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Use of LuxA sequences for investigation on Luciferases kinetics and characterization of luminous bacteria

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USE OF *Lux*A SEQUENCES FOR INVESTIGATION ON LUCIFERASES KINETICS AND CHARACTERIZATION OF LUMINOUS BACTERIA

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To: Dean Arthur W. Herriott
College of Arts and Sciences

This thesis, written by Rita Di Bonito, and entitled Use of *lux*A sequences for investigation on luciferases kinetics and characterization of luminous bacteria, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this thesis and recommend that it be approved.

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DEDICATION

I dedicate this thesis to my son, Daniel Bash that lightened my days with his smile and enthusiasm.

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ABSTRACT OF THE THESIS

USE OF *lux*A SEQUENCES FOR INVESTIGATION ON LUCIFERASES KINETICS AND CHARACTERIZATION OF LUMINOUS BACTERIA

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Known luminous bacteria belong to the genera *Vibrio, Photobacterium, Shewanella,* and *Photorhabdus.* The enzyme luciferase catalyzing the luminous reaction is composed by the α and β polypeptides and subunit α is responsible for substrate binding and catalytic activities. Luciferases are classified into "slow " of *Vibrio harveyi* and "fast" of *Photobacterium* sps. on the basis of enzyme kinetics. *Shewanella woodyi* has "intermediate" kinetics. This research has tested the hypothesis of existence of three kinetic classes by sequencing *lux*A gene (coding for α subunit) of new strains and comparing these clusters to phenotypic analysis and sequencing of 16S rRNA. Phenotypic analysis has shown strains distinct from the known. *Lux*A amino acids and nucleotides and 16S rRNA sequences have shown 5 major lineages corresponding to known species. A clade distinct from the known species was present. Geographic location and fish habitat didn't affect the distribution of strains.

TABLE OF CONTENTS

LIST OF TABLES

INTRODUCTION

Classification of luminous bacteria

The early classification of Beijerink (1889) placed all the luminous bacteria in the genus *Photobacterium*. Successive works have shown their heterogeneity and identified several distinct groups. The seventh edition of the Bergeys's Manual (Breed *et al.,* 1957) listed all the luminous bacteria under the genera *Photobacterium* and *Vibrio*, as originally proposed by Breed and Lessel (1954). More recent studies have placed the isolates from marine habitats in the genera *Vibrio, Photobacterium, Alteromonas* (Reichelt and Baumann, 1973; Baumann *et al.,* 1980; 1983). The luminous species originally placed in *Alteromonas* has been reassigned to *Shewanella*, which includes the only exclusively respiratory luminous isolates (MacDonell and Collwell, 1985). A number of new species of luminous bacteria has been recently added to this group. Luminous bacteria isolated from freshwater have been identified as *Vibrio cholerae* (West *et al.,* 1983) and luminous symbionts of entomopathogenic soil nematodes have been classified as *Xenorhabdus luminescens* (Thomas and Poinar, 1979). *Xenorhabdus luminescens* is the only terrestrial luminous bacterium and has recently been reassigned to the new genus of *Photorabdus* on the basis of DNA sequences (Boemare *et al.*, 1993). All the luminous bacteria are gram negative, facultatively anaerobic rods; their characteristics and habitats are reported in Table 1.

The identification of the luminous bacteria was based on a large number of phenotypic tests described by Reichelt and Baumann (1973); a more simplified system using substrate metabolism has been also proposed for characterization of isolates of luminous

Table 1. Species and habitats of known bioluminescent bacteria. All are gram negative rods (Meighen and Dunlap, 1993; Makemson *et al.* 1997)

SPECIES	HABITAT
Facultatively anaerobic rods	
Family Vibrionaceae	
Vibrio harveyi	Coastal and open seawater, surfaces and intestines of marine animals. Tropical
V. logei	Exoskeletron and lesions of crabs, intestine of marine animals. Psychrotrophic
Photobacterium leiognathi	Coastal seawater, Surfaces and intestine of marine animals, light organs of fish (Leiognathidae, Apogonidae) and squid (*Doryteuthis kensaki*)
P. phosphoreum	Intestine of marine animals, light organs of fish. Psychrotrophic
V. fischeri	Coastal seawater, intestine of marine animals, light organs of fish (Monocentridae) and squid (*Eupryma scolope)*
V. cholerae (some strains)	Brackish or freshwater
V. orientalis	Coastal seawater
V. splendidus biotype 1	Coastal seawater
V. vulnificus (some strains)	Coastal seawater
Family Enterobaceriaceae	
Photorhabdus luminescens	Symbiont of enthomopathogenic soil nematode (*Heterorhabditis* spp.)
Obligate respiratory rods	
Shewanella hanedai	Coastal seawater. Psychrophile
S. woodyi	Intermediate water, squid ink. Psychrotrophic

bacteria (Makemson *et al.* 1998). Studies based on immunological techniques (Bang *et al.,* 1981; Baumann et al., 1980) and DNA hybridization (Reichelt *et al.,* 1976, Grimont *et al.*, 1984), have been used to support the classification of luminous bacteria. The analysis of sequences of 16S rRNA has been used for phylogenetic studies of the genera *Vibrio, Photobacterium, Shewanella, Photorhabdus* and for the identification of new species, as reported by Kita-Tsukamoto *et al.,* 1993; Ruimy *et al.,* 1994; Gautier *et al.,* 1995; Makemson *et al.,* 1997; Liu *et al.,* 1997; Fisher- Le Saux *et al.,* 1998. Comparative studies have been conducted by 16S rRNA PCR/RFLP (restriction fragment lenght polymorphism), to separate members of the genera *Vibrio* and *Photobacterium* (Urakawa *et al.,* 1997; 1998). The use of species specific hybridation probes for the gene *lux*A has been also proposed for the identification of the major luminous groups in the Persian gulf (Wimpee *et al.,* 1991; Lee and Ruby, 1992; Nealson *et al.,* 1993). Such method, however, cannot identify *V. harveyi* from closely related species as *V. splendidus* (Lee and Ruby, 1992; Nealson *et al.*, 1993).

Ecology of luminous bacteria

Luminous bacteria live in seawater as plankton (Nealson, 1978; Ruby and Nealson, 1978; O'Brien and Sizemore, 1979; Yetinson and Shilo, 1979; Orndorff and Colwell, 1980; Ruby and Morin, 1979) or in water above sediments (Ramesh, 1989). Their distribution is dependent on seasonal, environmental and geographical factors (Ruby and Morin, 1979). Their planktonic presence can simply be related to the overflow from other niches (Makemson and Hermosa, 1999). Luminous bacteria have been isolated as saprophytes on the surfaces of dead marine animals (Harvey, 1952) or

parasites on the exoskeleton of crustaceans (Baross *et al.*, 1978) and in the hemolymph of invertebrates (Harvey, 1952). They produce extracellular enzymes such as chitinase (Spencer, 1961; Reichelt and Baumann, 1973) that enable their growth on the surface of marine animals. Luminous bacteria are present as symbionts in the gut of several fishes and crustaceans (Ruby and Morin, 1979; Ohwada et al., 1980; Makemson and Hermosa, 1999). The ecological significance of luminous bacteria in the fish gut is still undefined and their presence could be related to metabolic activities other than luminescence. In fact, they can utilize N-acetyl glucosamine, product of the chitinase activity (Reichelt and Baumann, 1973). The presence of luminous bacteria in the fecal material may also have a significance in the cycling of this material (Andrews *et al.*, 1984). A species-specific symbiosis is represented by isolates living in specialized organs of some fish. The bacteria isolated from fish luminous organs are *V. fischeri*, *P. leiognathi* and *P. phosphoreum.* In fish of the families Ceratidae and Anomalopidae the presence of bacteria – like bodies has been detected in luminous organs but the culture outside the host has not been successful (Haneda and Tsuji,1971; Hansen and Herring, 1977).

The genes for bacterial luciferases have been isolated from the light organs of *Kriptophanaron alfredi*, the unculturable symbiont of flashlight fish (Anomalopidae) cloned into *E.coli* and sequenced (Haygood and Cohn, 1986). They seem more related to the *lux*A amino acid sequences of *V. harveyi* (Meighen, 1991). Unculturable bacteroid formations have been observed also in the luminous colonial chordate *Pyrosoma* (Nealson and Hastings, 1992). In light organs luminous bacteria live as a pure culture at high density. The colonization probably takes place by small ducts connecting the light organs with the outside or with the fish gut (Tebo *et al.*,1979). Luminous bacteria growth

rates and luminescence are affected by oxygen concentration (Lloyd *et al.*, 1985) iron availability (Makemson and Hastings, 1982; Haygood and Nealson, 1985), and osmolarity (Dunlap 1985; Watanabe and Hastings, 1986). Fish must be able to control growth rate and luminescence of the symbionts in their light organs (Hastings *et al.*, 1987).

Luciferase and biochemistry of luminescence

During the luminescent reaction, reduced flavin mononucleotide ($FMNH_2$) and a long chain aliphatic aldehyde are oxidized, with the production of FMN, water, the corresponding fatty acid and light. The reaction is catalyzed by the enzyme luciferase, which is a mixed function of oxidase and proceeds through a series of intermediates leading to the formation of C4a hydroxyflavin. Light emission apparently occurs from this hydroxyflavin, which when dehydrated to FMN emits light (Hastings *et al.*, 1965; Hastings and Nealson, 1977; Shimomura *et al.*, 1972). During the reaction, molecular oxygen is cleaved, with incorporation of one atom of oxygen into a fatty acid and the other atom into water. The double oxidation of the substrates generates about 60 kcal mol^{-1}, utilized for the production of blue-green light (Fisher *et al.*, 1996). The reaction can be summarized by:

$$FMNH_2 + RCHO + O_2 \rightarrow FMN + H_2O + RCOOH + h\nu \ (\lambda \text{ max} = 490 \text{ nm})$$

The energetic cost of the light emission was extimated as 6 ATP molecules for each photon produced, supposing an efficiency of 100% (Hastings and Nealson, 1977).

