TAXONOMY OF THE MARINE,

LUMINOUS BACTERIA

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ABSTRACT

One hundred and seventy-three strains of marine, luminous bacteria isolated from sea water, surfaces and intestines of fish, as well as from the luminous organs of fish and squid were submitted to an extensive phenotypic characterization. A numerical analysis of the results grouped these strains into four clusters which were formed on the basis of overall phenotypic similarity. One cluster, which was given the designation Beneckea harveyi, consisted of strains which had a moles % GC content in their DNAs of 46.5 ± 1.3 and a single, sheathed, polar flagellum when grown in liquid medium. Most of these strains had unsheathed, peritrichous flagella in addition to the sheathed, polar flagellum when grown on solid medium. The two phenotypically similar clusters which were assigned the species designations Photobacterium phosphoreum and P. mandapamensis consisted of strains which had 1-3 unsheathed, polar flagella and moles % GC contents in their DNAs of 41.5 ± 0.7 and 42.9 ± 0.5, respectively. The cluster designated P. fischeri contained strains having 2-8 sheathed, polar flagella and a moles % GC content of 39.8 ± 1.1. These four species could be further distinguished on the basis of a number of nutritional properties as well as other phenotypic traits. The assignment of the luminous, marine bacteria to four species was supported by differences in the properties of the luminous system as well as differences in the pattern of regulation of aspartokinase activity which are discussed. The species B. harveyi was found to be phenotypically similar to a number of previously characterized,

non-luminous strains of Beneckea which should probably be assigned to this species.

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ASW = artificial sea water

ATCC = American Type Culture Collection

BM = basal medium

BMA = basal medium agar

GC = guanine plus cytosine

LA = luminous medium agar

LB = luminous medium broth

MA = Difco Marine Agar

NCMB = National Collection of Marine Bacteria

PHB = poly-β-hydroxybutyrate

S = similarity coefficient

YEB = yeast extract broth

..... Admiring in the gloomy shade
Those little drops of light

Edmund Waller (1606-1687)

INTRODUCTION

Luminous bacteria are readily isolated from the marine environment, being found in sea water, the surfaces and the intestines of marine animals, and in the specialized luminous organs of certain fish and cephalopods. Although the biochemistry and physiology of bacterial

luminescence has received considerable attention (Harvey, 1952; Hastings, 1968; McElroy, 1961; Strehler, 1955), the taxonomy and ecology of these organisms has, for the most part, been neglected. Beijerinck (1889) proposed that on the basis of overall phenotypic similarity, as well as the ability to luminesce, these organisms should be placed into a single genus, Photobacterium. As more knowledge was acquired concerning facultative anaerobes and new genera were described, it became apparent that members of the genus Photobacterium shared properties with non-luminous genera. The studies of Doudoroff (1942b) showed that marine, luminous bacteria performed a mixed acid fermentation of D-glucose and that some strains of P. phosphoreum produced 2,3-butyleneglycol, properties which are characteristic of many non-luminous organisms. In addition, the electron microscopic study of P. harveyi and P. fischeri by John et al. (1943) showed that the former species had a thick, polar flagellum and thin, peritrichous flagella while the latter had tufts of polar flagella. Based on the results of these and subsequent studies, various investigators suggested that certain species be excluded from the genus Photobacterium (Breed and Lessel, 1954) or that this genus be dismembered and all of its constituent species assigned to existing genera (Spencer, 1955). A meaningful resolution of this problem has not been possible since some of the luminous species as well as the non-luminous genera to which they were assigned have been poorly characterized. A further complication in the taxonomy of these organisms has been the profusion of species designations which have

been assigned to strains after inadequate phenotypic characterizations (Buchanan et al., 1966). An examination of this problem as well as a synonymization of various names was performed by Breed and Lessel (1954). A recent study by Hendri et al. (1970) reduced to synonymy 13 specific epithets and showed that luminous bacteria could be assigned to five species. These authors recognized that "harveyi", due to its distinctive flagellation, deserved a generic placement different from the other luminous bacteria and created the genus Lucibacterium to accommodate this species. In addition, Hendri et al. (1970) described a new species, phenotypically related to P. phosphoreum, which they designated P. mandapamensis. The remaining two species were designated Vibrio fischeri and V. albensis. The single existing strain of the latter species is a fresh water isolate while L. harveyi, P. phosphoreum, P. mandapamensis, and V. fischeri are of marine origin. The type of flagellation observed in L. harveyi has been found in a number of species of marine bacteria recently characterized by Baumann et al. (1971a) and assigned to the genus Beneckea. Strains of these species when grown on solid medium have a single, thick (sheathed), polar flagellum and thin (unsheathed), peritrichous flagella; when grown in liquid medium these strains have only a single, thick (sheathed), polar flagellum (Allen and Baumann, 1971).

The present study confirms and extends the results of Hendri et al. (1970) by showing that on the basis of an extensive phenotypic analysis, 173 strains of marine, luminous bacteria isolated from sea water, marine animals, and luminous organs of fish and cephalopods

obtained from diverse geographical locations could be assigned to the four species characterized by these authors.

MATERIALS AND METHODS

The majority of the methods used in this study are those of Stanier et al. (1966) which have been adapted to the characterization of marine bacteria and previously described by Baumann et al. (1971a, 1972). Only significant modifications as well as additional methods will be described.

Bacteriological media.

The following media were employed.

Artificial sea water (ASW) (MacLeod, 1968)

0.4	M	NaCl
0.1	M	$MgSO_4 \cdot 7H_2O$
0.2	M	KCl
0.02	M	$CaCl_2 2H_2O$

Basal medium (BM)

50	mM	Tris-HCl (pH 7.5)
19	mM	NH_4Cl
0.33	mM	$K_2HPO_4 \cdot 3H_2O$
0.1	mM	$FeSO_4 \cdot 7H_2O$
1.2		dilation ASW

Basal medium agar (BM)

This was prepared by separately sterilizing and then mixing equal volumes of double strength BM and 20 g/liter Oxoid Ionagar (Colab Labs., Inc., Chicago Heights, Illinois, U.S.A.).

Luminous medium broth (LB)

5	g/liter Difco Yeast Extract
5	g/liter Difco Tryptone
0.3%	glycerol
50	mM Tris
514	mM NaCl
23	mM NH_4Cl
23	mM $K_2HPO_4 \cdot 3H_2O$
15	mM KH_2PO_4
10	mM KCl
4	mM $MgSO_4 \cdot 7H_2O$
pH	adjusted to 7.2

Solid luminous medium (LA)

LB was supplemented with 10 mM $CaCO_3$ and 20 g/liter Difco Agar.

Marine Agar (MA)

This complex medium is a product of Difco Laboratories, Detroit, Michigan, U.S.A.

Methods of isolation. Some of the strains characterized in this study were obtained by an enrichment method or by direct isolation from the intestines of marine fish. The enrichment method (based on

suggestions of Dr. M. Doudoroff) involved partial immersion of fish or squid in ASW and an overnight incubation at 15°C. The luminescent growth which developed was streaked onto LA and incubated at 15°C. Luminous bacteria were isolated from the intestines of fish by streaking the intestinal contents onto LA and incubating at 15°C. Special precautions were taken in the dissections to prevent the contamination of the intestine with flora from the fish surface. The luminous colonies which appeared after one or two days incubation at 15°C were further purified by streaking onto LA and incubating at 25°C. Only one isolate from each fish was included in this study. Many of the strains received from other investigators had been isolated from luminous organs or the surfaces of fish and squid as well as from sea water using the methods described by Haneda (1966), Hastings and Mitchell (1971) and Nakaji and Kim (1968). With the exception of strain 383 (Photobacterium sepia, ATCC 15709), which failed to luminesce, the designation "luminous bacteria" as used in this study is applied only to those strains which were observed to luminesce on LA. This medium was chosen since luminescence was consistently better and persisted for a longer time on LA than on MA or YEB solidified with 2% Difco Agar.

In a limited number of ecological studies, samples of sea water were filtered through 0.22 μm filters (Millipore Corp., Bedford, Mass., U.S.A.) and the filters placed into 5 ml of half-strength ASW containing 50 mM Tris-HCl (pH 7.5). After vigorous agitation in a Vortex Mixer, measured aliquots were spread onto LB solidified with 4% Difco Agar, in order to prevent the overgrowth of the plate with swarming organisms (Nakaji and Kim, 1968), and incubated at 25°C. The

luminous colonies were subsequently purified on LA and tested for a selected number of diagnostic traits (Table 4).

