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Isolation and characterization of a visibly luminous variant of *Vibrio fischeri* strain ES114 from the sepiolid squid *Euprymna scolopes*

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Abstract *Vibrio fischeri* strains isolated from light organs of the sepiolid squid *Euprymna scolopes* are non-visibly luminous and fast growing in laboratory culture, whereas in the symbiosis they are visibly luminous and slow growing. A spontaneous, visibly luminous, slow-growing variant was isolated from a laboratory culture of the squid-symbiotic *V. fischeri* strain ES114. Taxonomic and DNA-homology analyses demonstrated that the variant was *V. fischeri* and was very similar to the original form. However, the variant grew at one-fourth the rate of the original form, produced 30,000-fold more luminescence, induced luminescence at a lower cell density, and produced a higher level of *V. fischeri* luminescence autoinducer. Regulation of luminescence, nonetheless, was similar in the two forms and typical of *V. fischeri* with respect to responses to autoinducer, glucose, the iron chelator ethylenediamine-di(*o*-hydroxyphenyl acetic acid), and 3':5'-cyclic AMP. Compared to the original form, cells of the variant were smaller, exhibited from zero to two polar, sheathed flagella instead of a tuft of three to eight flagella, produced a deeper yellow-orange pigment, did not acidify media containing glycerol, and produced a more distinct pellicle. The two forms also differed in the levels of several outer membrane and soluble proteins. These results establish a distinctive physiological, morphological, and biochemical dimorphism in *V. fischeri* ES114 in which the variant exhibits several traits similar to *V. fischeri* cells in the symbiotic state. The variant and its conversion from the original form in laboratory culture may provide insight into the properties of *V. fischeri* cells in the symbiosis and may serve as a model for elucidating the mecha-

nism for their pleiotropic conversion upon colonization of the squid.

Key words *Vibrio fischeri* · Spontaneous variant · Pleiotropic variant · Dimorphism · Symbiosis · Sepiolid squid · *Euprymna scolopes*

Introduction

Bioluminescent symbioses between light-producing marine bacteria and their squid and fish hosts are potentially valuable systems with which to define the cellular and molecular interactions in bacterial-animal mutualisms. The bacteria are harbored as extracellular pure cultures at high population densities in specialized gland-like tissues called "light organs," and the light they produce is used by the animal in a variety of luminous displays associated with avoiding predators, locating food, and finding mates. In many cases, the bacteria can be cultured under laboratory conditions, and in some cases, they have been shown to be amenable to genetic manipulation (Hastings et al. 1987; Dunlap and Greenberg 1991; Dunlap and Kuo 1992; Ruby and McFall-Ngai 1992; Graf et al. 1994; Kuo et al. 1994; Dunlap 1995).

In one of these associations, the symbiosis between *Vibrio fischeri* and the sepiolid squid *Euprymna scolopes* (McFall-Ngai and Ruby 1991), the bacteria exhibit a particularly pronounced dimorphism. In culture, and presumably also in the free-living state, strains isolated from light organs of *E. scolopes* are not visibly luminous, are motile by means of a tuft of polar sheathed flagella (Boettcher and Ruby 1990), grow rapidly, and exhibit less of the yellow-orange pigment produced by other *V. fischeri* strains (Baumann et al. 1984; P. V. Dunlap, personal observation). *V. fischeri* cells in the *E. scolopes* light organs, in contrast, produce a high level of luminescence, grow slowly, lack flagella, and are smaller than when grown in culture (Ruby and Asato 1993). Within 24 h of colonizing the squid, more than 95% of the *V. fischeri* cells exhibit the small size and absence of flagella characteristic of the

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symbiotic state (Ruby and Asato 1993). This distinctive dimorphism indicates that *V. fischeri* undergoes a pleiotropic conversion upon its colonization of the animal light organ, with the non-visibly luminous, fast-growing, flagellated form apparently better adapted to the free-living state, and the luminous, slow-growing, non-flagellated form better adapted to the symbiotic state. At present, the cellular and molecular events leading to this conversion in *V. fischeri*, or in other symbiotic luminous bacteria, are not known.

In this report, we describe a spontaneous variant of the squid-symbiotic *V. fischeri* strain ES114 that exhibits in laboratory culture physiological, morphological, and biochemical characteristics distinct from the original, non-visibly luminous form of ES114. In several of these characteristics, including visible luminescence, slower growth, and a decreased number of flagella, the variant resembles *V. fischeri* cells in the squid light organ. The variant and its conversion from the original form in laboratory culture may provide insight into the properties of *V. fischeri* in the symbiotic state and serve as a model for understanding the mechanism controlling its pleiotropic conversion upon colonization of the squid.

Materials and methods

Bacterial strains, media, and culture conditions

The strains used in this study were *Vibrio fischeri* MJ-1 (Ruby and Nealson 1976), MJ-100 (Dunlap and Kuo 1992), ES114 (Boettcher and Ruby 1990; designated here as ES114 original form), and the visibly luminous ES114 variant (this study); *V. harveyi* B392 (Reichelt and Baumann 1973); and *Photobacterium leiognathi* LR-1a (Dunlap 1985).