However, estimates of the quantum yield *in vivo* during the enzymatic reaction has given values between 0.01 and 1 photon for each cycle (Lee and Seliger, 1972; Makemson and Gordon, 1989), with ATP consumption estimated between 600 to 6 molecules of ATP for photon of emitted light. In a fully induced cell, the luminescent reaction produces 10^4 photons s^{-1} $cell^{-1}$ and consumes about 20% of the oxygen taken up (Makemson, 1986). The apparent consumption of energy in bioluminescence has been evaluated as 0.01% or less of the total energy consumed during growth and seems to be a minor component of the total energy output in a growing culture (Makemson and Gordon, 1989).

Flavin specificity in the reaction is quite restricted and the reduced FMN is the preferred substrate. Minor chemical modifications of $FMNH_2$ significantly decrease the activity (Meighen and Hastings, 1971). The luminescent reaction requires long chains aliphatic aldehydes for the production of light emission (Cormier and Strehler, 1953). Specificity for aldehyde chain length has been observed for luciferases of different isolates (Meinghen and Dunlap, 1993). Enzyme activity and reaction turnover rate have shown different relationships with aldehyde chain length (Hastings *et al.*, 1969).

The luciferases have been isolated and purified from several luminous bacteria and are heterodimers devoid of prostetic groups, metals or non-amino-acid residues (Hastings and Nelson 1977). They are formed by two non-identical subunits, α and β, with molecular masses of 41 kDa and 37 kDa respectively. The α and β polypeptides are encoded by two adjacent genes, *lux*A and *lux*B, situated in the *lux* operon. On the basis of their homologies, *lux*A and *lux*B seem to be arisen by gene duplication. (Illarinov *et al.*, 1990; Meighen, 1991).

The α subunit is responsible for the substrate binding and catalytic activities (Meinghen *et al.*, 1971; Cline and Hastings, 1972; 1974). Both the α and β subunits are necessary for the catalytic activity (Friedland and Hastings, 1967; Watanabe *et al.*, 1982). There is a single active center on the heterodimer that resides on the α subunits and binds one reduced flavin molecule (Fisher *et al.*, 1996).

The amino acid sequences of α and β subunits have been determined for several isolates of luminous bacteria and the sequence alignment has shown a degree of similarity between 54% and 88% for the α subunits and between 45% and 77% for the β subunits (Meighen, 1991). The relatively high conservation of the α amino acid sequences is in accord with the primary role of this subunit on the kinetic properties of the enzyme. The alignment of the α and β sequences of *V. harveyi* shows 32% of identity, with an insertion of 29 amino acids in the α subunit, between residue 258 and 259 of the β subunit. This region of the α subunit is sensitive to proteolitic digestion and a single proteolitic cleavage in the region of residues 274-291 produces the enzyme inactivation (Fisher *et al.*, 1996).

The bacterial luciferases have been classified into slow and fast, in respect to their reaction with C_{12} and C_{10} aldehydes (Meighen and Dunlap, 1993) obtained by one-catalytic-cycle assay (Hastings *et al.*, 1978). A significant difference is observed in the C_{12} decay rates: they are > 0.6 sec^{-1} for the fast luciferases and < 0.1 sec^{-1} for the slow luciferases. The fast luciferases have also C_{10} decay rates slightly faster or similar to C_{12}, while the slow luciferases are characterized by C_{10} decay rates between 0.2 and $0.4 \times sec^{-1}$. On the basis of luciferase kinetics the known luminous bacteria could be divided into two

groups characterized by slow luciferases (*V. harveyi*, *Ph. luminescens*) and fast luciferases (*V. fischeri*, *P. phosphoreum*, *P. leiognathi)* as reported in Table 2. Bacteria with intermediate luciferase on the basis of the enzyme kinetics have been described and identified as a new species, *Shewanella woodyi* (Makemson *et al.*, 1997; Table 2). *S. woodyi lux*A gene phylogenetic analysis has shown that the translated amino acids sequence is distinct from *V. harveyi* and *P. phosphoreum* (Makemson *et al.*, 1997).

Proteins that alter the wavelength of the emitted light have been isolated in some strains. *Photobacterium* spp. has lumazine, that in presence of luciferase shifts the peak of luminescence to about 480 nm (blue shift) and stimulates the level of luminescence (Gast and Lee, 1978; Small *et al.* 1980). Another protein responsible for the emission of yellow light ($\lambda \cong 540$ nm) has been isolated in Y-1, a strain of *Vibrio fischeri* (Ruby and Nealson, 1977; Daubrer *et al.*, 1987). The synthesis of aldehydes utilized as substrate in the luminescent reaction is catalyzed by a multienzyme fatty-acid reductase complex containing three proteins: a reductase, a transferase and a synthetase. They are three polypeptides encoded by the genes *luxC*, *lux*D and *lux*E, present in the *lux* operon of all the luminous bacteria (Mancini *et al.*, 1988; Meighen, 1991).

Lux genes organization

Lux genes have been cloned and sequenced in a number of luminescent isolates including *V. fischeri, V. harveyi, P. phosphoreum*, *P. leiognathi* and *Ph. luminescens* (Cohn *et al.*, 1983; Engebrecht *et al.*, 1984; Boylan *et al.*, 1989; Miyamoto *et al.*, 1986, Meighen, 1988; Miyamoto *et al.*, 1988a, b; Szittner and Meighen, 1990).
In all the cases the genes coding for luciferase subunits and fatty acid reductase

Table 2. Luciferase kinetics of the known species

CLASS (Species)	DECAY RATE (sec^{-1}) Aldehyde C_{10}	C_{12}
FAST *(P. phosphoreum)* *(V. fischeri)* *(P. leiognathi)*	> 0.6	≥ 0.6
SLOW *(V. harveyi)* *(Ph. luminescens)*	0.2-0.4	< 0.1
INTERMEDIATE *(Shewanella woodyi)*	0.2-0.4	0.2-0.4

complex are in the some order in the operon: *lux*CDABE. Other genes, adjacent or not, have been identified in specific strains and have different organization. An additional gene, *lux*F, is present in most *Photobacterium* species located between *lux*B and *lux*E (Mancini *et al.*, 1988; Illarionov *et al.*,1988). *Lux*F is absent in all the *Vibrio* and *Photorhabdus lux* operons (Meinghen, 1991). The *lux*F gene codes for a nonfluorescent protein (NFP) whose function is unknown. *Lux*F protein has an identity of 22.4 and 33.3% respectively with the α and β subunits (Fisher *et al.*, 1996). The gene *lux*F has probably generated by duplication of *lux*A or *lux*B and its function could be linked to some specific ecological niches of *Photobacterium* (Meighen, 1991). *Lux*G has been identified in the *lux* operon, downstream *lux*E of the *Vibrio* and *Photobacterium* isolates. This gene is induced during the development of luminescence, but its functions are still unknown (Soly *et al.*, 1988; Martin *et al.*, 1989 Swarzmann *et al.*, 1990a, 1990b). Downstream *lux*G, in *V. harveyi*, the gene *lux*H has been identified but a correlation between his expression and the luminescent activity has not been found (Martin *et al.*,1989).

Regulation of the bioluminescent reaction

In luminous bacteria the expression of bioluminescence is strictly dependent on the cell density. This fascinating phenomenon has been called autoinduction and can be considered a form of intracellular communication (Kaiser and Losik, 1993). Autoinduction, described also as "quorum sensing", was originally observed in *V. fischeri* and *V. harveyi* (Nelson *et al.*, 1970; Eberhard, 1972). In batch cultures of these bacteria, light production and expression of luminescence genes is low in the early phases of

growth but increases significantly only during the late exponential-early stationary phases. This density-dependent regulation of the *lux* operon is related to the production and excretion of diffusible factors called autoinducers, that accumulate in the media. Autoinducers must accumulate to a sufficient concentration for luciferase induction which is dependent upon a critical density of bacteria in the environment (Kaplan and Greenberg, 1985). Autoinducers of *V. fischeri* and *V. harveyi* did not cross-react, showing species specificity. (Nealson *et al.*, 1970; Eberhard, 1972). The *V. fischeri* autoinducer was first to be purified and identified as a *N*-(3-oxohexanoyl)-L-homoserine lactone (Eberhard *et al.*, 1981). Two genes for autoinduction (*lux*I and *lux*R) have been identified in *V. fischeri*, where they are linked to *lux*CDABEG (Engebrecht and Silverman, 1984; Devine *et al.*, 1988). *Lux*I is located at the promoter proximal end of the *lux* operon, adjacent to the *lux*C and is responsible of the synthesis of the autoinducer. The second gene, *lux*R, is adjacent to *lux*I but is transcribed in the opposite, left direction. *Lux*R encodes a protein that activates transcription of the *lux*I-G operon only in presence of autoinducer. At low cell densities the *lux*I-G operon is transcribed at basal levels but at high cell densities, the autoinducer can reach a concentration sufficient to bind *lux*R and activate the transcription of the *lux* operon to its maximum level. A second autoinducer, identified as N- octanoyl-homoserine-lactone has been identified in *V. fischeri* (Gilson et al., 1995).