Sources of strains examined. The strains characterized in this study have numerical designations of 331 to 503. In the strain list, the strain number is followed by the following information enclosed in parentheses: (1) method of isolation (if known), (2) source of isolation (if known), (3) geographic source of isolation (if known), (4) investigator who isolated strain → investigator from whom strain was received (if unstated, strain was isolated in this study), (5) designation under which strain was received. The following abbreviations have been used: E (enrichment), F (direct isolation from surfaces of marine animals), I (intestines of marine fish), S (symbionts from luminous organs), SW (direct isolation from sea water), H (Dr. J. W. Hastings), T (Dr. A. Thanos), and YH (Dr. Y. Haneda). With the exception of strains designated by ATCC numbers, all other strain numbers refer to the previous work of Baumann et al. (1971a, 1973). It should be noted that the strain numbering system (for strains 331-491) is one which groups together strains of high overall phenotypic similarity, irrespective of their origin, and was established post facto on the basis of a numerical analysis which is discussed later.

Strains assigned to Beneckea harveyi. 331 (F, grunt, Bermuda, H, BG 1-2); 332 (F, grunt, Bermuda, H, BG 3-4); 333 (SW, New Guinea, H, SH 1); 334 (SW, Israel, H, Ei 2); 335 (SW, Israel, H, Ei 14); 336 (H, PJ 2B); 337 (SW, Massachusetts, USA, H, AG 8); 338 (SW, Massachusetts, USA, H, MUD); 339 (SW, Israel, H, Ei 3); 340 (SW, Puerto Rico, H, PR 2); 341 (SW, Puerto Rico, H, PR 7-1); 342 (SW, Puerto Rico, H, PR 17-3); 343

SW, Israel, H, Ei 1); 344 (H, PP); 345 (Israel, H, Ei 4); 346 (SW, Puerto Rico, H, PR 10-1); 347 (SW, Puerto Rico, H, PR 10-3); 348 (F, fish, Puerto Rico, H, PR 11-1); 349 (F, Puerto Rico, H, PR 11-2); 350 (SW, Puerto Rico, H, PR 5); 351 (SW, Puerto Rico, H, PR 7-2); 352 (SW, Salton Sea, California, USA, Nakaji, DI-3); 353 (SW, Puerto Rico, H, PR 3); 354 (SW, Puerto Rico, H, PR 6); 355 (SW, Puerto Rico, H, PR 17-2); 356 (F, grunt, Bermuda, H, BG 3-3); 357 (F, grunt, Bermuda, H, BG 5); 358 (F, shad, Bermuda, H, BS 1); 359 (F, hogfish, Bermuda, H, BH 4); 360 (H, PJ 1D); 361 (SW, Portugal, H, P 4); 362 (F, hogfish, Bermuda, H, BH 1); 363 (SW, Massachusetts, USA, H, NO_3 1); 364 (F, grunt, Bermuda, H, BG 4); 365 (F, grunt, Bermuda, H, BG 3-2); 366 (SW, Massachusetts, USA, H, NO_3 3); 367 (F, hogfish, Bermuda, H, BH 2-1); 368 (SW, Portugal, H, P 6); 369 (F, shad, Bermuda, H, BS 2); 370 (F, grunt, Bermuda, H, BG 1-1); 371 (F, hogfish, Bermuda, H, BH 2-2); 372 (F, hogfish, Bermuda, H, BH 3); 373 (F, grunt, Bermuda, H, BG 2-1); 374 (F, grunt, Bermuda, H, BG 2-2); 375 (SW, Portugal, H, P 3); 376 (F, squid, California, USA, T, H 2); 377 (SW, Portugal, H, P 1); 378 (SW, Massachusetts, USA, H, ND 2); 379 (SW, Massachusetts, USA, H, ND 3); 380 (SW, Massachusetts, USA, H, ND 1); 381 (H, AF); 382 (H, AF M3); 383 (Photobacterium sepia ATCC 15709); 384 (P. harveyi ATCC 14126); 385 (Johnson, Achromobacter harveyi); 386 (SW, Portugal, H, P 2); 387 (SW, Portugal, H, P 7); 388 (SW, Massachusetts, USA, H, Nah); 389 (H, DF 2R); 390 (SW, Massachusetts, USA, H, W 18); 391 (SW, Massachusetts, USA, H, W 14); 392 (H, MAV); 393 (H, DF 2); 492 (Lucibacterium harveyi NCMB 24); 493 (L. harveyi NCMB 42).

Strains assigned to Photobacterium fischeri. 394 (T, H 4); 395 (P. pierantonii ATCC 14546); 396 (SW, Salton Sea, California, USA, Nakaji, BI-2); 397 (F, rock fish, California, USA, T, H 9); 398 (P. fischeri ATCC 7744); 399 (F, squid, Johnson, Achromobacter fischeri). Strains 61 to 66 have been previously described (Allen and Baumann, 1971; Baumann et al., 1971a).

Strains assigned to Photobacterium phosphoreum. 400 (E, Decapterus pinnulatus, Hawaii); 401 (E, Abula vulpes, Hawaii); 402 (I, Decapterus pinnulatus, Hawaii); 403 (E, Elops hawaiensis, Hawaii), 404 (S, Paratrychthus prosthenicus, Japan, YH → H, PPr-B); 405 (E, Priacanthus cruentatus, Hawaii); 406 (E, Decapterus pinnulatus, Hawaii); 407 (E, sardine, Hawaii); 408 (F, jack mackerel, California, USA, T H 7); 409 (I, Pristipomoides sieboldii, Hawaii); 410 (E, channel catfish, Hawaii); 411 (E, Mulloidichthys pflugeri, Hawaii); 412 (E, mullet, Hawaii); 413 (I, Pristipomoides microlepsis, Hawaii); 414 (E, Pristipomoides microlepsis, Hawaii); 415 (I, Epinephelus quernus, Hawaii); 416 (E, Pristipomoides sieboldii, Hawaii); 417 (I, Mulloidichthys samoensis, Hawaii); 418 (H, PPr-D); 419 (E, Parupeneus porphyreus, Hawaii); 420 (E, Acanthuris dussemieri, Hawaii); 421 (E, Trachurops crumenophthalmus, Hawaii); 422 (E, Naveta sp., Hawaii); 423 (E, Scarus sp., Hawaii); 424 (E, Pristipomoides microlepsis, Hawaii); 425 (E, Elops hawaiensis, Hawaii); 426 (E, Ablennes hians, Hawaii); 427 (E, Abula vulpes, Hawaii); 428 (E, Trachurops crumenophthalmus, Hawaii); 429 (I, Myripristis berndti , Hawaii); 430 (E, Mulloidichthys samoensis, Hawaii); 431 (Parupeneus porphyreus, Hawaii); 432 (I, Trachurops crumenophthalmus, Hawaii); 433 (F, tuna, California, USA, T, H 8); 434 (E, Scorpaenopsis

cacopsis, Hawaii); 435 (S, luminous fish organ, Japan, T, H 1); 436 (E, Trachurops crumenophthalmus, Hawaii); 437 (E, moray eel, Hawaii); 438 (S, Steindachneria argentea, Mississippi, USA, YH → H, SA 2B); 439 (P. phosphoreum ATCC 11040); 440 (E, squid, Hawaii); 441 (F, squid, Japan YH → H, H 2); 442 (E, moray eel, Hawaii); 443 (Shewan → H, P. phosphoreum PP-N); 444 (E, perch, Hawaii); 445 (E, Scarus sp., Hawaii); 446 (E, Kyphosus cinerascens, Hawaii); 447 (E, Abudefduf abdominalis, Hawaii); 448 (I, Abudefduf abdominalis, Hawaii); 449 (SW, Spain, T, H 3); 450 (E, Etelis marshi, Hawaii); 451 (E, Myripristis berndti, Hawaii); 452 (S, luminous fish organ, Japan, YH → H, PJ 3B); 453 (E, snapper, Hawaii); 454 (E, squid, Hawaii); 455 (E, Etelis marshi, Hawaii); 456 (I, Pristipomoides sieboldii, Hawaii); 457 (Hawaii, Mower, BBw); 458 (E, Caranx mate, Hawaii); 459 (Hawaii, Mower, BBs), 460 (E, Mulloidichthys samoensis, Hawaii); 461 (F, Mulloidichthys samoensis, Hawaii, Mower, Weke); 462 (Hawaii, Mower, BBm); 463 (S, luminous fish organ, Japan, YH → H, PJ 1B); 464 (S, Leiognathus splendens, New Guinea, H, SPC); 465 (F, fish, Japan, YH → H, CJ 1B); 494 (F, squid, Doudoroff, P. phosphoreum); 495 (Johnson, P. phosphoreum); 496 (F, squid, Nakamura, P. phosphoreum); 497 (P. phosphoreum NCMB 62); 498 (P. phosphoreum NCMB 67); 499 (P. phosphoreum NCMB 61); 500 (P. phosphoreum NCMB 68); 501 (P. phosphoreum NCMB 60).