For routine cultivation, LBS (Dunlap 1989) and SWT (Boettcher and Ruby 1990) media were used. For experimental broth cultures, an artificial seawater-Hepes (ASH) medium (Dunlap and Kuo 1992) was used. ASH contained 300 mM NaCl, 10 mM KCl, 50 mM MgSO₄, 10 mM CaCl₂, 50 mM Hepes (pH 7.25), 40 mM glycerol (unless otherwise indicated), 3 g yeast extract l⁻¹ (Difco, Detroit, Mich., USA), and 5 g tryptone l⁻¹ (Difco). ASH was filter-sterilized (0.2 µm pore size) and supplemented, as indicated, with filter-sterilized glucose (10 mM) (replacing glycerol), 3':5'-cyclic AMP (cAMP, 10 mM), ethylenediamine-di(*o*-hydroxyphenyl)-acetic acid (EDDHA, 10 µM), or with conditioned ASH medium (1:1). EDDHA was deferrated by the method of Rogers (1973).

Broth cultures were grown in 3-ml volumes in 13-mm diameter tubes or 5-ml volumes in 16-mm diameter tubes with aeration (250 rpm) in a gyratory water-bath shaker at 27°C. Growth was followed as the change in optical density at 660 nm (OD₆₆₀) with time. For luminescence studies, ASH broth and 13-mm diameter tubes were used, and cultures were initiated at an OD₆₆₀ of 0.005–0.010 from mid- to late-exponential phase cultures (OD₆₆₀ of 0.5–1.0) grown in ASH broth. An OD₆₆₀ of 0.20 corresponded to approximately 1.2 × 10⁸ cells ml⁻¹ for the ES114 original form and 1.3 × 10⁸ cells ml⁻¹ for the variant, as determined by plate counts on LBS agar. Light production was measured with a Pacific Instruments (Concord, Calif., USA) Model 124 photometer and with a Turner Designs (Sunnyvale, Calif., USA) Model 20e Lumimeter, as described previously (Dunlap and Greenberg 1985).

Conditioned medium, for determining the presence and effects of *V. fischeri* autoinducers (Eberhard et al. 1981; Kuo et al. 1994), was prepared by growing cells in ASH broth to an OD₆₆₀ of 1.0 and then removing the cells by centrifugation (10,000 × *g* for 10 min at 5°C) and filter sterilization (0.2 µm pore size) and was stored frozen at -20°C. To assay for autoinducer synthesis, a pre-

viously described sensitive autoinducer-bioassay system of *Escherichia coli* containing the *V. fischeri lux* genes (Dunlap and Ray 1989) was used.

Changes in the pH of growth media were assessed for cultures grown in ASH, LBS, or SWT broth with aeration, with the pH of the medium monitored periodically with a calibrated pH electrode. Pellicle formation was assessed with LBS broth cultures grown at room temperature (22–24°C) without shaking for several days. To screen for siderophore production, chrome azurol S (CAS) agar plates were prepared as described by Schwyn and Neilands (1987), except that the medium was modified to contain artificial seawater (300 mM NaCl, 10 mM KCl, 50 mM MgSO₄, 10 mM CaCl₂), NH₄Cl (15 mM), α-glycerophosphate (0.3 mM), and glycerol (40 mM). To screen for an *rpoS* mutation (Zambrano et al. 1993), hydrogen peroxide was added dropwise to colonies grown on LBS agar plates. Intact cell assays for the periplasmic 3':5'-cyclic nucleotide phosphodiesterase of *V. fischeri* were conducted as described previously (Dunlap et al. 1992).

Taxonomic and DNA-homology analyses

Determination of taxonomic traits followed the methods Reichelt and Baumann (1973), with comparisons made to *V. fischeri* MJ-1, *V. harveyi* B392, and *P. leiognathi* LR-1a. The ES114 cryptic plasmid (Boettcher et al. 1990) was visualized by standard plasmid purification and agarose gel electrophoresis procedures (Sambrook et al. 1989). Growth on 3':5'-cyclic AMP (cAMP) was determined as described previously (Dunlap and Callahan 1993). Glucose fermentation was determined with the indicator medium of Hugh and Leifson (1953) prepared with 70% natural seawater and containing glucose (20 mM).

DNA was extracted and purified essentially as described by Marmur (1961). The mol% G+C content of DNA was determined by the HPLC method of Katayama-Fujimura et al. (1984) with modifications. Specifically, denatured DNA, approximately 1 mg ml⁻¹ dissolved in distilled water, was digested with nuclease P1 (2 U ml⁻¹; Yamasa Shoyu, Choshi, Japan) for 30 min at 50°C. Reversed-phase HPLC was conducted with a Senshu Pad ODS-1251-N column (4.6 mm internal diameter × 250 mm). Nucleotides were eluted with 10 mM phosphate buffer (pH 7.0) at a flow rate of 1 ml min⁻¹ at 30°C and were monitored with a spectrophotometric detector at 260 nm. Nucleotide standards were from Yamasa Shoyu.