Studies in *V. harveyi* have shown the presence of two autoinducers. Autoinducer I (AI-1) has been identified as N-(3-Hydroxybutanoyl)-L-homoserine lactone and its synthesis depends on the activities of the products encoded by the *lux*L and *lux*M genes. AI-1 is detected by *lux*N, a sensor protein that functions as a two component hybrid

kinase (Bassler *et al.*, 1993). The second inducer of *V. harveyi* has not been identified yet and is called A1-2. The response to A1-2 depends on *lux*P, a periplasmatic ribose binding protein and *Lux*Q, a two component hybrid kinase similar to *lux*N (Bassler *et al.*, 1993, 1994a). AI-2 probably binds to *lux*P and the complex AI-2-*lux*P interacts with *lux*Q. Mutant analysis have shown that both induction systems act in parallel but can function indipendently from each other. The signaling from both sensor systems converge to the regulatory protein *lux*O, repressor of the *lux* CDABEGH operon (Bassler *et al.*, 1994b). *Lux*O function as repressor of the *lux* operon when phosphorylated at Asp47. The function of an additional phosphorelay protein *lux*U, positioned downstream of the *lux*N, *lux*P pathways and upstream of *lux*O has been identified (Freeman and Bassler 1999). In the model proposed by Freeman and Bassler (1999), the sensor kinases *lux*N and *lux*Q are phosphorylated at low inducer concentration. In this condition, a phosphotransfer cascade occurs to His58 of *lux*U, wich results in phosphorylation of Asp47 of *lux*O, and repression of the *lux* operon. The high inducer concentration produces a cascade reaction that results in dephosphorylation of *lux*O and derepression of *lux* CDABEGH transcription.

Several chemical and biochemical factors affect the expression of luminescence and are probably related to the response to environmental factors, but their relation with the density –dependent regulation systems is not known. The expression of luminescence in *V. harveyi* and *V. fischeri* is inhibited by glucose (Nealson *et al.*, 1972, Nealson and Hastings, 1979), by iron (Makemson and Hastings, 1982) and requires cAMP and cAMP receptor protein (Nealson *et al.*, 1972; Dunlap and Greenberg, 1985). The induction of luminescence in *V. harveyi* is also stimulated by the addition of arginine (Nealson *et al.*,

1970). Thus, regulatory systems respond to environmental factors in addition to the response from autoinductors.

HYPOTHESIS

The hypothesis is that there exist three kinetic classes of luciferases: "slow", "fast" and "intermediate". This study will test the hypothesis by sequencing the *lux*A gene of new isolates from Oman and Florida. These *lux*A sequences and their translated amino acid sequence products will be compared to known sequences present in Genebank.

Further phenotypic analysis will be conducted by analysis of substrate utilization patterns (BIOLOG plates), and a phylogenetic study will analyze sequences of the new isolates's 16S rRNA gene to compare the results to the trees obtained from *lux*A nucleotides and amino acids.

MATERIAL AND METHODS

Bacterial Media and Strains

The natural isolates were cultured at room temperature in Glycerol Marine Broth (GMB, Makemson and Hermosa, 1999) or Glycerol Marine Agar (GMA) composed of 0.5% Bacto-Peptone, 0.1% Yeast Extract, 0.02%-Glycerol phosphate, 0.3% Glycerol, in 75% artificial seawater (ASW) and a final pH of 7.5. The 1X composition of artificial

seawater was: 0.517 mol/l NaCl, 0.025mol/l $MgSO_4$, 0.025 mol/l $MgCl_2$ and 0.01 mol/l KCl. ASW was prepared in a 5X stock and diluted to the media to reach the concentration of 0.75 X. For GMA, 1.5% of Bacto-Agar was added before sterilization.

The isolates from Florida were collected from fish caught and immediately refrigerated for a period of 1 – 3 days. The intestines were removed aseptically and the content was placed in a sterile petri dish. A loopfull of this material was streaked on the surface of a GMA plate and incubated at room temperature in the dark. After 24 hours, the presence of luminous colonies was detected. The luminous colonies were re-streaked 2 or 3 times in order to obtain a pure culture of the isolates. All the isolates were mantained in GMA and checked for purity and luminescence. The list of the isolates is shown in Table 3.

Phenotypic characterization of the isolates

Phenotypic characterization was conducted by using BIOLOG GN plates (BIOLOG, Hayward, CA) that are based on 95 tests of substrate utilization. The strains were grown overnight on GMA plates and checked for purity. Cells were removed with a sterile swab and suspended in 25 ml of 75% sterile ASW. Optical density of the suspension was measured by a Spectronic 20 at 660 nm and adjusted at absorbance of 0.25. The cell suspension was used to inoculate a BIOLOG GN plate, using 125 µl /well. The plates were incubated at 25°C and the reaction observed at 24 and 48 hours. The

Table 3. List of the isolates characterized in this research

ISOLATES	FISH SPECIES	HABITAT (*)	LOCATION
ID3, ID2, 2D, 3D2, 3D3,	*Coryphaena hippurus* (Dolphin)	P	South Florida
SCI	*Mycteroperca phenax* (Scamp)	P	South Florida
2YSC, 2YSD, 2YSG, 3YSB, YSI	*Ocyrus chrysurus* (yellowtail snapper)	P	South Florida
KI	*Menticirrhus saxatilis* (King fish)	P	South Florida
Agr2	*Epinephelus sp.* (Grouper)	P	South Florida
Flcarpio 1	*Floridichthis carpio* (Gold spotted killfish)	D	Florida Bay
LPAR3	*Lucania parva* (rain water killfish)	D	Florida Bay
MSB	*Lutjanus analis*) (mutton snapper)	D	Florida Bay
Pin2	*Lagodon rhomboides* (juvenile pinfish)	D	Florida Bay
Tod1, TodB	*Opsanus beta* (gulf todfish)	D	Florida Bay
IM3, IMK3, IMK4, IMK5,	*Rastrelliger kanagurta* indian makarel	P	Oman
YSC1	*Selaroides leptolepis* (Yellow stripe scad)	P	Oman
Gsanl, GmD5, Gm3A	*Mulloidichthus flavolineatus* (yellow stripe goatfish)	D	Oman
MSC3, MSC5, Raj3, RAJ3	*Decapierus macarellus* (Makerel scad)	P	Oman
LO3	*Leiognathus fasciatus* (ponyfish)	P	Oman
Sar1, Sar2, Sard13	*Sardinella longiceps* (Oil sardine)	P	Oman
SUP1	Tidal pool, beach		Oman
SH4	Coastal seawater, sink hole		Oman

(*) Habitat of fish: P= pelagic, D=demersal

positive reactions after 48 hours were recorded and the data added to a STATISTICA (Stat Soft. Tulsa, OK) database. Cluster analysis was conducted by the Ward's Method using euclidean distances in STATISTICA.

The strains were grown overnight in GMA in petri dishes. The cells were gently scraped from the surface of the plate and placed in a 1 ml of 0.05M phosphate buffer, pH 7.0. The suspension was sonicated with a micro-tip sonicator for 3 x 15 seconds to release the enzyme and kept on ice to prevent the over heating.

Luciferase assay was conducted on the luciferase present in cell extracts, by measuring the luminescence intensity and its decay rate in presence of decanal or dodecanal aldeydes as substrates, according to the standard method described by Hastings (1978).

In a scintillation vial containing 1 ml of phosphate buffer pH 7.0 and 0.02% bovine serum albumin were added 3 µl of C10 aldeyde (5mM in ethanol) and 10 µl of the enzyme suspension. Then the vial was placed into the photometer housing and 1 ml of 0.05mM $FMNH_2$ were rapidily injected. The luminous intensity over the time was recorded. These tests were repeated using C12 aldeyde and the graphs obtained were used for the calculation of decay rates.

DNA extraction

For the DNA extraction the strains were cultured in Glycerol Marine Broth on a shaker at 25°C. After 24 hours, 1 ml of culture was aseptically removed, placed in a 1.5 ml eppendorf tube and centrifuged at 14,000 rpm for 15 min. The supernatant was discarded and the cell from the pellet were suspended in 200 µl of the extraction buffer

obtained from Promega (Madison, WI). DNA was extracted using the Wizard ™ DNA purification kit, (Promega, WI) and resuspended in 200 µl of Tris-EDTA pH 8.0 (100 mM Tris·Cl, 1mM EDTA).

RNase treatment was performed by incubating the DNA at 37°C for 1 hour with 1 µl of RNase A (10mg/ml solution) obtained from SIGMA, St Louis, MO. The DNA samples were kept at 4°C until use.

DNA presence was evaluated by electrophoresis of 2 µl of DNA in 1% of agarose Fisher at 70V for 2 hours. DNA in the gel was stained with ethidium bromide, visualized with UV and photographed. DNA concentration was evaluated by fluorometry, using the DyNA Quant 200 (Hoefer, Amersham, Piscataway, NJ).