Strains assigned to Photobacterium mandapamensis. 466 (Cormier → H, DAG); 467 (S, Lutjanus rivulatus, Japan, YH → H, LR B); 468 (S, Lutjanus rivulatus, Japan, YH → H, LR D); 469 (S, Equulites novaehollandiae, New Guinea, H, NG 4); 470 (I, Polydactylis sexfilis, Hawaii); 471 (I, Epinephelus quernus, Hawaii); 472 (I, Pristipomoides

sieboldii, Hawaii); 473 (E, Polydactylis sexfilis, Hawaii); 474 (E, squid, Hawaii); 475 (I, Pristipomoides sieboldii, Hawaii) 476 (S, Loligo sp., Moreton Bay, Australia, YH → H, SQ ID); 477 (P. mandapamensis NCMB 391); 478 (I, Polydactylis sexfilis, Hawaii); 479 (E, Scorpaenopsis cacopsis, Hawaii); 480 (SW, Indonesia, YH → H, H 3B, ATCC 27561); 481 (SW, Indonesia, YH → H, H 3D); 482 (S, Siphamia sp., Indonesia, YH → H, 22); 483 (S, Loligo sp., Moreton Bay, Australia, SQ 1); 484 (SW, Indonesia, YH → H, H 4B); 485 (SW, New Guinea, H, SH 21); 486 (F, squid, Philippines, YH → H, H 1); 487 (S, pony fish, New Guinea, H, L 4D); 488 (S, Equula equula, New Guinea, H, NG 6); 489 (SW, Indonesia, YH → H, H 4D); 490 (S, Steindachneria argentea, Mississippi, USA, YH → H, SA 1); 491 (S, pony fish, New Guinea, H, L 4); 502 (S, Equulites novaehollandiae, New Guinea, H, BA); 503 (S, Equula equula, New Guinea, H, NG 5).

Maintenance of strains. The strains were maintained on LA slants and transferred monthly. Strains assigned to Beneckea harveyi, Photobacterium fischeri, and P. mandapamensis lost viability much more rapidly at 4°C than at 20-22°C and were maintained at the latter temperature. Conversely, strains assigned to P. phosphoreum lost viability more rapidly at 20-22°C and were maintained at 4°C.

Temperature of cultivation. Unless otherwise stated, strains 495 and ATCC 15381 were incubated at 15°C and all the remaining strains at 25°C. The ability to grow at different temperatures was tested in LB using the methods previously dscribed (Baumann et al., 1971a).

Growth factor requirements and the utilization of carbon compounds. Strains which were unable to grow in BM containing 0.2% D-glucose were

tested for growth in a medium consisting of BM, 0.2% D-glucose, 5 g Difco Vitamin-free Casamino Acids, 20 mg L-tryptophan, 40 mg L-methionine, and 40 mg L-cysteine per liter. The specific amino acid requirements of the strains which grew on the latter medium were determined by replica plating onto BMA containing 0.2% D-glucose and five mixtures of filter-sterilized amino acids, each amino acid at a concentration of 20 mg/liter. The amino acid mixtures consisted of L-glutamate, L-arginine, and L-proline; L-aspartate, L-methionine, L-threonine, L-isoleucine, and L-lysine; L-phenylalanine, L-tyrosine, and L-tryptophan; L-serine, L-cysteine, and glycine; L-alanine, L-valine, L-leucine, and L-histidine. Strains which required more than a single amino acid were tested for growth with all possible combinations of the amino acids present in the required mixture(s). The results of these and other experiments indicated that a mixture of 10 mg/liter of L-methionine, L-arginine, L-histidine, L-leucine, L-isoleucine, and L-valine was able to meet the amino acid requirements of strains 400-402, 407, 439, 441-444, 449-463, 465, and 476. This amino acid mixture was included in BMA when these strains were tested for their ability to utilize different organic compounds as principal sources of carbon and energy and when these strains were tested for a specific sodium ion requirement. The latter test was performed as previously described (Baumann et al., 1971a) in media containing 0.2% glycerol; a few strains which were not able to utilize glycerol were tested in media containing 0.2% D-glucose. Strains 398, 399, 492, 494, 495, 497, 499-503, and ATCC 15382 were received after the screening for amino acid requirements was performed. Since these strains did not

give adequate growth on BMA containing 0.2% D-glucose and the mixture of amino acids which fulfilled the requirements of the previously screened strains, and since some of these strains required yeast extract, their ability to utilize organic compounds as principal sources of carbon and energy was tested on BMA supplemented with 0.1 g/liter Difco Yeast Extract. This amount was not sufficient to produce visible growth on media which lacked an added utilizable carbon and energy source. With the exception of DL-kynurenine, kynurenate and trigonelline, all the organic compounds screened by Baumann et al. (1971a) were tested for their ability to serve as sole or principal sources of carbon and energy.

Oxidase test and cytochrome spectra. The oxidase test was performed as described by Stanier et al. (1966). Strains which gave a negative or a slow oxidase reaction were retested by adding a drop of toluene prior to the addition of N,N'-dimethylphenylenediamine (Baumann et al., 1972). Differential oxidized/reduced cytochrome spectra were performed at room temperature as previously described (Stanier et al., 1966).

Cell shape, motility and the gram-stain. Morphological observations and the gram-stain were performed on all strains grown on LB as well as on representative strains grown in YEB as previously described (Baumann et al., 1971a).

Mode of flagellation. Strains in exponential phase of growth in LB and/or cells harvested from MA were stained for flagella by the Leifson method (Baumann et al., 1971a; Liefson, 1960). In addition,

representative strains were grown on LB or MA, negatively stained, and examined by means of the electron microscope (Allen and Baumann, 1971).

Reserve material. Tests for the presence of poly-β-hydroxybutyric acid (PHB) were performed by the method of Williamson and Wilkinson (1958) using 7-9 g wet weight of cells. Following digestion of the cells by 5% sodium hypochlorite for one hr at 20-22°C, the residue was collected by centrifugation, dissolved in chloroform, and PHB precipitated by the addition of acetone. The extraction by chloroform and precipitation by acetone was repeated twice, after which the PHB was dried and its identity verified by conversion to crotonic acid as described by Slepecky and Law (1960). Cells were stained with Sudan black B (Nutritional Aniline and Chemical Co., Inc., New York, U.S.A.) as described by Burdon (1946). Ultrathin sections of representative strains were prepared by the method of Ryter and Kellenberger (1958) and examined by electron microscopy (Allen and Baumann, 1971).

Fermentation of carbohydrates. The methods used have been previously described (Baumann et al., 1971a). Quantitative determinations of acetoin and/or diacetyl and 2,3-butyleneglycol in the culture media were performed by the methods of Neish (1952).

DNA base composition. Cells growing exponentially in LB were harvested by centrifugation and washed twice in half-strength ASW containing 50 mM Tris-HCl (pH 7.5). Three to four grams wet weight of cells were suspended in 30 ml of a solution containing 0.15 M NaCl, 0.1 M EDTA (pH 8.0), and 15 mg/ml lysozyme (3x crystalline, Sigma Chemical Co., St. Louis, Missouri, U.S.A.) and incubated at 37°C for 30 min. The subsequent procedures used for the isolation of DNA have

been described by Ballard *et al.* (1970). The increase in absorbance at 260 nm due to thermal denaturation of DNA in 1/10 standard saline citrate was determined using a Gilford 2400 S Spectrophotometer (Gilford Instrument Labs., Inc., Oberlin, Ohio, U.S.A.) as described by Mandel and Marmur (1968). The midpoint of the transition in the absorbance was determined simultaneously for three DNA samples, with the DNA of *P. fischeri* (strain 61) as an internal standard. The moles % GC contents of the samples relative to the moles % GC content of *P. fischeri* (strain 61) was determined from the formula of Mandel *et al.* (1970). The moles % GC content in the DNA of *P. fischeri* (strain 61) was determined relative to the values of strain 64 (*P. fischeri*), strains 68 and 70 (group B-2), and strains 113 and 115 (*B. parahaemolytica*), which had been determined previously by buoyant density measurements in caesium chloride density gradients (Baumann *et al.*, 1971a). [The values reported by Baumann *et al.* (1971a) for the moles % GC contents in the DNAs of strains 61 and 64 of *P. fischeri* were interchanged; the correct GC contents of strains 61 and 64 are 39.3 and 40.3 moles %, respectively.] A standard deviation of ± 0.1% was obtained for five determinations of the moles % GC content in the DNA of *B. harveyi* (strain 384), relative to *P. fischeri* (strain 61). An indication of the purity of the DNAs was obtained from a comparison of the values of the moles % GC contents from thermal denaturation experiments and the values computed from the ratio of the absorbance at 260 and 280 nm of DNA denatured with 0.1 M acetic acid (Fredericq *et al.*, 1961). The values determined by the latter method were generally within ± 1.5% of those obtained by thermal denaturation. Two DNA

preparations of the same strain which had deviations of +5% and -0.8% were found to have an identical response to thermal denaturation.