DNA homology determinations followed the quantitative dot-blot hybridization method of Ezaki et al. (1988) using photobiotin-labeled DNA probes and the BluGENE nonradioactive nucleic acid detection system (Bethesda Research Laboratories, Gaithersburg, Md., USA), in accordance with the manufacturer's instructions. Quantification of hybridization spots with a Shimadzu CS-9000 computing densitometer followed the method of Hiraishi et al. (1991). To minimize the variation inherent to the DNA hybridization methodology, due in part to differences in the amounts of immobilized DNA, 5–8 replicates were conducted for each hybridization.

Microscopy

Cells were examined from exponential-phase ASH broth cultures (OD₆₆₀ of approximately 0.20–0.50). Flagellar arrangement and structure were determined using a Philips 300 transmission electron microscope. Cell preparations were gently pelleted and resuspended in 2% filter-sterilized NaCl, and then negatively stained with 1.0% aqueous uranyl acetate on Formvar-coated grids. Cell morphology was determined from preparations on agar-coated glass slides (Pfennig and Wagener 1986) with a Zeiss standard microscope using phase-contrast illumination and a 100 × Neofluar oil immersion objective.

Analysis of proteins

Outer membrane and soluble-protein fractions were prepared by differential solubilization with *N*-lauroylsarcosine, as described by

Lümmen and Winkler (1986). Cells grown to stationary phase in 25 ml of LBS broth were harvested by centrifugation ($10,000 \times g$ for 10 min at room temperature), washed once with 3% NaCl containing 50 mM Tris-HCl (pH 7.5), and resuspended in 10 mM Tris-HCl (pH 8.0). After sonication and incubation with 1% *N*-laurylsarcosine for 30 min at room temperature, intact cells were removed by centrifugation ($2,000 \times g$ for 10 min at room temperature). Outer membranes were separated from the soluble fraction by centrifugation ($45,000 \times g$ for 90 min at 5°C) and washed once with deionized water. Proteins of the outer membrane fraction, solubilized in 1% Triton X-100 at 32°C for 4 h, and of the *N*-laurylsarcosine-soluble fraction were separated by SDS-PAGE according to the method of Laemmli (1970) using 6% stacking and 12% separating gels and were visualized by staining with Coomassie Blue R-250.

Chemicals

Buffers (Hepes, Pipes, Tris), CAS, cAMP, EDDHA, and hexadecyltrimethylammonium bromide (used in CAS agar) were purchased from Sigma (St. Louis, Mo., USA).

Results

Isolation of a visibly luminous variant of *V. fischeri* ES114

Spontaneous, visibly luminous variants arose in an SWT agar slant culture of the non-visibly luminous *V. fischeri* ES114 during prolonged storage of the culture. When initially sampled, the culture gave rise only to colonies of the original, non-visibly luminous form of ES114. After several months of storage, however, the culture was found to give rise also to colonies of the visibly luminous variant. With repeated sampling of the culture over time, the proportion of the variant appeared to increase as the culture aged, up to a point at which the culture lost viability.

Taxonomic and DNA-based analyses demonstrated that the variant was *V. fischeri* and that the variant and the original form were very similar. Specifically, no differences between the two forms were found in taxonomic traits that distinguish *V. fischeri* from other species of luminous bacteria (Allen and Baumann 1971; Boettcher and Ruby 1990; Dunlap and Callahan 1993) including: synthesis of *V. fischeri* luminescence autoinducer; presence of a tuft of sheathed polar flagella; production of lipase; inability to grow at 4°C ; ability to utilize cellobiose, mal-

tose, mannitol, and cAMP, and inability to utilize gluconate and lactate as sole carbon and energy sources; except that the variant was unable to grow at 35°C . Furthermore, both forms exhibited a large cryptic plasmid, a trait thought to be specific for *V. fischeri* symbiotic with *Euprymna scolopes* (Boettcher et al. 1990). The DNA mol% G+C values of the original form and the variant were identical, and their DNAs hybridized to each other at a very high level (Table 1).

The variant and the original form generally were stable under routine laboratory culture. Occasionally, however, apparent interconversions of the two forms were observed; cultures of the variant strain gave rise to weakly luminous, large, pale colonies, and cultures of the original form gave rise to visibly luminous, slow-growing, yellow-pigmented colonies. The conditions necessary to force the conversions to occur, however, have not yet been elucidated.