PCR amplification and sequencing of *lux*A gene

In order to amplify the gene *lux*A, new primers have been designed and used in conjunction with the primers already available. Sequences of the genes *lux*D and *lux*B, flanking *lux*A of *V. harveyi, P. leiognathi P. phosphoreum, V. fischeri, Ph. luminescens* have been obtained from Genebank and aligned. The forward primer *lux*d and the reverse primers LuxBcon, LuxBdeg were designed on the zones of uniqueness by using the program OSP (Miller and Green, 1991). LuxBcon is a consensus primer, and LuxBdeg is a degenerate primer. The forward primers 127f, 275f and the reverse primer 1007r are degenerate primers (Van Ert, pers. comm.) derived from primers designed on conserved regions of *lux*A (Wimpee, 1991). The sequences of the primers used are reported in Table 4. PCR amplification of *lux*A were performed in 0.5 ml tubes in a final reaction

Table 4. List and sequences of the primers

Primer	sequence 5' to 3'	Location and direction	Reference
Luxd	GTCTYTCWGCTCGWRTYGCYTATGA	luxD, forward	This thesis
LuxBcon	AGGAAGAATAATCCAAATTCAT	luxB reverse	This thesis
LuxBdeg	AGDAARAATAAYCCDAAAWTTCAT	luxB reverse	This thesis
127f	GAICAITTIACIGAGTTTGG	luxA forward	Van Ert, pers. comm.
275f	TIYTIGATCAAITGTCIAAAGGICG	luxA forward	Van Ert, pers. comm.
1007r	ATTTCITCTTCAGIICCATTIGCTTCAAAICC	luxA reverse	Van Ert, pers. comm.
8f	AGAGTTTGATCMTGGCTCAG	16S rRNA forward	Weisburg, et al. 1991
1492r	TACGGYTACCTTGTTACGACTT	16S rRNA reverse	Weisburg, et al. 1991
530f	GTGCCAGCMGCCGCGG	16S rRNA forward	Lane et al. 1985
926f	AAACTYAAAKGAATTGACGG	16S rRNA forward	Lane et al. 1985
519r	GWATTACCGCGGCKGCTG	16S rRNA reverse	Lane et al. 1985
907r	CCGTCAATTCMTTTRAGTTT	16S rRNA reverse	Lane et al. 1985

volume of 50 µl containing 60 pMoles of each primer, 200 µM of each dNTP, 1 Unit of Taq polymerase (Promega) and 5 µl of the 10X buffer recomended (Promega). The template was used at the concentrations of 5, 7.5, 10 and 15 ng for reaction and a drop of mineral oil was added to prevent the evaporation. The amplification reactions were performed to 30 cycles by using Biometra UNO thermal cycler with the program: 94 °C for 60 sec. (denaturation), 50 °C for 60 sec. (annealing), 72 °C for 120 sec (extention), and a final extention period of 8 minutes at 72 °C. The annealing temperatures of 37°C or 40°C were used in some cases instead of 50°C. The primers used were: Luxd, 127f and 275f (forward) and 1007r, LuxBcon, LuxBdeg (reverse). Reaction mixtures containing template DNA from non luminous isolates (*E. coli*) and sterile distilled water were added as negative control. After amplification, the mineral oil was carefully removed and 10 µl of each final reaction mixture was examined by electrophoresis through 1% agarose gel. PCR products in gels were stained with ethidium bromide, visualized by UV transillumination and photographed. Amplified DNA was purified by using the kit GeneCleanIII™. The reaction mixture was incubated with 5 µl of glass milk beds for 15 min and centrifugated at 14.000 rpm for 5 min. The supernatant was removed and the glassmilk containing DNA was washed 3 times with the Acid Wash Solution provided by the kit. Then, DNA was eluted from the pellet by three extractions with 5 µl of 1/10 TE buffer. After evaluation of DNA concentration by fluorometry, samples were frozen at –20°C until the sequencing. Sequencing of both strands of the amplified fragments was performed by using the automated sequencer ABI Prism 377 (Applied Biosystems, Foster City, CA) following the instructions of the manufacturer. For the sequencing

reaction, 40 ng of template DNA was incubated with 1.6 pmoles of the specific primer and 4 µl of the ABI PRISM BigDye™ Sequencing Terminator Kit, containing the AmpliTaq DNA polymerase, the deoxynucleoside triphosphates, di-deoxy-Rhodamine dye terminators, $MgCl_2$ and reaction buffer. Purified water was added for a final volume of 10 µl. The amplification was conducted in a thermal cycler with 25 cycles of the following program: 96 °C for 10 sec. (denaturation), 50 °C for 5 sec. (annealing), 60 °C for 4 min 30 sec. (extension), rapid ramp to 4 °C. The amplification products were purified from non incorporated fluorescent dyes by precipitation in 30 µl of ice cold 100% ethanol and 5 µl of 7.5 M ammonium acetate for 1 hour at -20°C. After centrifugation of the samples for 30 min at 14,000 rpm, the pellet was washed 2 times with 70% ethanol and dryed in a vacuum centrifuge. The samples were resuspended in 5µl a loading buffer (deionized formamide and 25 mM EDTA-blue dextran at a ratio of 5:1) and denaturated at 95°C for 5 minutes. After that, they were loaded in the sequencing gel (5% acryl: bis acrylamide gel 29:1 (Amresco, Solon, OH) mounted in the electrophoresis chamber. The gel was covered with TBE buffer (22.5 mM Tris-borate, 0.5 mM EDTA) and electrophoresis conducted with the module: Run 2X (1680 volts for 7 hours, 1200 laser-scans/hour). The signals of the scans were automatically recorded, converted into electropherograms and sequences with the Sequence Analysis software provided by the sequencer.

PCR amplification and sequencing of the gene 16s rRNA

The PCR amplification of the gene 16S rRNA was obtained by using the primers 8f and 1492r, described by Weisburg (1991) and the protocol already described. Both strands of the amplified fragments were sequenced by using the primers 8f, 1492r, 530f, 907r 902f, 518r, (Table 4, Lane *et al.* 1985, Weisburg *et al.*, 1991) according to the method already described.

Analysis of the sequences

The editing and the assemblage of the sequences were performed with the Autoassembler (Applied Biosystems) and a consensus sequence was obtained from each fragment sequenced. Sequences of *lux*A obtained were translated into amino acid sequences by using the program Sequence Navigator (Applied Biosystems). Alignment of the amino acid sequences was done with ClustalX (Higgins, EMBL, Heidelberg, Germany). Sequences of DNA from 16S rRNA gene were also aligned with ClustalX. Phylogenetic analysis was conducted by using PAUP (Sinauer Associates, MA).

RESULTS

Phenotypic characterization

A phenotypic characterization of the isolates Imk3, Imk4, Imk5, YSCI, HSC2, HSC3, GmD5, Gm3A, IM3, from Oman and 3D2, KI, Agr2, Pin2, Tod1, TodB, Flcarpio1, MSB, 2YSC, 2YSD, 2YSG, SCI, YSI, ID3, 3D3, 3YSB, 2D3, ID2 from

Florida has been performed by using the BIOLOG GN plates. The results have been added to the data previously obtained by Makemson and Hermosa (1999) from the analysis of the known luminous species, some related species and a group of new isolates from Oman, that represent the database for the present work. The cluster analysis of luminous isolates previously reported by Makemson and Hermosa (1999) shows the presence of two major groups, corresponding respectively to groups possessing "slow" and "fast or intermediate" luciferases, with several sub-clusters of isolates in each group. Oman luminous bacteria formed six clusters distinct from the known species. Cluster analysis obtained by adding the strains of this study to the database is reported in Figure 1. IMK3, IMK4, YSCI form a distinct cluster in the group of "slow" luciferases, which includes two other groups distinct from the known species, including respectively MSC5, Pin2 and HSC2, HSC3, SUP1, Flcarpio1 and RAJ3. The clustersof YSI, ID3, 3D3 is also distinct from the known species and included in the groups of "fast and intermediate" luciferases. All the other isolates were in clusters with the known species.

PCR amplification of the *lux*A gene

In order to amplify the *lux*A gene from the new isolates, three new primers have been designed on the basis of the alignment of DNA sequences from *lux*D, *lux*A and *lux*B of the species *V. harveyi*, *P. phosphoreum* and *V. fischeri*, obtained from Genebank. The new primers (Luxd, LuxBcon, LuxBdeg) and the primers 127f, 275f, 1007r have been used for the PCR amplification of *lux*A from *DNA* of *V. harveyi, V. fischeri, P. leiognathi, P. phosphoreum, S. woodyi* and some of the new isolates. In a preliminary experiment, different concentrations of DNA and annealing temperatures were tested.

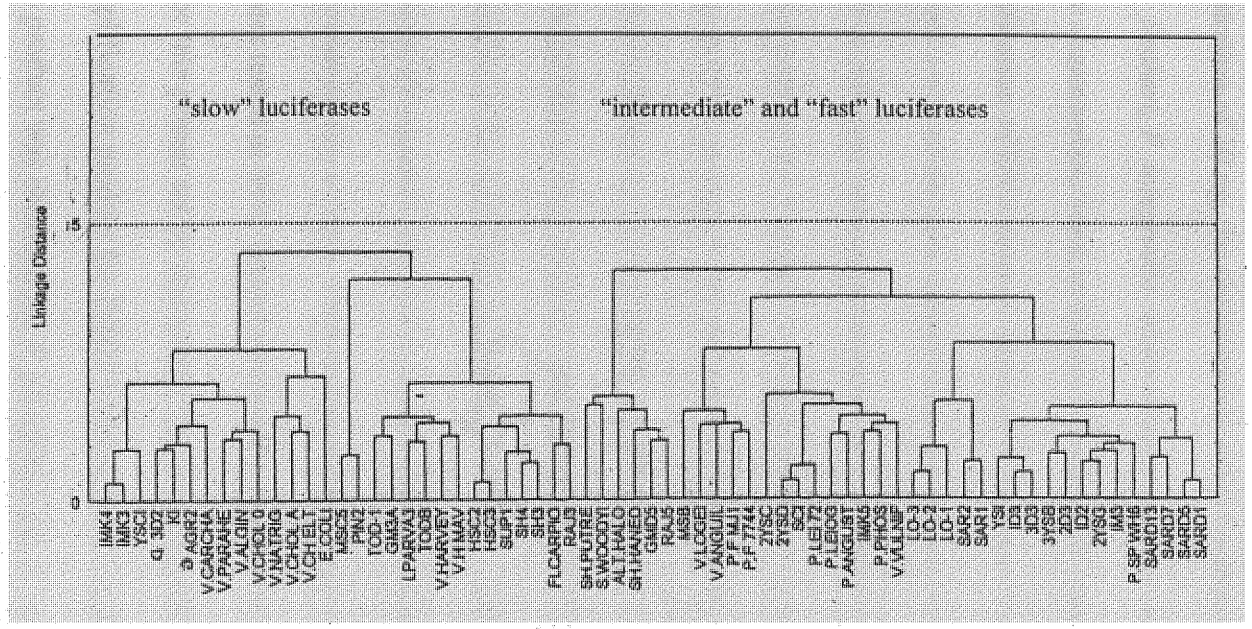

Figure 1
Cluster analysis of substrate metabolism (95 characters, BIOLOG GN plates) of luminous bacteria from Oman and Florida obtained by Ward's Method - Euclidean distances (STATISTICA soft.)