Enzymes of glucose and gluconate catabolism. The methods used for the cultivation of strains, preparation of cell-free extracts, and enzyme assays have been previously described (Baumann and Baumann, 1973a; Baumann et al., 1973).

RESULTS

Numerical analysis of the nutritional and physiological properties. The physiological properties of the strains studied as well as the results of a nutritional screening which tested the ability of these strains to utilize 147 carbon compounds as sole or principal sources of carbon and energy are given in Tables 1, 2, and 3. With the exception of the Voges-Proskauer reaction and the production of acetoin and/or diacetyl and 2,3-butyleneglycol, these data together with the data for strains 61-66 (Baumann et al. 1971a) were submitted to a numerical analysis by Leslie Bryant (Department of Microbiology, University of Queensland, Brisbane, Australia) with programs for a GE 225 computer. The estimation of similarity was based on the inclusion of both positive and negative characters, using the simple similarity coefficient described by Sokal and Sneath (1963). On the basis of the numerical analysis, the strains were separated into four major clusters which were designated Beneckea harveyi, Photobacterium fischeri, P. phosphoreum, and P. mandapamensis (Fig. 1). The reasons for these assignments are given in the Discussion. Additional strains, which were characterized after the numerical analysis had been performed, were assigned to B. harveyi (strains 492 and 493), P. phosphoreum (strains 494-501), and P. mandapamensis (strains 502 and 503). The data for these strains is included in Tables 1, 2, and 3 as well as in Table 4 which lists selected traits of use in differentiating the four species. The nutritional versatility of these species is shown in Fig. 2.

Table 1

Some physiological traits of *B. harveyi*, *P. fischeri*, *P. phosphor*

Trait	*B. harveyi*		*P. fischeri*		*P.*
	Percent positive strains	Positive or negative strains	Percent positive strains[a]	Positive or negative strains[b]	Percent positive strains
Gas from D-glucose	0		0		89
Voges-Proskauer reaction	8	(+): 336,371, 380,388, 389	25		97
Production of acetoin and/or diacetyl[c]	0		0		92
Production of 2,3 butyleneglycol[d]	0		0		84
Growth at 4°C	0		0		96

Table 1

of *B. harveyi*, *P. fischeri*, *P. phosphoreum* and *P. mandapamensis*

	P. fischeri		*P. phosphoreum*		*P. mandapamensis*	
ve	Percent positive strains[a]	Positive or negative strains[b]	Percent positive strains	Positive or negative strains	Percent positive strains	Positive or negative strains
	0		89	(-): 449,461-464,494, 495,501	7	(+): 477,490
371, 388,	25		97	(-): 441,449	93	(-): 471,479
	0		92	(-): 402,439, 441,449, 457,464	50	(+): 472,474-478,480-484,488-490,502
	0		84	(-): 406,413, 414,419, 429-431, 439,441, 448,449, 464	11	(+): 476-478
	0		96	(-): 464,500, 501	0	

Table 1. (Continued) Some physiogical traits of B. harveyi. P. fischeri

Trait	B. harveyi		P. fischeri		P.
	Percent positive strains	Positive or negative strains	Percent positive strains[a]	Positive or negative strains[b]	Percent positiv strains
Growth at 25°C	100		100		99
Growth at 30°C	100		92	(-): 394	85
Growth at 35°C	100		67	(-): 394,398, 399	0
Growth at 40°C	57	(-): 336-338, 356-363, 365-380, 387	0		0
Growth at 45°C	0		0		0
Nitrate to Nitrite	100		75	(-): 399	93
Oxidase[e]	100		100		7

iogical traits of B. harveyi. P. fischeri, P. phosphoreum and P. mandapamensis

	P. fischeri		P. phosphoreum		P. mandapamensis	
ve	Percent positive strains[a]	Positive or negative strains[b]	Percent positive strains	Positive or negative strains	Percent positive strains	Positive or negative strains
	100		99	(-): 495	100	
	92	(-): 394	85	(-): 408,427, 435,439, 456,496-501	100	
	67	(-): 394,398, 399	0		96	(-): 477
338, 363, 380,	0		0		0	
	0		0		0	
	75	(-): 399	93	(-): 439,449, 494,500, 501	96	(-): 491
	100		7	(+): 400,401, 420,438, 458	64	(-): 466-469, 480,482, 488-490, 502

Table 1. (Continued) Some physiological traits of *B. harveyi*. *P. fisch*

Trait	*B. harveyi*		*P. fischeri*		*P.*
	Percent positive strains	Positive or negative strains	Percent positive strains[a]	Positive or negative strains[b]	Percent positiv strains
Amylase	100		0		0
Lipase	100		92		0
Gelatinase	98	(-): 381	8	(+): 394	0
Chitinase	100		75	(-): 396,398, 399	97
Alginase	37	(+): 334-339, 343-349, 358-362, 367-369, 376,380, 383	0		0

[a]Includes strains 61-66 of *P. fischeri* which have been previously characterized (Bauma

[b]Does not include strains 61-66.

[c]Positive strains produced 0.3 to 1.3 µg acetoin and/or diacetyl per ml of culture med

siological traits of *B. harveyi*, *P. fischeri*, *P. phosphoreum* and *P. mandapamensis*

ve	*P. fischeri*		*P. phosphoreum*		*P. mandapamesis*	
	Percent positive strains[a]	Positive or negative strains[b]	Percent positive strains	Positive or negative strains	Percent positive strains	Positive or negative strains
	0		0		0	
	92		0		82	(-): 469,485, 488,491, 502
	8	(+): 394	0		0	
	75	(-): 396,398, 399	97	(-): 441,463	96	(-): 503
339, 349, 362, 369, 380,	0		0		0	

have been previously characterized (Baumann *et al.*, 1971a).

coin and/or diacetyl per ml of culture medium.

Table 1. (Continued) Some physiological traits of *B. harveyi*, *P. fisch*

[d]Positive strains produced 30 to 110 µg 2,3 butyleneglycol per ml of culture medium.

[e]Includes strains which are positive after the use of toluene.

siological traits of B. harveyi, P. fischeri, P. phosphoreum and P. mandapamensis

utyleneglycol per ml of culture medium.

e use of toluene.

Table 2

Substrates utilized by strains of B. harveyi as sole or principal sources of carbon and energy

Substrate	% of positive strains	Positive or negative strains
D-Ribose	100	
L-Arabinose	6	(+): 346, 347, 349, 492
D-Glucose	100	
D-Mannose	100	
D-Galactose	83	(-): 331-335, 339, 381, 383, 387, 393, 492
D-Fructose	100	
Sucrose	49	(-): 331-333, 336-338, 350-352, 356-377, 386, 387
Trehalose	100	
Maltose	100	
Celloibose	100	
Salicin	55	(+): 331, 332, 334, 336-339, 343-347, 349, 356-377, 492
D-Gluconate	95	(-): 378-380
D-Glucuronate	100	
N-Acetylglucosamine	100	
Acetate	100	
Propionate	100	
Caproate	42	(+): 340, 341, 343-364, 373, 374, 493
Heptanoate	100	

Table 2. (Continued) Substrates utilized by strains of B. harveyi as sole or principal sources of carbon and energy

Substrate	% of positive strains	Positive or negative strains
Caprylate	65	(-): 335-339, 376-393
Pelargonate	63	(-): 335-339, 363, 366, 372, 376-388, 392, 393, 493
Caprate	77	(-): 335-339, 380-382, 385, 387, 388, 390-393
Succinate	100	
Fumarate	100	
DL-Malate	100	
DL-Lactate	100	
DL-Glycerate	89	(-): 379, 381-385, 493
Citrate	100	
α-Ketoglutarate	98	(-): 355
Pyruvate	100	
Aconitate	98	(-): 371
Mannitol	100	
Glycerol	88	(-): 379, 381-385, 492, 493
Propanol	49	(+): 340-342, 346-349, 356-363, 365-371, 373-375, 382, 388-393
Quinate	11	(+): 333, 350-352, 378-380
Glycine	45	(+): 340-355, 362-364, 371, 378-380, 382, 384, 385, 391, 492, 493

Table 2. (Continued) Substrates utilized by strains of *B. harveyi* as sole or principal sources of carbon and energy

Substrate	% of positive strains	Positive or negative strains
L-α-Alanine	66	(-): 331, 334-339, 365, 369, 373-376, 381, 382, 386-388, 390-393
D-α-Alanine	100	
L-Serine	80	(-): 331, 333, 335, 365, 373-375, 381, 386-389, 392
L-Threonine	100	
L-Aspartate	86	(-): 340-342, 346, 347, 351, 353-355
L-Glutamate	100	
L-Arginine	86	(-): 334-339, 372, 381,382
L-Citrulline	28	(+): 331, 333-338, 342, 345, 348, 349, 378-380, 385, 388, 492, 493
L-Histidine	15	(+): 336-338, 348, 349, 356, 364, 378, 492, 493
L-Proline	100	
L-Tyrosine	100	