Regulation of luminescence

Luminescence in the variant and the original form exhibited a pattern of expression characteristic of autoinduction in *V. fischeri* (Nealson 1977; Rosson and Nealson 1981). Upon inoculation of cells into ASH broth, in which the variant grew at one-fourth the rate of the original form,

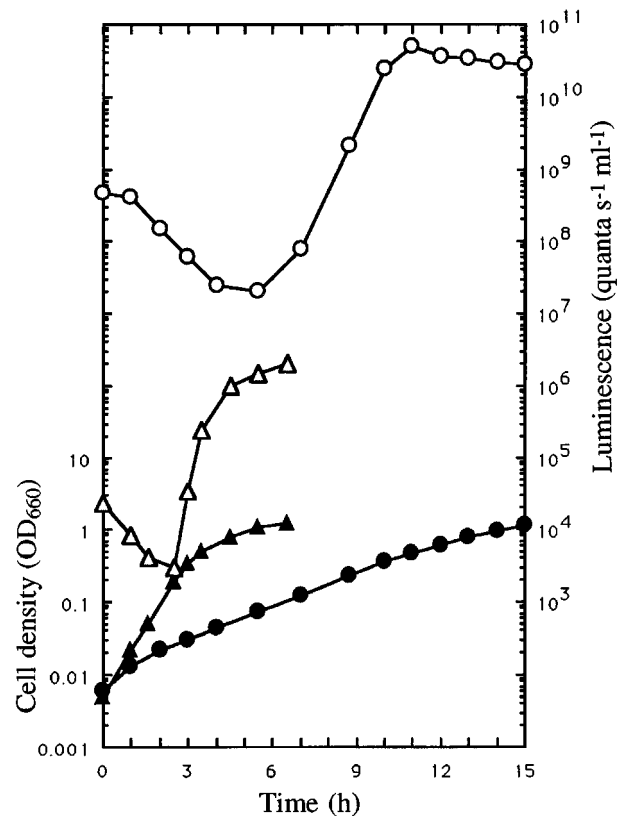


Fig. 1 Growth and luminescence of the variant and original form of *Vibrio fischeri* ES114 (circles variant, triangles original form, filled symbols cell density, open symbols luminescence)

Table 1 Mol %G+C and DNA homology of the variant and original form of *Vibrio fischeri* ES114

Strain	% G+C ^b	% Homology of reference DNA from strain ^a		
		ES114 Variant	ES114 Original	MJ-1
ES114 Variant	38.9	100	92.5	78.5
ES114 Original	38.9	92.5	100	82.7
MJ-1	38.7	78.5	82.8	100

^aAverage of five to eight replicates

^bAverage of three replicates

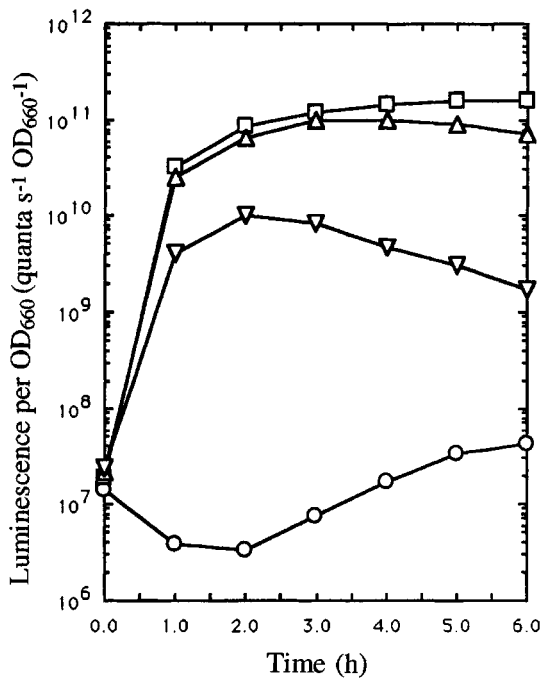


Fig. 2 Bioassay for autoinducer synthesis by the variant and original form of *Vibrio fischeri* ES114. The luminescence response of the bioassay strain *Escherichia coli* PD100 containing pPD749 and pJR551 (Dunlap and Ray 1989) to unconditioned medium (open circles), and to medium conditioned by the ES114 original form (inverted open triangles), the ES114 variant (open triangles), and *V. fischeri* MJ-1 (open squares)

light levels produced by both strains declined during the first 3–5 h of growth and then induced to levels 1,000-fold greater than the uninduced levels (Fig. 1). Compared to the original form, luminescence of the variant was approximately 30,000-fold higher, at a level approximately 15-fold less than that produced by the strongly luminous *V. fischeri* strain, MJ-1. Furthermore, induction of luminescence occurred at a substantially lower OD_{660} in the variant (Fig. 1). Consistent with the variant's higher production of light and lower OD_{660} at induction, medium conditioned by the variant contained substantially more autoinducer, nearly as much as that detected in medium conditioned by MJ-1 (Fig. 2). Since growth rate is generally inversely related to light production and autoinducer synthesis in *V. fischeri* (Dunlap and Greenberg 1991; Dunlap 1992), the unusually rapid growth of the original form (Fig. 2) might account in part for its levels of luminescence and autoinducer lower than that of the slower-growing variant.