PCR amplification has been conducted with the primers 275f/1007r and the annealing temperature of 50 °C, using the DNA of *V. harveyi, V. fischeri, P. leiognathi* and P. *phosphoreum* at the concentrations of 5ng and 10 ng/50ml of reaction mixture. At the concentration of 10 ng, the characteristic amplification product was obtained for *V. harveyi, V. fischeri, P. leiognathi*, while using 5 ng, only the *V. harveyi* and *P. leiognathi* showed the characteristic band in the gel, but with less intensity. Table 5 shows results of PCR amplification of DNA from *V. harveyi, V. fischeri, P. leiognathi, P. phosphoreum, S. woodyi* and some of the new isolates with the primers 127f/1007r and 275f/1007 and Luxd/1007r at different annealing temperatures. At 50°C, primers 127f/1007r gave amplification product for 5 of the 17 isolates analyzed. Lowering the annealing temperature at 37°C produced amplification product for six of seven negatives tested. At this temperature, however, several bands of different size from the expected (not specific amplification products) were present in the gel. Primers 275/1007r gave a product for 14 of the 19 strains at the annealing temperature of 50 °C. The annealing temperature of 37°C increased the number of positive samples and amplification was obtained for *P. phosphoreum* and *S. woodyi*. Primers Luxd/1007r gave amplification product for 11 of the 19 isolates tested at 50°C annealing temperature and no significant improvement was observed for this primer set by decreasing the annealing temperature. In fact, at 40 °C there is amplification product for IMK3 but absence of amplification for GmD5 and IMK5. No product was obtained for *P. phosphoreum, S. woodyi*, Gsanl, MSC3, Sar1, Sar2, Sard13. Negative PCR samples were used as template for a second PCR amplifications with the primers Luxd/1007r but no results were obtained. The primer

Table 5. Results of PCR amplification of *lux*A gene from DNA of luminous bacteria using different primers and annealing temperatures.

STRAINS	PRIMERS (*)					
	127f/1007r (888bp)		275f/1007r (740bp)		Luxdf/1007r (1600bp)	
	37°C	50°C	37°C	50°C	40°C	50°C
V. harveyi	+	-	+	+	+	+
V.fischeri	+	-	+	+	+	+
P. leiognathi	+	+	+	+	nd	+
P. posphoreum	+	-	+	-	-	-
S.woodyi MS32	+	+	+	-	-	-
Imk3	+	-	nd	+	+	-
Msc3	+	-	nd	+	-	-
ImK4	+	nd	nd	+	+	+
Gsanl	-	-	-	-	-	-
GmD5	+	-	nd	+	-	+
Lo3	nd	+-	nd	+	+	+
Im3	+-	nd	nd	+	+	+
Sar1	+-	-	nd	+-	-	-
Sar2	nd	-	nd	-	-	-
Sar13	nd	-	nd	-	-	-
Imk5	nd	+	nd	+	-	+
Msc5	nd	+	nd	+	+	+
Raj3	nd	-	nd	+	+	+
SH4	nd	-	nd	+	+	+

(*) size of the amplification product +- positive, with low product

275f was used in conjunction with the reverse primers LuxBcon and LuxBdeg for amplification of DNA from *V. harveyi, V. fischeri, P. leiognathi*, and *P. phosphoreum* at the annealing temperature of 37°C. A band of about 840 bp, corresponding to the expected product, was present only with the reverse primer LuxBcon for *V. fischeri.* Lower annealing temperature increased the number of positive samples by allowing the annealing when the primers didn't perfectly match the DNA template, but produced reduced yield of the expected product or several bands of non specific amplification. The amplification products obtained at 37 or 40 °C were separated on Nusieve agarose gel and the DNA of the specific band was cut out and purified before sequencing. The DNA obtained had a low concentration (about 8 - 10 ng/ml) and did not gave good results when used for sequencing. The annealing temperature of 50°C improved the results for all the positive samples, by eliminating the not specific bands and increasing the concentration of specific amplified product as shown in Figure 2. Amplified DNA obtained at 50°C had a concentration ranging from 20 to 60 ng/ml and did not require purification from not specific products before sequencing. Primers 275f/1007r gave amplification product at 50°C for the higher number of isolates in the preliminary experiments and were then used for all the isolates of this study.

Analysis of *lux*A sequences

A portion of the *lux*A has been obtained by PCR with the primers 127f/1007r from *S. woodyi* MS32 and with the primers 275f/1007r from the isolates YSC1, IMK3, MSB, SH4, LO3, MSC5, IMK4, Gm3A, SUP1, 2YSG, RAJ3, Pin2, Tod1, IMK5, SCI,

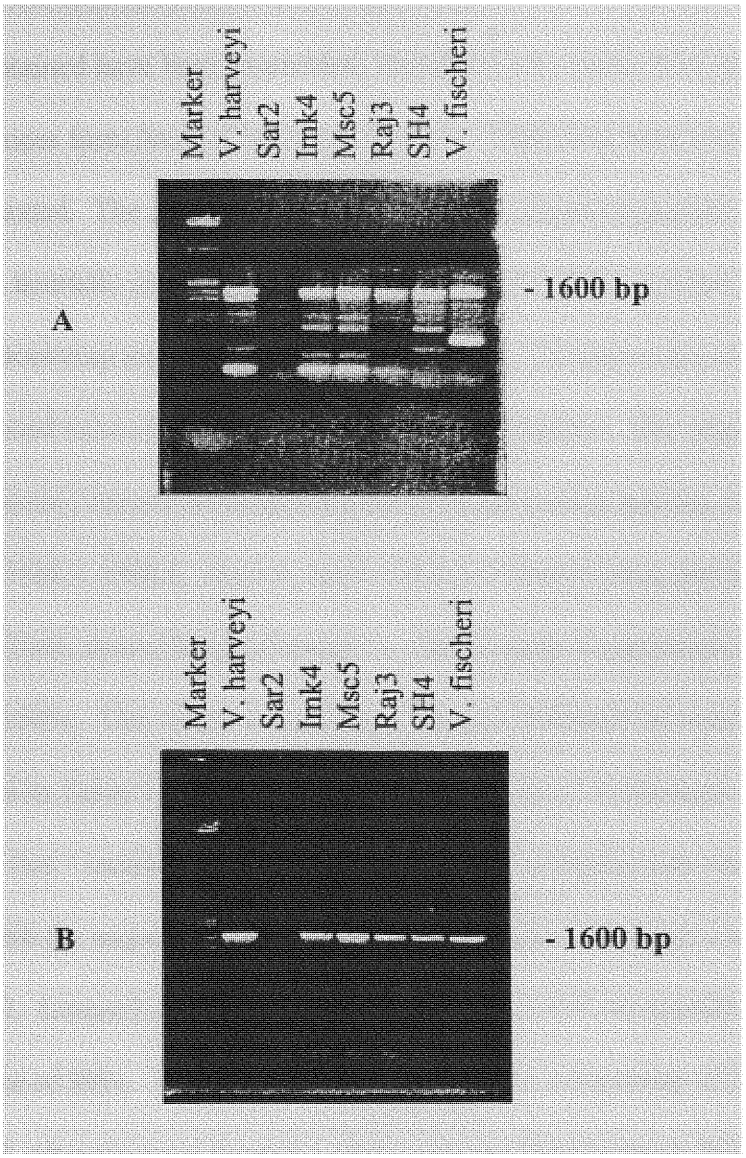

Figure 2
PCR amplification of luminous strains wth the primers Luxd/1007r at the annealing temperatures of 40 °C (A) and 50 °C (B).

GMD5, 3D2, KI. Both strands of the amplified product have been sequenced and the DNA sequences obtained have been assembled, translated into amino acid sequences and aligned. The sequences from *lux*A of luminous bacteria (Table 6A) have been added to the alignment generated. The phylogenetic analysis of the aligned sequences, was conducted with the heuristic method in PAUP. In order to verify the consistency of the clades obtained, a bootstrap analysis was conducted. Figure 3A shows one of the 6 most parsimonious unrooted trees obtained from amino acids sequences. The sequence of *V. harveyi* (slow luciferase) formed a lineage distinct from the known species, including the new strains YSC1, IMK3, IMK4, Gm3A, Sup1 and RAJ3 from Oman, and MSB, Lucpar3, Pin2 and Tod1, from Florida (lineage 1). This lineage is supported by a bootstrap value of 100%. The values of luciferase kinetics obtained for some of these isolates are reported in Table 7 and show the presence of "slow" luciferases. The sequences of the " fast " luciferases of *P. leiognathi* and *P. phosphoreum* were in two diferent lineages with the sequences of some of the new isolates (lineages 2 and 3). Strains GmD5 3D2, Lucpar3 and KI, were in the lineage 2 of *P. leiognathi*, supported by a bootstrap value of 89%. This lineage was supported by a bootstrap of 54 % from the isolate IMK5. *P. phosphoreum* formed a clade with the Florida strain SCI (lineage 3) supported by a value of bootstrap of 100%. The "intermediate" luciferase *S. woodyi* MS32, was in a lineage distinct from all the other strains (lineage 4). Phylogenetic analysis has been conducted by using the alignment of the luxA nucleotide sequences of the same isolates and Fig 3B shows one of the 45 most parsimonious trees, along with branch lengths and bootstrap values. This tree confirmed the lineages 1-4 obtained from the analysis of the the amino acid sequences, with lineage 4 of *S. woodyi* including also

Table 6. List of DNA and amino acids sequences obtained from Genebank

A - *Lux*A (nucleotides and amino acids) and 16s rRNA genes

Species	Abbreviation
Vibrio harveyi	Vh
V. fischeri	Vf
V. cholerae	Vcho
Photobacterium phosphoreum	Pp
P. leiognathi	Pl
Photorhabdus luminescens	Plum
Shewanella hanedai	Sh
Kriptophanaron alfredi-symbiont	Kal

B - 16S rRNA gene

Species	Abbreviation
Escherichia coli	Eco
P. angustum	Pang
S. woodyi MS32	Sw
S. putrefaciens	Sput
V. splendidus	Vsplend
V. carchariae	Vcarch
V. anguillarum	Vanguil
V. parhaemolyticus	Vparah
V. vulnificus	Vvulnific
V. orientalis	Vorient
V. logei	Vlo

Table 7. Luciferases kinetics of the new strains

STRAIN	DECAY RATE (sec^{-1}) Aldehyde	
	C_{10}	C_{12}
* *S. woodyi* MS32	0.263	0.293
* LO3	0.300	0.020
* SH4	0.338	0.053
* Sup1	0.272	0.056
° MSC3	0.300	0.060
° MSC5	0.380	0.037
° Imk3	0.230	0.030
° Imk4	0.140	0.015
° Raj3	0.270	0.070
° Pin2	0.230	0.069

* Makemson, pers. comm.