Table 3

Substrates utilized by P. fischeri, P. phosphoreum, and P. mandapamensis as sole or principal sources of carbon and energy

Substrate	P. fischeri		P. phosphoreum		P. mandapamensis	
	Percent positive strains[a]	Positive or negative strains[b]	Percent positive strains	Positive or negative strains	Percent positive strains	Positive or negative strains
D-Ribose	100		65	(-): 407,408,419, 420,425-431, 437,440,441, 448,450,451, 453,454,460, 462-465,497, 501	93	(-): 483,503
D-Glucose	100		100		100	
D-Mannose	92		100		100	
D-Galactose	100		99	(-): 435	100	
D-Fructose	100		100		100	
Sucrose	8	(+): 395	0		0	
Trehalose	17	(+): 394,396	0		0	
Maltose	92		99	(-): 464	0	

Table 3. (Continued) Substrates utilized by *P. fischeri*, *P. phosphoreum*, and *P. mandapamensis* as sole or principal sources of carbon and energy

Substrate	*P. fischeri*		*P. phosphoreum*		*P. mandapamensis*	
	Percent positive strains[a]	Positive or negative strains[b]	Percent positive strains	Positive or negative strains	Percent positive strains	Positive or negative strains
Cellobiose	92		0		0	
Salicin	42	(+): 395-399	0		0	
D-Gluconate	0		85	(-): 409-412,428, 431,439,465, 494,500,501	100	
D-Glucuronate	0		49	(+): 400-408,412, 427,434,435, 438,444,445, 450-462,494-496,498-501	0	
N-Acetylgluco-samine	100		100		96	(-): 503
Acetate	0		0		86	(-): 488-491
Caprate	0		0		43	(+): 466,467,470-474,476,479, 484,486,487
Succinate	100		81	(-): 428-439,500 501	82	(-): 485-487,491, 502

Table 3. (Continued) Substrates utilized by *P. fischeri*, *P. phosphoreum*, and *P. mandapamensis* as sole or principal sources of carbon and energy

Substrate	*P. fischeri*		*P. phosphoreum*		*P. mandapamensis*	
	Percent positive strains[a]	Positive or negative strains[b]	Percent positive strains	Positive or negative strains	Percent positive strains	Positive or negative strains
Fumarate	100		81	(-): 428-439,500, 501	82	(-): 485-487,491, 502
DL-Malate	8	(+): 399	20	(+): 440,441,450-454,457,461-463,465,494, 495,499	64	(-): 466-469,484, 485,487,488, 491,502
DL-Lactate	0		18	(+): 413,414,440, 444-447,464, 465,494,496, 498,499	100	
DL-Glycerate	67	(-): 396,396	92	(-): 420,440,441, 465,494,500	75	(-): 476-479,482, 488,503
Citrate	42	(+): 397-399	0		0	
α-Ketoglutarate	0		0		4	(+): 477
Pyruvate	0		0		96	(-): 478
Aconitate	17	(+): 397	0		0	

Table 3. (Continued) Substrates utilized by P. fischeri, P. phosphoreum, and P. mandapamensis as sole or principal sources of carbon and energy

Substrate	P. fischeri		P. phosphoreum		P. mandapamensis	
	Percent positive strains[a]	Positive or negative strains[b]	Percent positive strains	Positive or negative strains	Percent positive strains	Positive or negative strains
Mannitol	92		0		0	
Glycerol	100		100		100	
L-α-Alanine	8	(+): 399	26	(+): 444,450-452, 454,457-465, 494,495,497-499	61	(-): 466-471,488-491,503
L-Serine	8	(+): 399	35	(+): 439,442-444, 450-465,494-497,499,500	75	(-): 467,483,488-491,503
L-Threonine	17	(+): 396,397	5	(+): 462,494,495 497	43	(+): 466-468,470-474,478,479, 484,487
L-Aspartate	67		77	(-): 411,412,421-429,436-438, 440,441,494	86	(-): 484-487

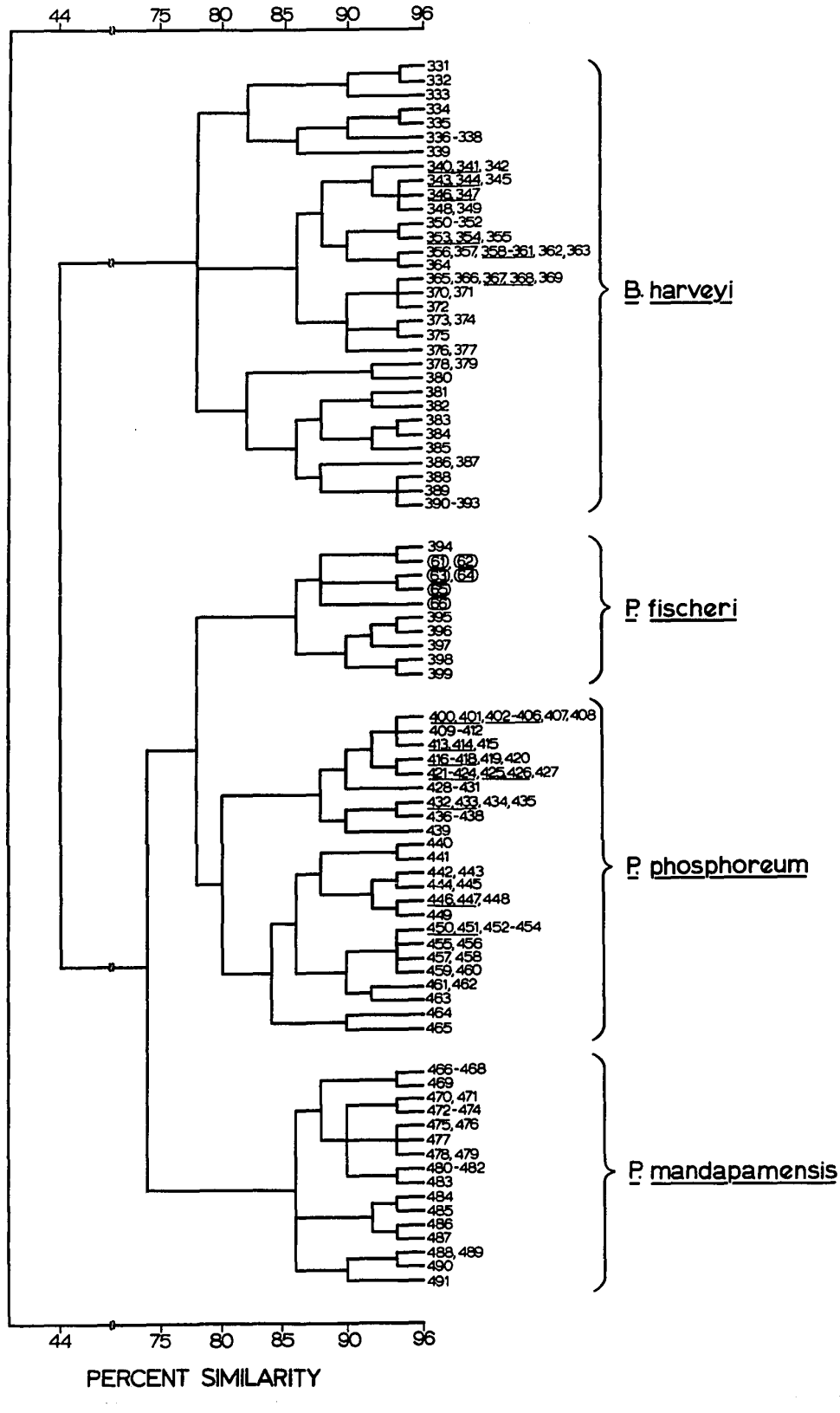
Table 3. (Continued) Substrates utilized by P. fischeri, P. phosphoreum, and P. mandapamensis as sole or principal sources of carbon and energy

Substrate	P. fischeri		P. phosphoreum		P. mandapamensis	
	Percent positive strains[a]	Positive or negative strains[b]	Percent positive strains	Positive or negative strains	Percent positive strains	Positive or negative strains
L-Glutamate	75		38	(+): 431,439,441, 443,444,446-449,452-458, 461-465,494-497,499,501	68	(-): 472,474,483-486,488,490, 491
L-Proline	100		4	(+): 461,464,465	96	(-): 502

[a]Includes strains 61-66 of P. fischeri which have been previously characterized by Baumann et al., 1971a).

[b]Does not include strains 61-66.

Fig. 1. Numerical analysis of luminous strains. Circled numbers refer to strains previously characterized by Baumann et al. (1971a). Strains which were identical in their nutritional properties are underlined.