The variant and the original form responded similarly to factors that regulate luminescence in *V. fischeri* and did so in the manner described for *V. fischeri* MJ-1 (Dunlap and Greenberg 1991). In both strains, the addition of autoinducers (via conditioned medium) shortened the lag before induction of luminescence, the presence of glucose delayed induction while enhancing growth, the presence of the iron chelator EDDHA restricted the growth rate and led to induction of luminescence at a lower OD_{660} , and the

addition of cAMP enhanced luminescence by a small amount (Fig. 3). These results are partially at variance with results of an earlier report on the original form in which glucose and EDDHA, atypically for *V. fischeri*, had little or no influence on induction of luminescence (Boettcher and Ruby 1990). The variance might be due to methodological differences or to physiological variability in strain ES114.

Cell morphology of the variant

Cells of the variant were significantly smaller than original-form cells (Table 2), with the size difference most readily seen in cells that had just divided (Fig. 4). With respect to flagellation, a substantial percentage of the variant cells, 27% compared to 4% for the original form, lacked flagella. Furthermore, 95% of the variant cells that did contain flagella (73% of the 200 cells examined) exhibited only one or two polar, sheathed flagella, whereas 91% of the original form cells that were flagellated (96% of the 200 cells examined) exhibited a tuft of 3 or more polar, sheathed flagella (Table 2; Fig. 5) typical of *V. fischeri* (Allen and Baumann 1971). Possibly consistent with a reduced torque due to fewer flagella and with a slower growth rate, cells of the variant migrated less rapidly through viscous media than cells of the original form (Table 2).

Altered response of the variant to glycerol

Growth of *V. fischeri* in complete media containing glycerol typically leads to a decrease in the pH of the medium (Hill 1928; Giese 1943; P. V. Dunlap, unpublished observation). Growth of the ES114 original form and strain MJ-1 led to a decrease in the pH of ASH broth (40 mM glycerol), despite the presence of buffer (50 mM HEPES). In contrast, growth of the variant led to an increase in the pH of this medium (Table 2). The difference between the variant and original form in affecting the pH of the medium was observed also for cells grown in LBS and SWT broth, which also contained glycerol. Presence of glycerol was necessary for this difference; growth of the variant, original form and MJ-1 led to a gradual increase in the pH of ASH broth prepared without glycerol. These results suggest that the variant is altered in its metabolism of glycerol. The variant was not altered, however, in its ability to ferment glucose; growth of all three strains led to a sharp decrease in the pH of ASH-broth-containing glucose (data not shown), and all three strains gave strong positive reactions characteristic of *V. fischeri* in fermentation-indicator medium containing glucose. Furthermore, cultures of the variant in ASH broth containing glycerol, in which the pH was increased, retained viability for several days, whereas those of the original form, in which the pH was decreased (Table 2), sometimes exhibited a complete loss of viability in this time.

Fig. 3 Effects of autoinducer, ethylenediamine-di(*o*-hydroxyphenyl)-acetic acid (EDDHA), glucose, and cAMP on luminescence of the variant and original form of *Vibrio fischeri* ES114. Variant (upper set of symbols), original form (lower set of symbols). Open circles No addition, open squares autoinducer added (as conditioned medium, mixed one-to-one with fresh medium), open diamonds glucose added (to 10 mM), inverted open triangles EDDHA added (to 10 μ M), open triangles cAMP added (to 10 mM)