° This study

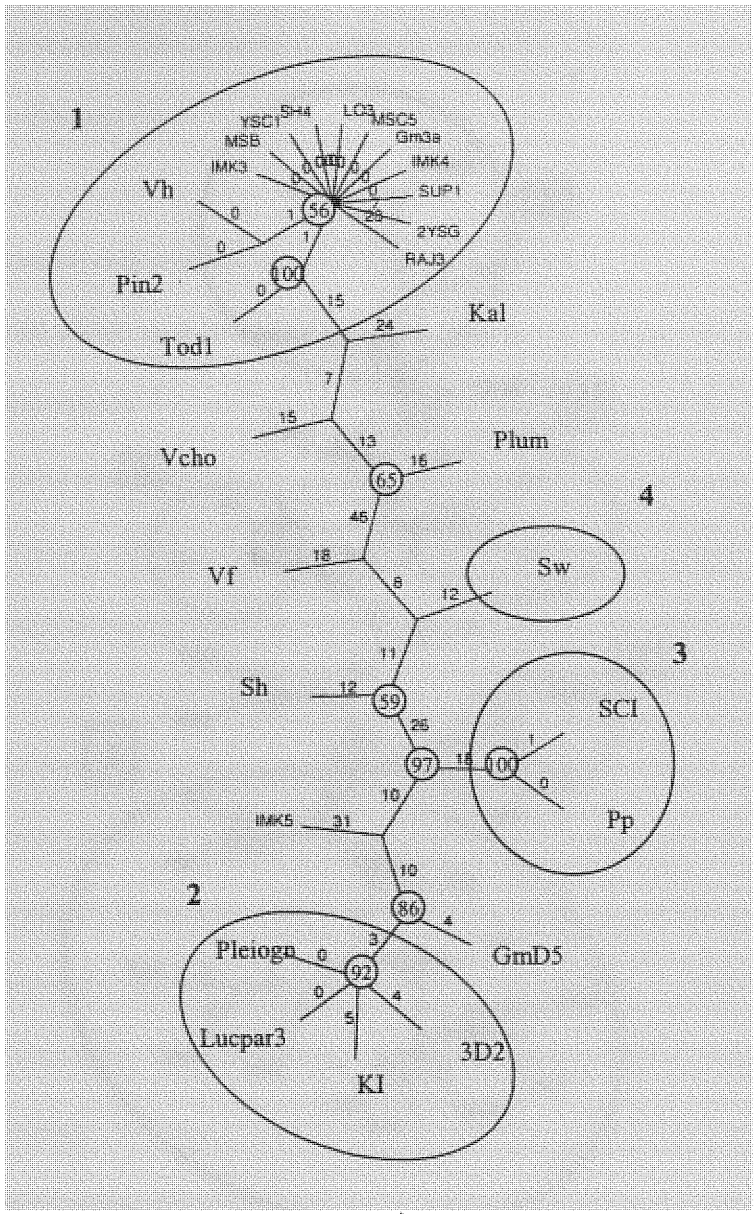

Figure 3A
One of the 6 most parsimonious tree obtained from *lux*A amino acids sequences of new luminous bacteria and *S. woodyi*. Parsimony analysis with heuristic search has been conducted by using the program PAUP. Branch lengths are above branches, values of bootstrap are in circles. Sequences from Genebank: Vh (*Vibrio harveyi*), Vcho (*V. cholerae*), Vf (*V. fischeri*), Sh (*Shewanella hanedai),* Pp *(Photobacterium phosphoreum),* Pleiogn *(P. leiognathi),* Kal *(Kriptophanaron alfredi*-symbiont), Plumin (*Photorhabdus luminescens*).

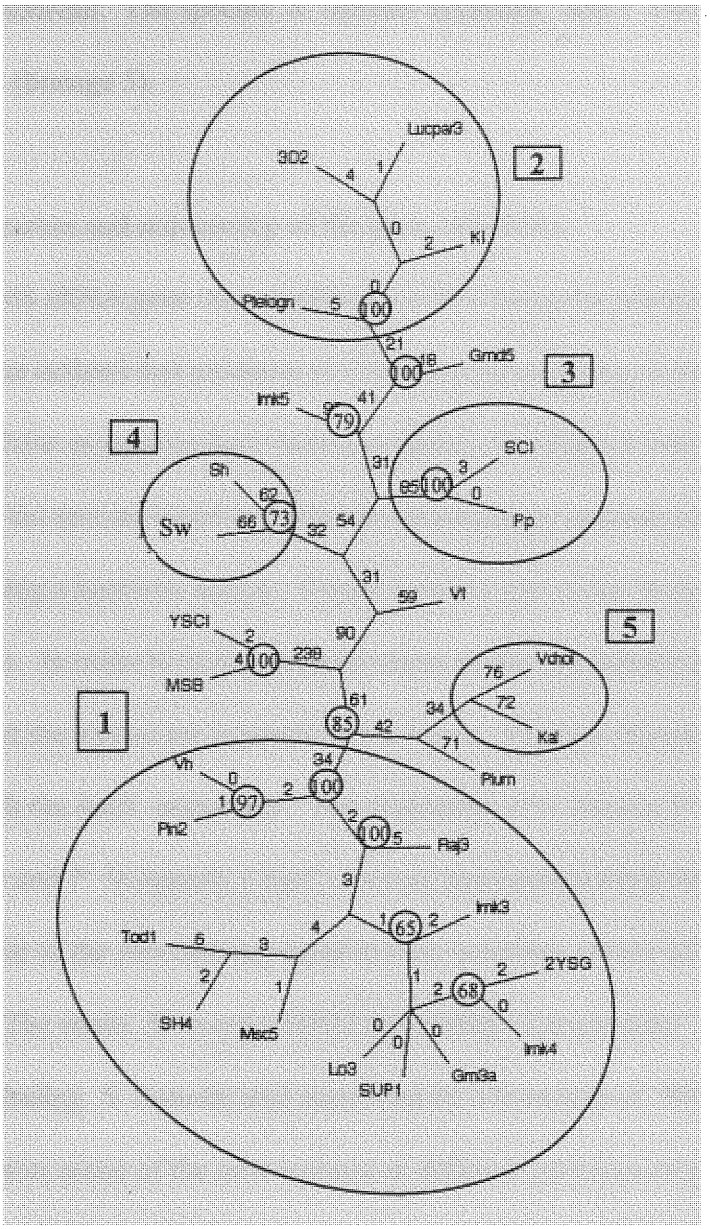

Figure 3B

One of the 45 most parsimonious trees obtained from *lux*A nucleotides sequences of new luminous bacteria and *S. woodyi*. Parsimony analysis with heuristic search has been conducted by using the program PAUP. Branch lenghts are above branches, values of bootstrap are in circles. Sequences from Genebank: Vh (*Vibrio harveyi*), Vcho (*V. cholerae*), Vf (*V. fischeri*), Sh (*Shewanella hanedai),* Pp *(Photobacterium phosphoreum),* Pleiogn *(P. leiognathi),* Kal *(Kriptophanaron alfredi*-symbiont), Plumin (*Photorhabdus luminescens*).

the strain *S. hanedai*. The species *K. alfredi*- symbiont, and *V. cholerae* were in the same lineage (lineage 5).

