experiments.

Induction of luciferase could be detected in cultures of *V. salmonicida* grown with or without added VAI-1 (Fig. 1) if aldehyde was added to an aliquot of the culture immediately before placing the aliquot in a photometer. The addition of VAI-1 to growing cells of *V. salmonicida* induced their luminescence approximately 100-fold above the method's detection limit. Nevertheless, the luciferase of these cells apparently was still severely limited by their inability to supply adequate substrate aldehyde. Thus, the luminescence of VAI-1-induced *V. salmonicida* cells, measured in the presence of added decyl aldehyde, increased an additional 100-fold (Fig. 1) to a level that is comparable to fully induced *V. fischeri* ES114 cells.

The ability of *V. salmonicida* to respond to *V. fischeri* autoinducer is consistent with these two species' phylogenetic proximity (Wiik et al. 1995; Fidopiastis et al. 1998). Similarly, the luminescence of the closely related species *V. logei* can also be induced in the presence of VAI-1 (data not shown), and *V. logei* has been shown to produce VAI-1 (E.P. Greenberg, University of Iowa, Iowa City, Iowa, USA; personal communication). Thus, these three closely related bacteria all respond to the same acyl-HSL autoinducer molecule. This finding is an example of how cells of several closely related species that can co-occur in nature might together act like a "quorum" of conspecific cells (Greenberg et al. 1979; Bassler et al. 1997; Visick and Ruby 1998).

Interestingly, the addition of VAI-1 apparently induced aldehyde synthesis in *V. salmonicida*, resulting in light emission (Fig. 1); however, this effect was not detected until approximately midway through exponential growth (i.e., at an OD_{600} of 0.4). The apparent temporal difference in expression of luciferase and aldehyde synthesis genes suggests that expression of these two sets of genes in *V. salmonicida* is subject to different regulatory controls.

Our work with *V. salmonicida* has led us to consider the following question: what is the cellular consequence of being a luminous bacterium that does not produce light? A "dark" pathway, in which luciferase (in the absence of aldehyde) catalyzes only a partial reduction of O_2 by $FMNH_2$, results in essentially no light production and leads to the formation of intracellular hydrogen peroxide (Nealson and Hastings 1977; Gonzalez-Flecha and Dem-

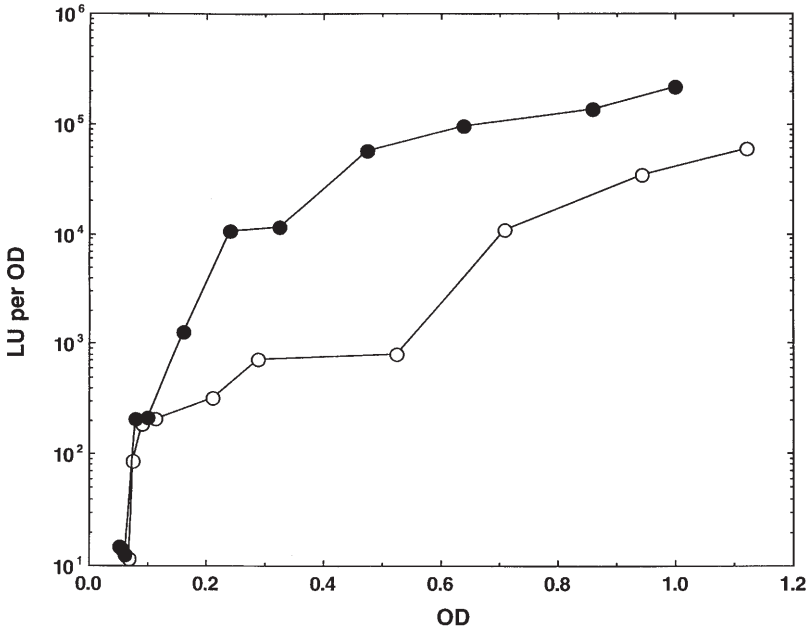

Fig. 2 Luminescence of *Vibrio fischeri* ES114 cells grown in seawater-tryptone-yeast extract medium either with (●) or without (○) the addition of an ethyl acetate extract of *Vibrio salmonicida* spent-culture medium (1 luminescence unit [LU] = 1.7×10^3 quanta/s; the detection limit was 10^0 LU/OD)