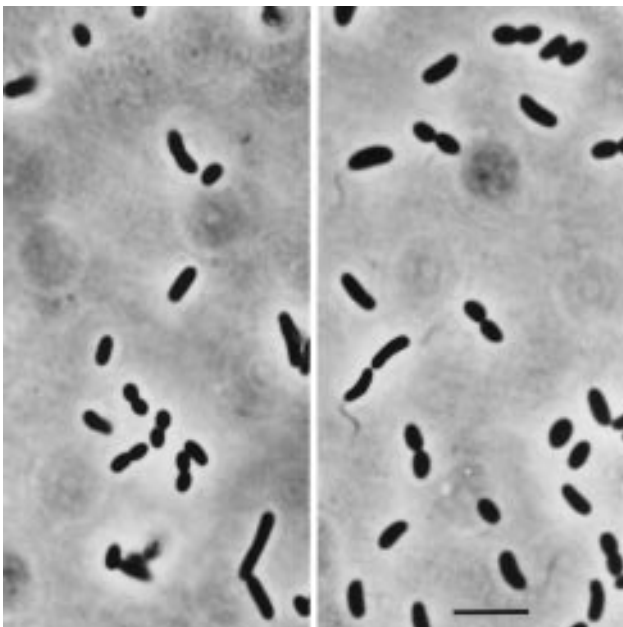
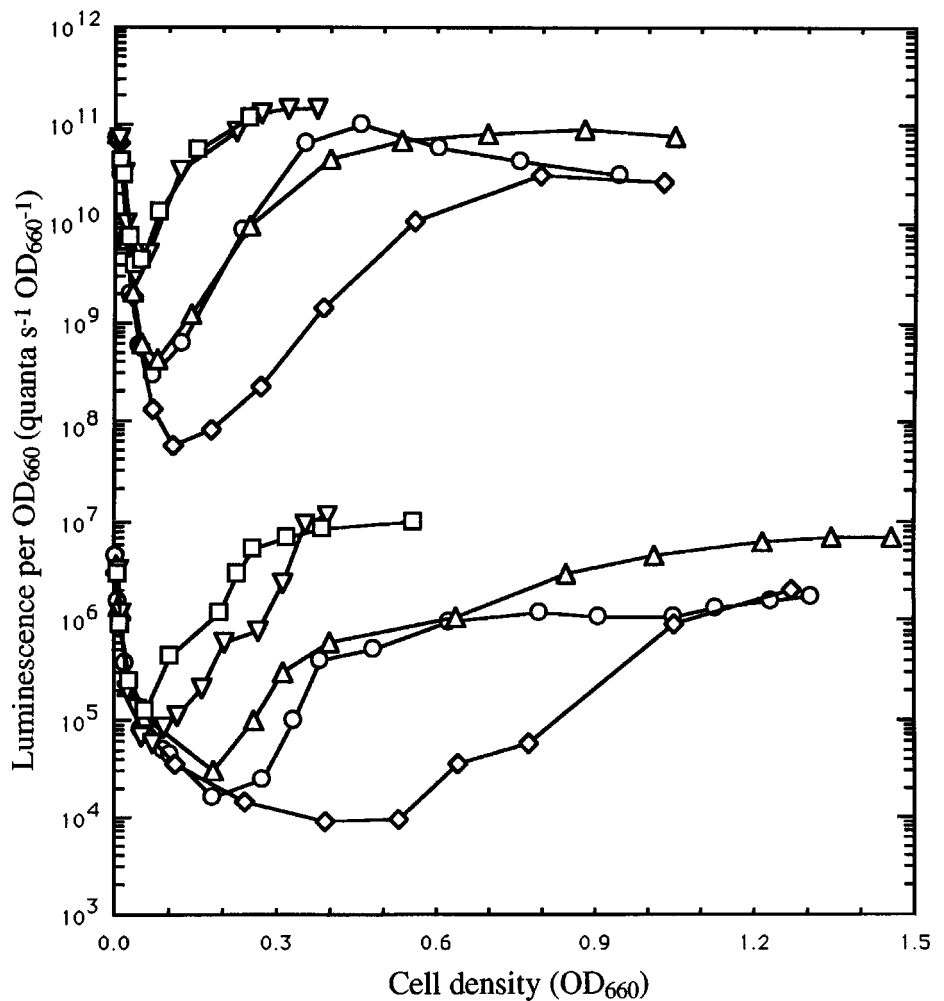


Fig. 4 Comparison of cell sizes of variant and original forms of *Vibrio fischeri* ES114. Left panel Variant, right panel original form. Bar 5 μ m. The difference in cell size was seen most readily in cells that just completed division

Pigmentation

V. fischeri produces a distinctive yellow-orange pigment, possibly riboflavin (Doudoroff 1938; Giese 1943; Baumann et al. 1984). Colonies of the ES114 variant on LBS or SWT agar plates were a deep yellow-orange, similar in color to colonies of *V. fischeri* MJ-1. Colonies of the original form, however, were substantially less yellow-orange on LBS agar and generally were pale on SWT agar (Table 2). The pigmentation difference between the variant and original form was observed also for cells grown in ASH, LBS, and SWT broth. Previous studies have shown that loss of the pigment, or failure to produce it, and loss of viability of the culture correlate with decreases in pH of the medium during growth on complete media containing glycerol or glucose; these effects apparently relate to excretion of organic acids and sensitivity of *V. fischeri* to the resulting low pH (Hill 1928; Hill and Shoup 1929; Giese 1943; Ruby and Neilson 1977; P. V. Dunlap, unpublished observation).

Analysis of proteins

The variant exhibited fewer or lower levels of some outer membrane proteins (Fig. 6A), as also demonstrated for



Fig. 5 Flagellation of the variant and original form of *Vibrio fischeri* ES114 (upper panel variant, lower panel original form). For the variant, 54 of 200 cells examined (see Materials and methods) lacked flagella, 139 had one or two flagella, and 7 had a tuft of three or more flagella. For the original form, 9 of 200 cells examined lacked flagella, 18 had one or two flagella, and 173 had a tuft of three or more flagella. Bar 1 μM

antibiotic resistance mutants of *Photobacterium phosphoreum* (Lümmen and Winkler 1986). Consistent with a difference in the surface of the cell, and possibly reflecting a greater sensitivity to oxygen leading to cell clumping, the variant also developed a more distinct pellicle in static broth culture than the original form (Table 2). Besides outer membrane proteins, differences between the variant and original strain were also observed for several *N*-lauroylsarcosine-soluble proteins (Fig. 6B).