44
75
80
85
90
96
331
332
333
334
335
336-338
339
340,341,342
343,344,345
346,347
348,349
350-352
353,354,355
356,357,358-361,362,363
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365,366,367,368,369
370,371
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373,374
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376,377
378,379
380
381
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383
384
385
386,387
388
389
390-393
B. harveyi
394
(61),(62)
(63),(64)
(65)
(66)
395
396
397
398
399
P. fischeri
400,401,402-406,407,408
409-412
413,414,415
416-418,419,420
421-424,425,426,427
428-431
432,433,434,435
436-438
439
440
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442,443
444,445
446,447,448
449
450,451,452-454
455,456
457,458
459,460
461,462
463
464
465
P. phosphoreum
466-468
469
470,471
472-474
475,476
477
478,479
480-482
483
484
485
486
487
488,489
490
491
P. mandapamensis
44
75
80
85
90
96
PERCENT SIMILARITY

Table 4

Some distinguishing properties of species of marine, luminous bacteria[a]

	B. harveyi	P. fischeri	P. phosphoreum	P. mandapamensis
Number of strains	65	12	74	28
Moles %GC	46.5 ± 1.3	39.8 ± 1.1	41.5 ± 0.7	42.9 ± 0.5
Flagellation[b]	P → Pr	P	P	P
Number of polar flagella	1	2-8	1-3	1-3
Sheathed polar flagella	+	+	-	-
Straight rods[c]	62	11	+	27
PHB-Accumulation	-	-	+	+
Growth at 4°C	-	-	71	-
Growth at 35°C	+	8	-	27
Gas from D-glucose	-	-	66	2
Amylase	+	-	-	-
Lipase	+	11	-	23
Gelatinase	64	1	-	-
Maltose	+	11	73	-
Cellobiose	+	11	-	-
D-Gluconate	62	-	63	+
Acetate	+	-	-	24
Propionate	+	-	-	-
Heptanoate	+	-	-	-
DL-Lactate	+	-	13	+

Table 4. (Continued) Some distinguishing properties of species of marine, luminous bacteria[a]

	B. harveyi	P. fischeri	P. phosphoreum	P. mandapamensis
α-Ketoglutarate	64	-	-	1
Pyruvate	+	-	-	27
Mannitol	+	11	-	-
D-α-Alanine	+	-	-	-
L-Proline	+	+	3	27
L-Tyrosine	+	-	-	-

[a] + = All strains positive; - = all strains negative; numbers indicate number of positive strains; underlined numbers indicate that the number represents 80% or more of the strains.

[b] P = Polar flagellation; Pr = 50 strains of B. harveyi, when grown on solid medium, have unsheathed peritrichous flagella in addition to the sheathed polar flagellum. When grown in liquid medium strains of B. harveyi have a single, sheahted, polar flagellum.

[c] + = All straight rods; numbers indicate number of straight rods, the remaining strains are curved rods.

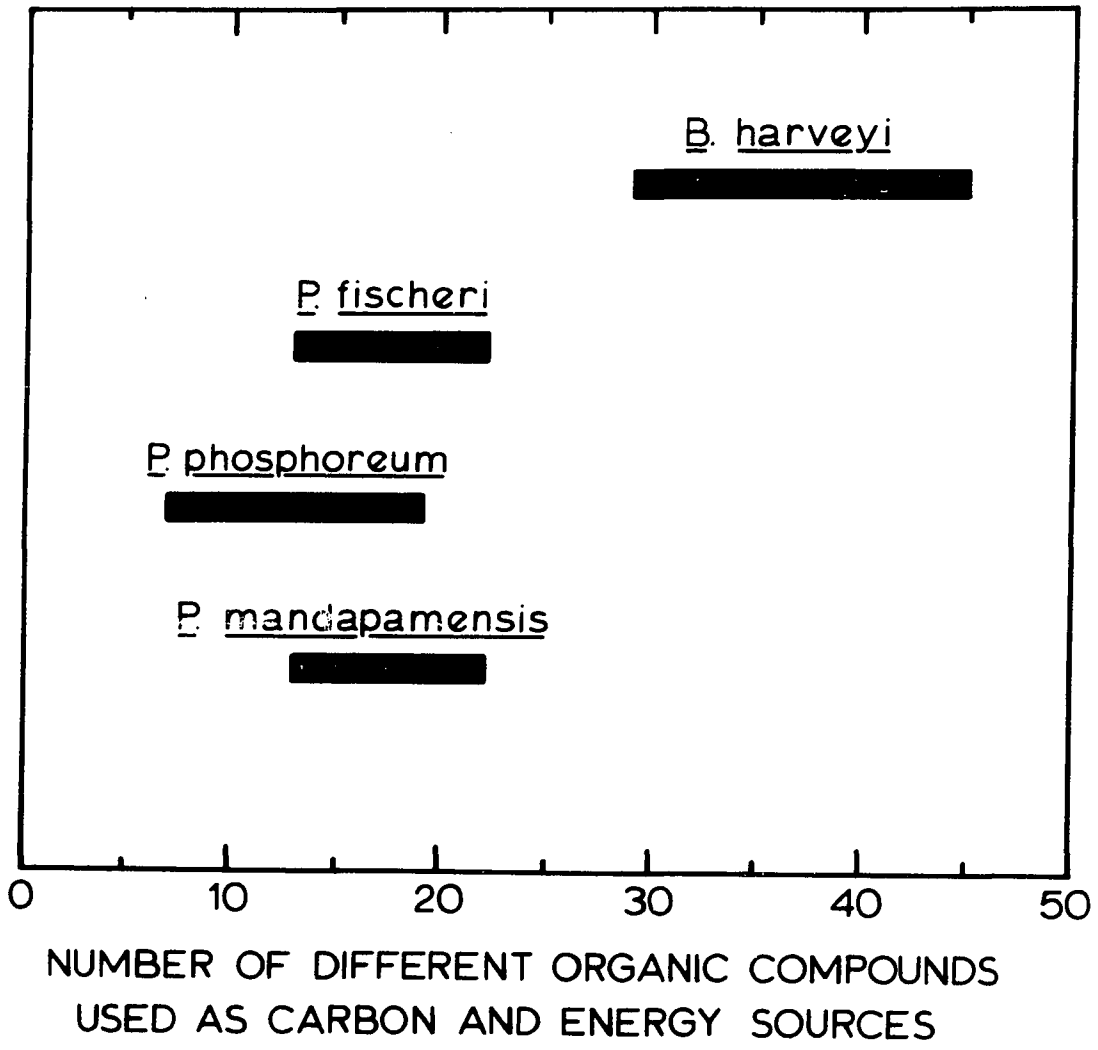


Fig. 2. Nutritional versatility of the luminous species studied.

A subsequent numerical analysis was performed which included all the strains of B. harveyi, 30 representative strains of B. campbellii, 10 strains of B. parahaemolytica, and 10 strains of B. alginolytica as well as all the strains in the remaining species and groups of Beneckea (Baumann et al., 1971a, b, 1973). The results of this analysis indicated that groups C-1, 2, and 3 (B. neptuna) were linked to the strains of B. harveyi at an S-value of 76%. A dendrogram of this cluster is presented in Fig. 3 and the traits of use in distinguishing it from other species and groups of Beneckea are presented in Table 5.

Sodium and organic growth factor requirements. Of the 179 strains included in this study, 142 had no organic growth factor requirements since they were able to grow in BM containing 0.2% D-glucose or 0.2% glycerol. The growth factor requirements of the remaining strains are presented in Table 6. As seen from this table, 24 strains of P. phosphoreum required L-methionine either alone or in combination with other amino acids. The requirement for L-methionine by some strains of this species has been previously reported by Doudoroff (1942a). Each of the four species contained a few strains which were not able to grow on BM containing 0.2% D-glucose and the six amino acids listed in Table 6. Eight of these strains were, however, able to grow on media supplemented with the complete amino acid mixture, while three strains grew only in media containing yeast extract (Table 6). Strains able to grow in BM containing 0.2% D-glucose or 0.2% glycerol and, when required, the mixture of the six amino acids listed in Table 6, reached a final trubidity of 106 to 350 Klett units. When tested for growth in a similar medium which differed from BM in the replacement of

Fig. 3. Dendrogram of the cluster consisting of luminous strains (B. harveyi) and non-luminous strains of Beneckea (groups C-1, 2, and 3) which have been previously characterized (Baumann et al., 1971a; 1973).