ple 1994). Our results with *V. salmonicida* suggest that this species may have significant dark-pathway activity in the absence of exogenous aldehyde, and thus might be subjecting itself to oxidative stress. Interestingly, *V. salmonicida* appears to counteract this potentially growth-limiting oxidative stress by producing unusually abundant levels of catalase activity, which could be readily detected when a drop of hydrogen peroxide was added to isolated colonies on an agar surface (data not shown).

Ethyl acetate extracts of spent *V. salmonicida* medium enhanced specific luminescence in growing cultures of *V. fischeri* ES114, a natural underproducer of VAI-1 (Boettcher and Ruby 1990; Gray and Greenberg 1992), on the average approximately tenfold (Fig. 2). This effect suggests that *V. salmonicida* produces a functional acyl-HSL autoinducer that can augment the luminescence induction effect of the endogenously produced autoinducer(s) (Kuo et al. 1994) of strain ES114. However, while addition of *V. salmonicida* culture extract to cells of *V. salmonicida* enhanced their level of luminescence up to threefold (data not shown), the enhancement by exogenous aldehyde remained, suggesting that *V. salmonicida* cells do not produce in culture an acyl-HSL that can fully induce sufficient aldehyde synthesis activity. Interestingly, among the three *Vibrio* species tested, the lowest OD_{600} at which luminescence was induced occurred at different levels of added VAI-1 (Table 2). This observation suggests that (1) the structure of VAI-1 may be different from that of the acyl-HSL(s) naturally produced by *V. logei* and *V. salmonicida*, and/or (2) these species may have acyl-HSL receptor proteins (LuxR homologues) with lower affinities for VAI-1 than does *V. fischeri* LuxR.

In summary, we have demonstrated that *V. salmonicida* (1) produces a typical bacterial luciferase with decay kinetics similar to those of light-organ symbionts of squids and fishes, (2) possesses the intrinsic ability to synthesize aldehyde, (3) can induce both luciferase and aldehyde synthesis genes in the presence of the acyl-HSL VAI-1, (4) produces an autoinducer activity that only slightly induces its own luciferase gene expression and does not detectably induce aldehyde synthesis, and (5) secretes an activity that can induce luminescence in cultures of *V. fischeri*. Both the precise organization and regulation of *lux* genes in *V. salmonicida* and their significance to the ecology of this bacterium remain to be revealed. *V. salmonicida* is typically found as a pathogen associated with decaying wound tissue of fishes (Sørum et al. 1988) and may produce hydrogen peroxide as a means of causing host tissue damage, as has been reported for pathogenic mycoplasmas (Razin 1986). We speculate that the suppression of light emission in *V. salmonicida* and the subsequent formation of hydrogen peroxide that may occur in the absence of an externally provided organic aldehyde could be a significant clue to its mechanisms of vir-

Table 2 Representative results of three experiments to determine the effect of *Vibrio fischeri* autoinducer *N*-(3-oxo-hexanoyl)-L-homoserine lactone (VAI-1) addition on the optical density (OD_{600}) at which luminescence is induced. Each strain was grown with shaking at 16 °C in seawater-tryptone-yeast extract medium, and culture OD_{600} and luminescence were monitored. For *Vibrio salmonicida* only, decyl aldehyde (50 ng/ml) was added immediately before luminescence was measured. The lowest OD_{600} at which the specific activity of luminescence began to increase is reported as the point of induction

Strain	Optical density at induction			
	Concentration of added VAI-1 (ng/ml)			
	0	2.4	24	240
Vibrio fischeri ES114	> 0.6	0.04	0.04	0.04
Vibrio logei SR6	0.8	0.15	0.06	0.04
Vibrio salmonicida 2262	0.4	0.4	0.12	0.12

ulence and perhaps even to the functional evolution of bacterial luciferase.

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