PCR amplification and sequencing of the gene 16S rRNA

The 16S rRNA gene was amplified by PCR using the primers 8f/1492r from DNA of all the isolates and products of 1500bp, corresponding to the entire gene, were sequenced. These sequences have been assembled, and aligned with the 16S rRNA sequences of the species listed in Table 6B. Phylogenetic analysis has been conducted by parsimony using a heuristic search. Figure 4 shows one of the 36 most parsimonious unrooted trees. In order to verify the robustness of the tree, bootstrap test has been performed and the values are reported in the figure. This tree shows that lineages 1-5 conincide with the *lux*A sequences. The sequences of *V. harveyi* ("slow" luciferase), *P. phosphoreum* and *P. leiognathi* ('fast") formed three independent lineages (1-3) including some of the new strains, supported by bootstrap values of 95%, 86% and 83% respectively. The strain *S. woodyi* MS32 possessing "intermediate" luciferase formed lineage 4, including *S. hanedai* and supported by a bootstrap of 96%. But, the 16S rRNA tree places some of the new isolates at different positions respect to *lux*A sequence trees. Gm3A and 2YSG, present in the lineage 1 (with *V. harveyi*) in the *lux*A trees, seem more related to lineages 2 and 3 containing *P. phosphoreum* and *P. leiognathi* (bootstrap value of 95%) and IMK3 branched in a clade with *S. woodyi* and *S. hanedai* (bootstrap 100%). The 16S rRNA tree also shows the presence of a new lineage (clade 6: PIN2, Raj3, Tod1, 3D2, SCI, Lucpar3, Agr2) distinct from the known species and supported by a bootstrap of 100%. Some of these strains were grouped with the

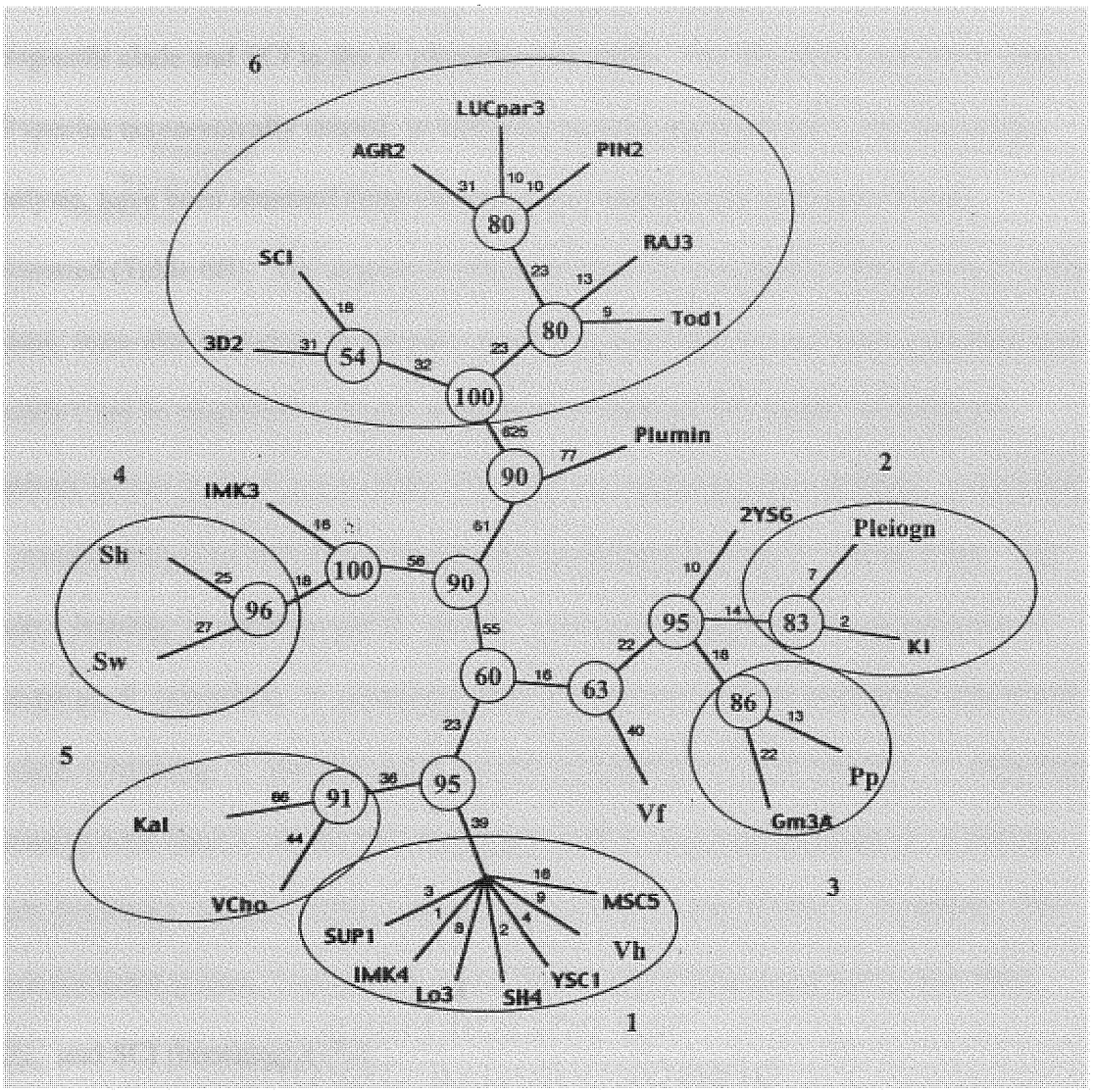

Figure 4
One of the 36 most parsimonious trees from 16S rRNA sequences of new luminous bacteria. Parsimony analysis with heuristic search has been conducted by using the program PAUP. Branch lengths are above branches, values of bootstrap are in circles. Sequences from Genebank: Vh (*Vibrio harveyi*), Vcho (*V. cholerae*), Vf (*V. fischeri*), Sh (*Shewanella hanedai),* Sw (*S. woodyi),* Pp *(Photobacterium phosphoreum),* Pleiogn *(P. leiognathi),* Kal *(Kriptophanaron alfredi*-symbiont), Plumin (*Photorhabdus luminescens*).

known species in the tree generated from *lux*A amino acids and nucleotides sequences. Pin2, Raj3, Tod1 were in the *V. harveyi* clade, 3D2 and Lucpar3 were in the *P. leiognathi* clade and SCI in the *P. phosphoreum* clade. The 16s rRNA sequences from 10 species taxonomically related to luminous bacteria and included in the phenotypic analysis, have been obtained from Genebank and added to the alignment already generated (Table 6B). The sequences obtained from the new strains IM3 and Sar2, from Oman, were also included. In the phenotypic analysis, Sar2 is in a cluster with Sar1 and couldn't not be amplified with any of the *lux*A primers of this study. Phylogenetic analysis shown in Fig 5 is one of the 100 most parsimonious trees obtained. In this tree, *V. parahaemolyticus* and *V. carchariae* were associated with the *V. harveyi* clade. The clade contining *V. cholerae* and *K. alfredi* - symbiont included also *V. anguillarum, V. vulnificus, V. splendidus, V. orientalis* while *Ph. luminescens* was associated with *E.coli.* A clade containing *S. hanedai* and *S. woodyi* included also *S. putrefaciens. P. angustum* clustered with Gm3A and the new strain IM3 with *P. leiognathi* (lineage 2). A distinct clade (bootstrap value of 100%) was not related to any other species in Genebank. This clade included at least 2 subgroups: Agr2, Pin2, Lucpar3 (bootstrap 84%) and Sar2, 3D2, and SCI (bootstrap 81%).

Sar1, Sard 13 and Flcarpio1 were not included in the 16S rRNA tree shown in Fig 4 and 5 because only a sequence of about 800 bp was obtained. Sar1 and Sar13 did not give amplification with any of the *lux* primers. In order to characterize these isolates a new alignment has been produced by using a fragment of the same size for all the isolates of Fig 4. In this alignment, the isolates *Bacillus subtilis* and *B. pumilus* were added as an outgroup. Fig 6 shows a rooted consensus tree obtained by heuristic search