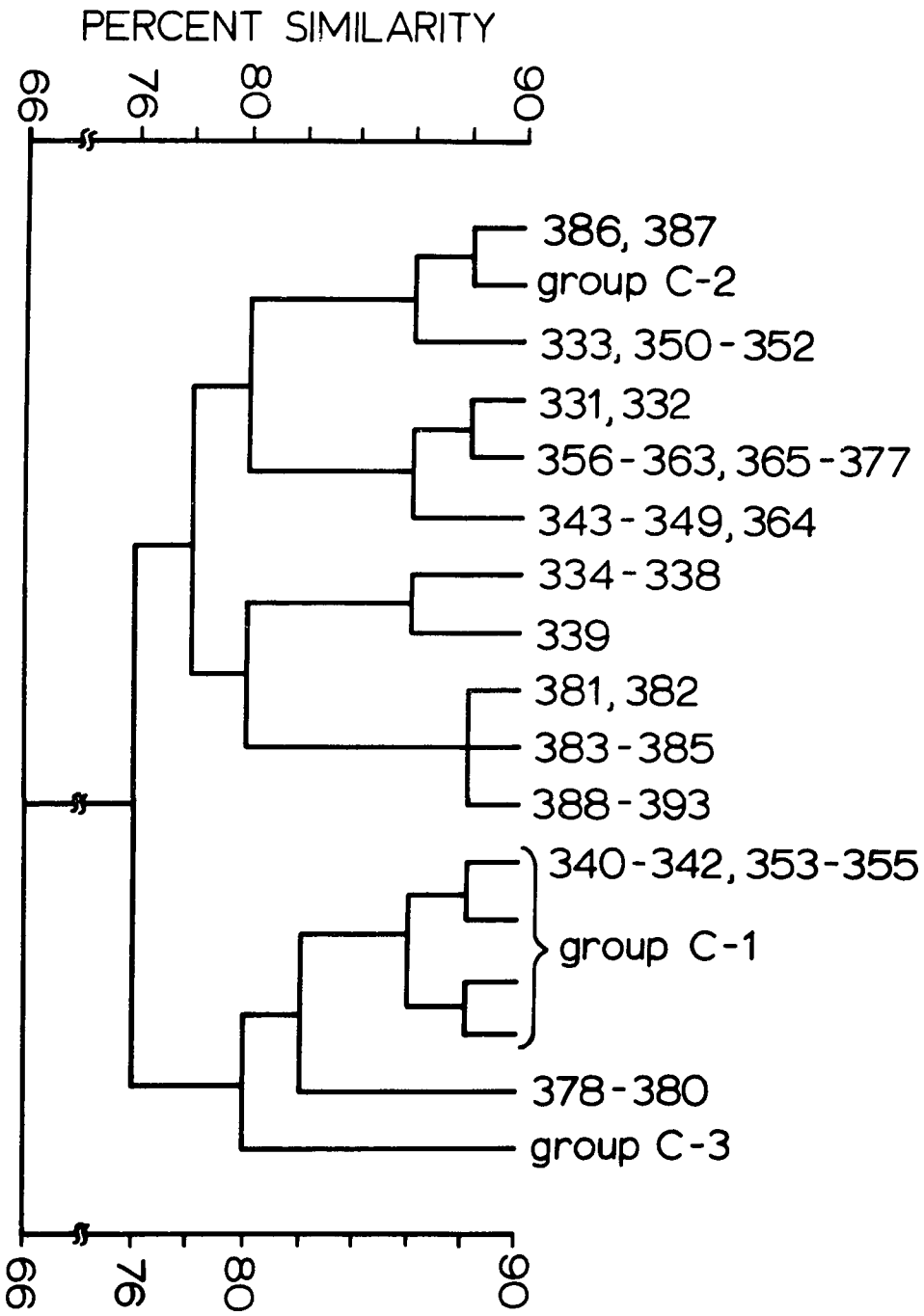

PERCENT SIMILARITY
99
96
80
90
386, 387
group C-2
333, 350-352
331, 332
356-363, 365-377
343-349, 364
334-338
339
381, 382
383-385
388-393
340-342, 353-355
group C-1
378-380
group C-3
99
96
80
90

Table 5

Selected traits of use in differentiating the combination of B. harveyi and groups C-1, 2, and 3 from other species of the genus Beneckea[a]

	B. harveyi and groups C-1, 2, and 3	B. campbellii	B. parahaemolytica	B. alginolytica	B. pelagia	B. nigrapulchrituda	B. nereida	B. natriegens	strains 84, 85	group E-3[b]
Number of strains	106	60	132	30	11	14	6	6	2	2
PHB-Accumulation	-	-	-	-	-	7	+	+	+	+
2,3-Butyleneglycol production	-	-	-	+	-	-	-	-	-	-
D-Galactose	92	-	128	4	+	+	-	+	+	-
Cellobiose	102	20	8	-	-	+	-	4	-	+
Lactose	-	-	-	-	-	+	-	-	-	-
D-Gluconate	102	5	+	+	+	5	+	+	+	+
D-Glucuronate	102	2	82	-	-	+	-	3	-	-
Butyrate	-	-	103	+	-	-	+	+	-	+
Valerate	-	-	8	26	-	-	+	+	+	+
DL-β-Hydroxybutyrate	-	-	-	-	-	+	+	+	+	+
DL-Glycerate	96	24	131	+	-	+	-	5	+	-
α-Ketoglutarate	104	57	130	+	-	+	+	+	+	+
Ethanol	-	-	117	16	5	5	+	+	1	1
L-Leucine	2	-	129	+	-	-	+	5	-	1
L-Arginine	93	-	119	29	+	-	+	+	+	1
L-Histidine	17	6	125	+	7	13	5	+	1	+
Putrescine	-	-	112	25	+	-	+	+	-	+

[a] + = All strains positive; - = all strains negative; numbers indicate number of positive strains; underlined numbers indicate that the number represents 80% or more of the strains.

[b] A group of facultative anaerobes undesignated with respect to genus (Baumann et al., 1971a).

Table 6

Organic growth factor requirements of *B. harveyi*, *P. fischeri*, *P. phosphoreum*, and *P. mandapamensis*

Additions allowing growth on BMA containing 0.2% D-glucose	*B. harveyi*	*P. fischeri*	*P. phosphoreum*	*P. mandapamensis*
L-Methionine	-	-	400-402, 407, 442 444, 450-460, 465	-
L-Methionine, L-histidine	-	-	441, 443	-
L-Methionine, L-leucine	-	-	463	-
L-Methionine, L-leucine, L-valine	-	-	439	-
L-Methionine, L-isoleucine, L-valine	-	-	461, 462	-
L-Histidine	-	-	449	-
L-Arginine	-	-	-	476
Complete amino acid mixture[a]	-	399	494, 495, 499-501	502, 503
Yeast extract (0.1 g/liter)[b]	492	398	497	-

Table 6. (Continued) Organic growth factor requirements of *B. harveyi*, *P. fischeri*, *P. phosphoreum*, and *P. mandapamensis*

[a]Contained Difco Casamino Acids, L-tryptophan, L-methionine, and L-cysteine. Strains able to grow on this medium were unable to grow on a medium supplemented with the six amino acids listed in this table.

[b]Strains able to grow on this medium were unable to grow on a medium supplemented with the complete amino acid mixture.

the sodium (200 mM) by equimolar amounts of potassium, the final turbidity was 0-15 Klett units, indicating that these strains had a requirement for sodium ion.

Fermentation. All strains fermented D-glucose with the production of acid and lowered the pH of the culture medium to 4.8-5.5. Eighty-nine percent of the strains of P. phosphoreum and 7% of the strains of P. mandapamensis produced gas during the fermentation of D-glucose (Table 1); gas was not produced by any of the strains of B. harveyi or P. fischeri. The majority of the strains of P. phosphoreum and P. mandapamensis (97% and 93%, respectively) gave a positive Vogues-Proskauer reaction while only 8% of B. harveyi and 25% of P. fischeri were positive for this trait (Table 1). The Vogues-Proskauer reaction in the latter two species as well as in some strains of P. phosphoreum and P. mandapamensis was weak but reproducible. A quantitative assay for acetoin and/or diacetyl as well as 2,3-butyleneglycol indicated that the culture medium of strains of B. harveyi and P. fischeri did not contain detectable levels of these compounds (less than 0.3 µg/ml acetoin and/or diacetyl and less than 30 µg/ml 2,3-butyleneglycol). Acetoin and/or diacetyl was, however, detected in 92% of the strains of P. phosphoreum and 50% of the strains of P. mandapamensis while 2,3-butyleneglycol was detected in 84% of the former and 11% of the latter species (Table 1). In general, the culture medium of strains which gave a weak Vogues-Proskauer reaction contained low or undetectable levels of acetoin and/or diacetyl. Similar discrepancies have been previously noted in some strains of Beneckea and P. fischeri (Baumann et al., 1971a). A strain of Serratia marcescens, which was

used as a control, gave a strong Vogues-Proskauer reaction and produced 1.3 µg acetoin and/or diacetyl and 288 µg 2,3-butyleneglycol per ml of the culture medium.

Temperature. The ability of strains of marine, luminous bacteria to grow at different temperatures is presented in Table 1. Most of the strains were able to grow at 25° and 30°C, while only strains of P. phosphoreum were able to grow at 4°C.