Table 2 Traits distinguishing the variant and original form of *Vibrio fischeri* ES114^a

Trait	Variant	Original Form
Growth at 35°C	–	+
Doubling time (h)	2	0.5
Luminescence (quanta s ⁻¹ ml ⁻¹) ^b	3 × 10 ¹⁰	1 × 10 ⁶
OD ₆₆₀ at luminescence induction	0.08	0.18
Autoinducer synthesis	High ^c	Low
Cell size (approximate, in μm) ^d	1.0 × 1.5	1.2 × 1.7
Flagellation	0–2 polar	3–8 polar
Migration rate (mm h ⁻¹) ^e	0.5–0.8	4.0–5.0
Yellow-orange pigment ^f	+++	+
pH change of medium ^g	7.5–7.6	4.9–5.0
Pellicle formation ^h	+++	+

^a The two forms also differed in outer membrane and *N*-lauroylsarcosine-soluble proteins (Fig. 6); see text

^b At OD₆₆₀ of 1.0

^c Similar to *V. fischeri* MJ-1

^d For freshly divided cells

^e In LBS plates prepared with 0.2% agar

^f On LBS or SWT agar plates at peak intensity (variant at 4–6 days, original form at 1–2 days)

^g In ASH broth (40 mM glycerol, initial pH 7.25), after 48 h

^h In static broth culture

Miscellaneous attributes

No significant difference between the two forms was found in the level of periplasmic 3':5'-cyclic nucleotide phosphodiesterase, which has been hypothesized to play a key role in cellular interactions between *V. fischeri* and its animal hosts (Dunlap and Callahan 1993). Furthermore, no obvious differences were noted between the two forms in the blue-to-orange color change of CAS marine agar or in the extent of bubbling upon the addition of hydrogen peroxide to colonies on LBS plates, indicating that mutations in genes for siderophore production (Schwyn and Neilands 1987) or in the *rpoS* gene (Zambrano et al. 1993), respectively, are not likely to account for the differences between the variant and original form.

Discussion

This study describes a distinct physiological, morphological and biochemical dimorphism in *V. fischeri* colonizing light organs of the coastal Hawaiian squid *E. scolopes*. From a laboratory stock culture of the representative squid-symbiotic strain ES114, a spontaneous variant was isolated that exhibited several traits distinct from the original strain and more similar to *V. fischeri* cells in the symbiosis, including the production of visible light, slower growth, a reduced number of flagella, and smaller cell size. The similarity of the variant to *V. fischeri* cells in the symbiosis suggests that the variant, atypically for *E. scolopes* symbionts in laboratory culture, expresses the phenotype of cells in the symbiotic state. The similarity is approximate, however; *V. fischeri* cells in the squid light organ

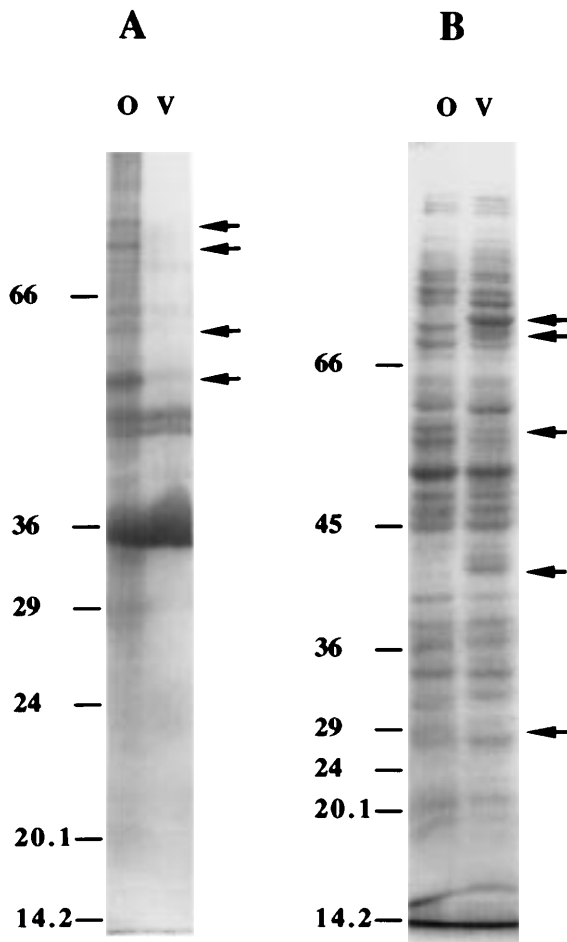


Fig. 6A, B Protein profiles of the variant and original form of *Vibrio fischeri* ES114. **A** Outer membrane proteins and **B** *N*-lauroylsarcosine-soluble proteins of the original form (O) and the variant (V). Lanes were loaded with equal amounts of total protein. Protein molecular mass standards (in kDa): bovine serum albumin (66), ovalbumin (45; not used in A), glyceraldehyde-3-phosphate dehydrogenase (36), carbonic anhydrase (29), trypsinogen (24), trypsin inhibitor (20.1), and α -lactalbumin (14.2)

grow much more slowly than the variant in culture, and they almost completely lack flagella (Ruby and Asato 1993; Table 2). Nonetheless, the distinctive nature of the dimorphism in culture may parallel the pleiotropic conversion of *V. fischeri* upon its colonization of the animal light organ. As suggested for the similarly strongly dimorphic *Photobacterium luminescens* (Smigielski et al. 1994), the ES114 variant form may be better adapted to the symbiotic state, and the original form better adapted to free-living existence.