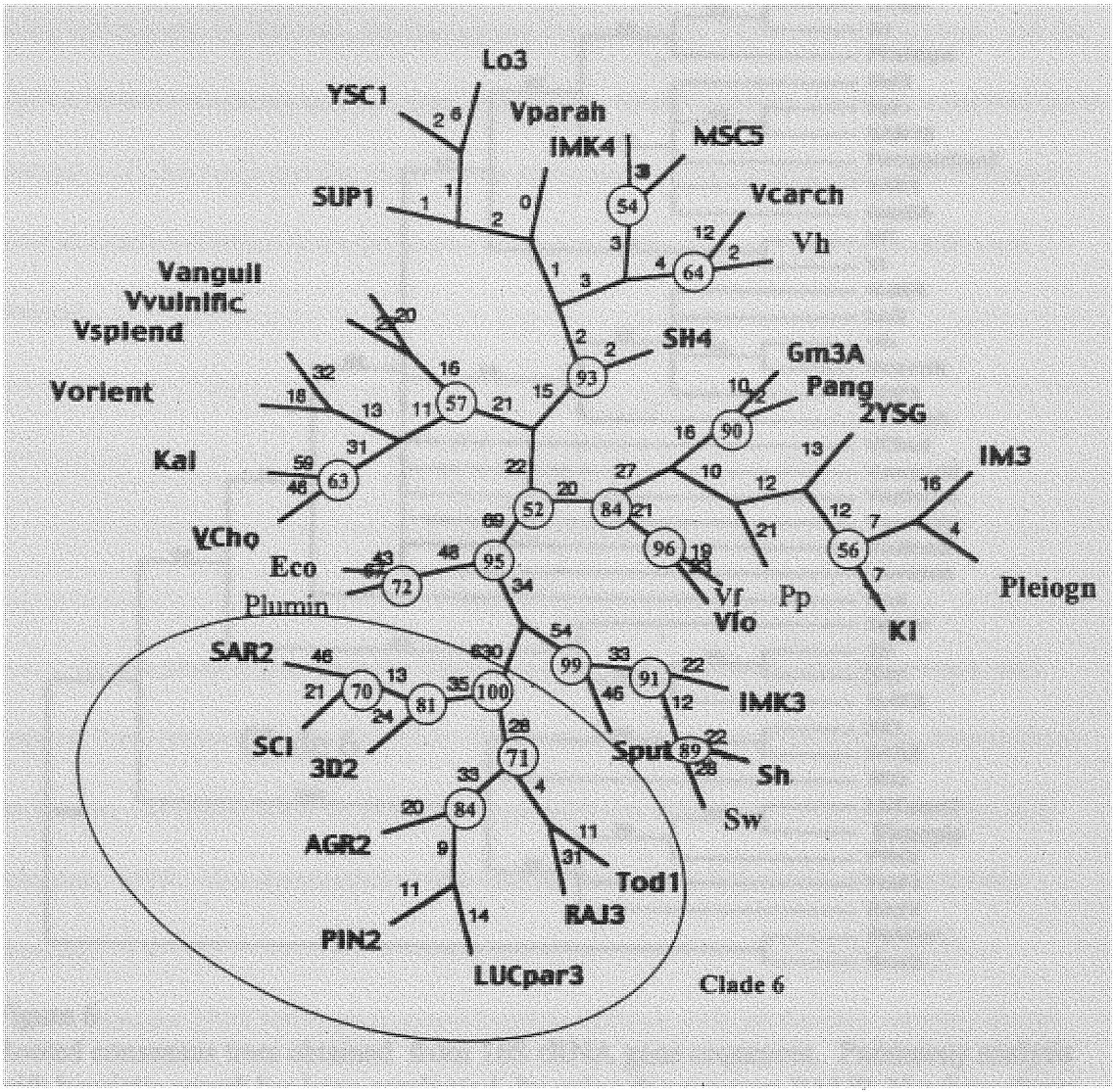

Figure 5
One of the 100 most parsimonius trees obtained from 16S rRNA sequences of new luminous bacteria. Parsimony analysis with heuristic search has been conducted by using the program PAUP. Branch lenghts are above branches, values of bootstrap are in circles. Sequences from Genebank: Vh (*Vibrio harveyi*), Vcho (*V. cholerae*), Vf (*V. fischeri*), Sh (*Shewanella hanedai),* Sw (*S. woodyi),* Pp *(Photobacterium phosphoreum),* Pleiogn *(P. leiognathi),* Kal *(Kriptophanaron alfredi*-symbiont), Plumin (*Photorhabdus luminescens*), Eco (*E.coli*), Vcarch (*V. carchariae*), Vanguil (*V. anguillarum*), Vvulnific (*V. vulnificus*), Vsplend (*V.splendidus*), Vorient (*V. orientalis*), Sput (*S. putrefaciens*), Vlo (*V. logei*), Pang (*P. angustum*).

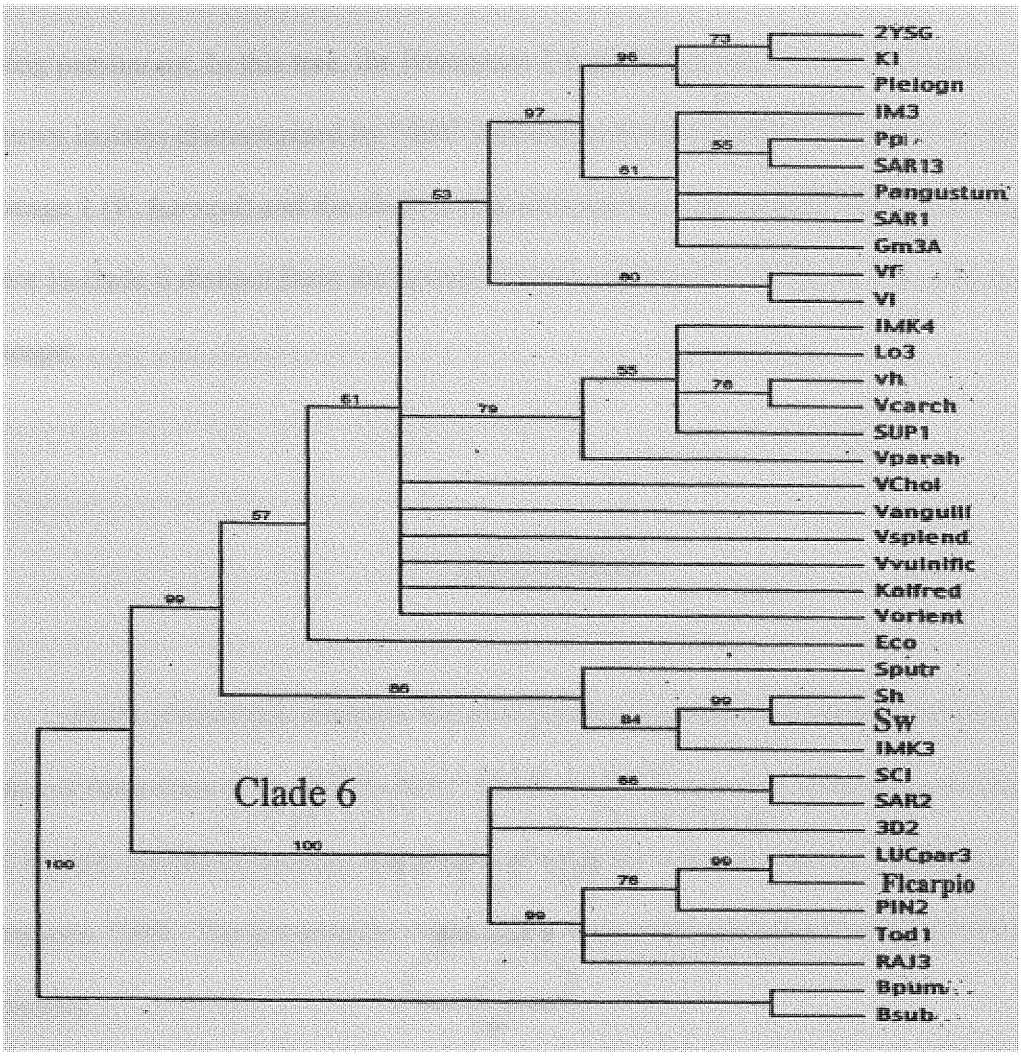

Figure 6

Rooted consensus trees obtained from 16S rRNA gene sequences. Parsimony analysis with heuristic search has been conducted by using the program PAUP. Numbers above branches are the values of bootstrap. Sequences from Genebank: Vh (*Vibrio harveyi*), Vcho (*V. cholerae*), Vf (*V. fischeri*) Sh (*Shewanella hanedai),* Sw (*S. woodyi),* Pp *(Photobacterium phosphoreum),* Pleiogn *(P. leiognathi),* Kal *(Kriptophanaron alfredi*-symbiont), Plumin (*Photorhabdus luminescens*), Eco (*E.coli*), Vcarch (*V. carchariae*), Vanguil (*V. anguillarum*), Vvulnific (*V. vulnificus*), Vsplend (*V.splendidus*), Vorient (*V. orientalis*), Sput (*S. putrefaciens*), Vlo (*V. logei*), Pang (*P. angustum*), Bsub (*Bacillus subtilis*), Bpum (*B.pumilus*).

using parsimony. In this tree, the lineageses 1-6 present in Fig 4 were conserved. All the known isolates characterized by "fast" luciferases formed distinct lineages separated from lineage 1 (*V. harveyi*) and the clade 6 was also present. The isolates Sar2 and Flcarpio1 were in clade 6 distinct from the known isolates and Sard13 was in *P. phosphoreum* lineage.

DISCUSSION

In the phenotypic analysis, the new strains clustered with known species, and some of them formed distinct clusters. The presence in fish guts of isolates distinct from the known luminous bacteria was in accord with the data reported by Makemson and Hermosa, 1999. The geographic origin and habitat of the fish didn't affect clusters obtained.

Primers 275f/1007r gave amplification of *lux*A with all the new strains, except Sar2, Sar13 and Gsnl as well as the known species *P. phosphoreum.* This absence of amplification product can be explained with variability in the DNA sequences, that don't match the primers used.

The alignment of *lux*A amino acids sequences of known species shown by Meighen (1991) presents two groups corresponding to the "slow" kinetics of *V. harveyi* and the "fast" of *V. fischeri, P. phosphoreum and P. leiognathi.* In the analysis of this study, the "slow" luciferase of *V. harveyi* formed a lineage with some of the new strains, distinct from any other know species. The"fast" luciferases *V. fischeri, P. phosphoreum*

and P. leiognathi didn't form an homogeneous group but three different lineages along with some of the new isolates also. The sequence of the "intermediate" *S. woodyi* formed a lineage distinct from the known species and the new strains. Geographic location and fish habitat didn't affect clustering in the trees generated from *lux*A sequences. Strains from pelagic and demersal fish of both locations clustered with the "slow" kinetics of *V. harveyi* and the "fast" of *P. phosphoreum* or *P. leiognathi,* with lineage 1 of *V. harveyi* containing most of the strains.

The trees generated from 16S rRNA sequences (Figures 4, 5, 6) presented the major lineages corresponding to the known species as shown in *Lux*A. The 16s rRNA tree has shown that clade 6 (Agr2, Pin2, Raj3, Tod1, 3D2, SCI, Lucpar3, Flcarpio1) is distinct from all the known luminous and not luminous species included in this study. The strains of this latter clade distinct from the known were isolated from pelagic and demersal fish of Florida, except Raj3, which came from coastal water in Oman. This clade could include new taxa. Geographic location and fish habitat didn't affect composition of clades in the trees from 16s rRNA sequences, as shown from clade 6, containing strains isolated from demersal and pelagic fish from both locations.

CONCLUSIONS

The phenotypic analysis showed that luminous bacteria isolated from fish guts of Oman and Florida clustered with all the known species but some strains were distinct from knowns.

A portion of *lux*A gene was amplified from *S. woodyi* with "intermediate" kinetics, and from 19 new strains with the primers 127f/1007r and 275f/1007r.

The phylogenetic trees from nucleotides sequences and derived amino acids sequences of *lux*A showed that:

- The "slow" luciferases of *V. harveyi* and the "fast" of *P. phosphoreum, P. leiognathi, V. fischeri* formed four distinct lineages.
- The "intermediate" *S. woodyi* was a lineage distinct from the "fast" and "slow" luciferases.
- The new strains clustered into 3 major clades corresponding respectively to *V. harveyi, P. phosphoreum* and *P. leiognathi.*

The phylogenetic analysis from the 16S rRNA sequences showed that:

- *Lux*A lineages shown in the *lux*A analyses were replicated in 16S rRNA lineages.
- Some new isolates formed a clade distinct from known luminous and non-luminous species.

Geographic location and fish habitat didn't affect the distribution of strains in the phenotypic and phylogenetic analyses.

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