The variant most likely did not originate by contamination of the original ES114 culture, either by another species of luminous bacteria or by another strain of *V. fischeri*. Taxonomic analysis demonstrated that the variant is *V. fischeri*, as supported by the identical mol% G+C contents of the variant and original form, which closely matched that of the well-studied *V. fischeri* strain, MJ-1. Furthermore, DNA of the variant and original form exhib-

ited greater than 92% homology, a level well above the 70–80% homology characteristic of two strains of the same species (Johnson 1984; Sneath 1984). The variation in results inherent to DNA hybridization methodology (Boemare et al. 1993) (see Materials and methods) most likely accounts for the less than 100% homology and precludes knowing with absolute certainty if the two strains are identical. Small and localized changes in the genome of a strain, through an inversion, a deletion, or the insertion of a plasmid, probably would not be detected with this method. Nonetheless, the method is sensitive enough to distinguish the variant and original form of ES114 from MJ-1 (Table 1). Moreover, the variant is phenotypically distinct from all other *V. fischeri* strains we have handled, including strains recently isolated from the squid's habitat (Lee and Ruby 1994).

Most likely, the variant arose by a conversion of original-form cells during the prolonged incubation of the original culture, with the proportion of variant to original form increasing over time as more of the original-form cells either converted or died. Preliminary results suggesting interconversion of the two forms in laboratory culture are consistent with this interpretation, which implies a selective advantage for the variant under the conditions developing over time in the tube containing the original ES114 culture. The conditions important for this conversion (e.g., darkness, desiccation, nutrient deprivation, etc.), however, have not yet been identified. Methods to select separately for the variant and original form, which would permit quantitative analysis of the conversion, may be necessary to identify those conditions. In this regard, Giese (1943), who studied a possibly similar conversion of dim to bright strains of *V. fischeri* (*Achromobacter fischeri*), found differences between the strains in tolerance to and utilization of acids produced during growth on glycerol-containing media. It is possible, therefore, that differences in the response to acids could provide a selective advantage to the ES114 variant during storage of the original culture. Whether such differences in acid tolerance or utilization relate to the conversion of the original form to the visibly luminous, slow-growing, non-flagellated form observed in the squid light organ is an intriguing, but not yet obvious issue.

Other luminous bacteria (e.g., *Vibrio harveyi*, *P. leiognathi*, *P. phosphoreum*) spontaneously produce dim or bright variants in culture (Nealson and Hastings 1979), and in some cases those occurring as luminescent symbionts of animals have been reported to exhibit a dimorphism in some ways similar to, but generally less pronounced than that seen for *V. fischeri* symbiotic with *E. scolopes* (Nealson 1979; Tebo et al. 1979; Dunlap 1984). As with the ES114 variant and original form, a particularly distinct dimorphism is seen with *P. luminescens*, which is symbiotic with entomopathogenic nematodes (Bleakley and Nealson 1988; Boemare et al. 1993). However, the conditions and mechanisms controlling variant formation in culture and the pleiotropic conversion of cells in ecological transitions between the symbiotic and free-living states at present are not understood (Doudoroff 1938; Giese 1943;

Nealson and Hastings 1979; Bleakley and Nealson 1988; Silverman et al. 1989).

The pleiotropic nature of the differences between the ES114 variant and original form, including the expression of several membrane-associated and soluble proteins, indicates the involvement and differential expression of several genes. The pleiotropic nature of the differences also would be consistent with a single regulatory element controlling the conversion from one form to the other, as proposed for conversions between primary- and secondary-form variants of *P. luminescens* (Bleakley and Nealson 1988). This regulatory element presumably would be involved in controlling the pleiotropic conversion of *V. fischeri* cells in their transitions between the free-living and symbiotic states, and a mutation in it, one that prevents cells of the variant from converting back to the original form, could account for the stable occurrence of the variants in the original ES114 culture. Identifying this element, consequently, would provide substantial insight into the molecular basis for variant formation in culture and the pleiotropic conversion of bacteria in nature. Regardless of this possibility, however, the variant and its conversion from the original form in laboratory culture may provide insight into the properties of *V. fischeri* in the symbiotic state and serve as a useful model system for elucidating the mechanism controlling its pleiotropic conversion upon colonization of the squid.

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