Extracellular enzymes. Most of the strains produced a chitinase (Table 1). All of the strains of B. harveyi and most of the strains of P. fischeri and P. mandapamensis produced a lipase; none of the strains of P. phosphoreum produced this enzyme. Amylase production was restricted to B. harveyi while gelatinase was produced by all members of this species as well as by one strain of P. fischeri. Alginase was produced by 37% of the strains of B. harveyi; this property was not found in any of the remaining species.

Other physiological tests. Most of the strains were able to reduce nitrate to nitrite (Table 1) and none of the strains was able to denitrify or grow chemolithotrophically with molecular hydrogen as the source of energy and carbon dioxide as the source of carbon. Strains 333, 350-352, and 378-380 (B. harveyi), when grown on quinate, degraded protocatechuate by means of a meta cleavage. Representative strains were tested for the presence of a constitutive arginine dihydrolase system by assaying for the production of ornithine (Baumann et al., 1971a). The arginine dihydrolase system was present in strains 378 and 380 (B. harveyi) and absent in strains 331, 333, 334, 339, 340, 350-352, 373, 376, 379, 381, 384, 386, 392, 492, 493 (B. harveyi); 394-398 (P.

fischeri); 409, 421, 436, 440, 446, 455, 459, 463-465, 494, 496-501 (P. phosphoreum); 466, 474, 477, 479, 480, 485, 488-491 (P. mandapamensis).

Pigment production. Strains of P. fischeri (61-66 and 394-399) when grown on LA, produced a light yellow, cell-associated pigment. Strains 331-393, 492, and 493 of B. harveyi, when grown on this medium, produced a soluble, light brown pigment after a 6-14 day incubation at 20-22°C. The light brown pigment was not produced when these strains were grown on a minimal medium.

Gram stain, cell shape, motility, and flagellation. All of the strains were gram-negative. Strains 378-380 (B. harveyi), 394 (P. fischeri), and 483 (P. mandapamensis) were curved rods; the remaining strains were straight rods. With the exception of strains 395 of P. fischeri and 443, 456, 461, 463, and 499 of P. phosphoreum, all strains were motile. Figs. 11-14 are photomicrographs of strains representative of the four species in exponential phase of growth. In early stationary phase, strains 400-491 and 494-503 (P. phosphoreum and P. mandapamensis) became very short rods or even spheres. After an incubation of 1-2 days involution forms were observed in all cultures of luminous bacteria.

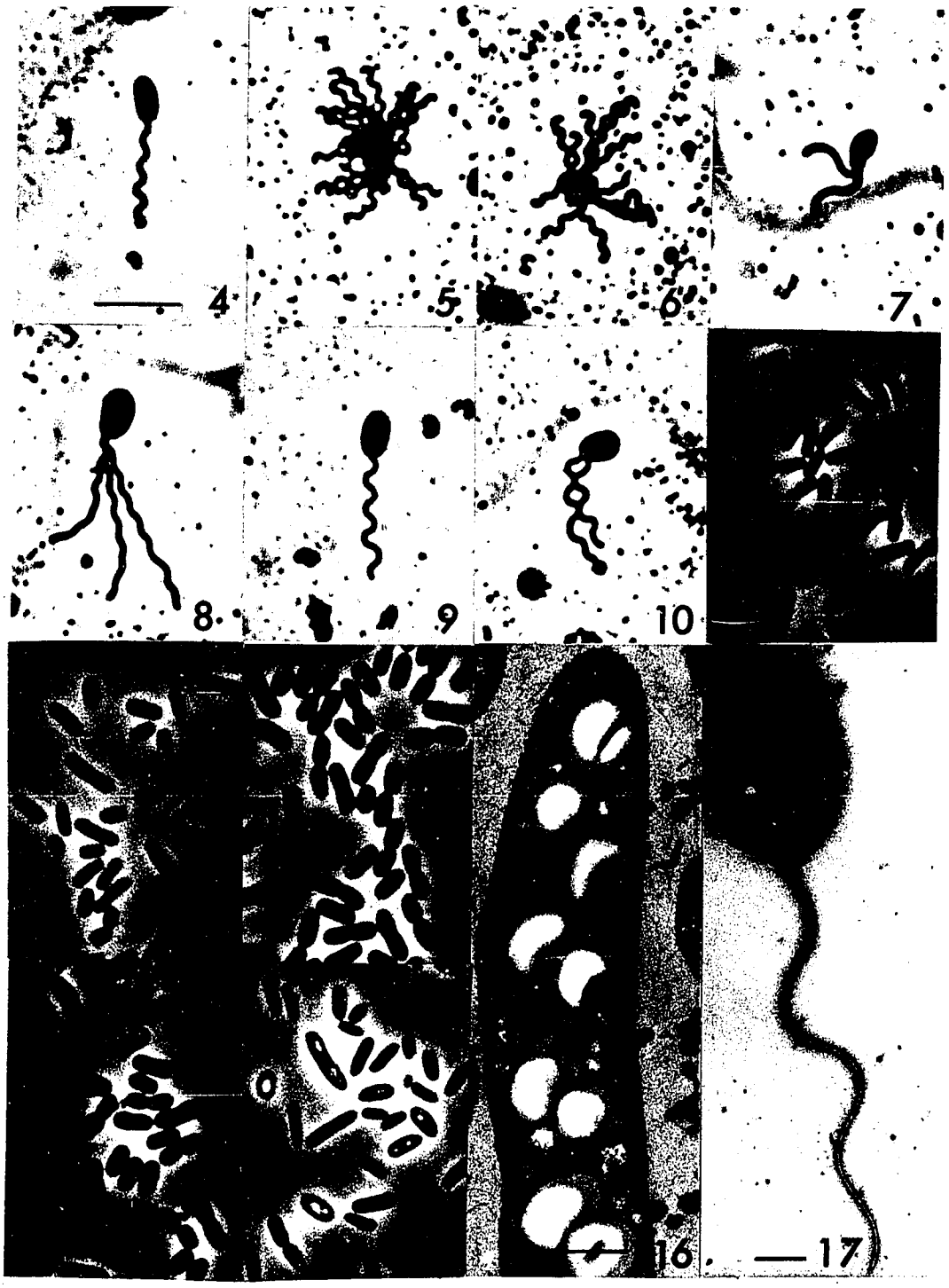
Twenty-five representative strains of B. harveyi, when grown in liquid medium and stained by the Leifson method, had a single polar flagellum (Fig. 4). When grown on solid medium, 50 strains of this species were peritrichously flagellated (Figs. 5 and 6) while 15 strains (334, 339, 378-380, 382, 387-393, 492, and 493) had single polar flagella. Strains 340, 341, and 353-355 which were peritrichously

Figs. 4-10. Leifson flagella stains. X2,500. Marker in Fig. 4 represents 5 μm. Fig. 4. This photomicrograph is representative of all strains of B. harveyi grown in liquid medium. The actual strain used is 348. Figs. 5 and 6. B. harveyi, strains 342 and 371, respectively, grown on solid medium. Fig. 7. P. fischeri, strain 397. Fig. 8. P. phosphoreum, strain 421. Figs. 9 and 10. P. mandapamensis, strains 470 and 481, respectively.

Figs. 11-15. Phase contrast micrographs. X2,000. Marker in Fig. 11 represents 5 μm. Figs. 11-14. Cells in exponential phase of growth in YEB. Fig. 11. B. harveyi, strain 392. Fig. 12. P. fischeri, strain 396. Fig. 13. P. phosphoreum, strain 404. Fig. 14. P. mandapamensis, strain 480. Fig. 15. P. mandapamensis, strain 477, in early stationary phase of growth in BM containing 0.2% D-glucose. Note the accumulation of refractile PHB inclusions.

Fig. 16. Ultrathin section of P. phosphoreum, strain 409, in early stationary phase of growth in BM containing 0.2% D-glucose. The PHB inclusions which appear in this cell are absent when this strain is grown in YEB. X23,000. Marker represents 0.5 μm.

Fig. 17. Electron micrograph of B. harveyi, strain 392, having a single, sheathed, polar flagellum. Exponential phase of growth in liquid medium. Negatively stained. X15,000. Marker represents 0.5 μm.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flagellated swarmed on LA. Examination of six representative strains of B. harveyi by means of the electron microscope indicated that, when grown in liquid medium, the strains had a single, sheathed, polar flagellum 24-30 nm in diameter (Fig. 17). When grown on solid medium, the strains had unsheathed, peritrichous flagella 14 to 16 nm in diameter in addition to the sheathed, polar flagellum (Fig. 18). This type of flagellation has been previously observed in B. harveyi (Hendrie et al., 1970; Hodgkiss and Shewan, 1968; Johnson et al., 1943; Reichelt and Baumann, 1972) and in several species of the genus Beneckea (Allen and Baumann, 1971; Baumann and Baumann, 1973c; Baumann et al., 1971a, 1973). With the exception of strain 395, which was non-motile, strains 394 and 396-399 of P. fischeri had tufts of 2-6 polar flagella when grown in liquid or solid medium and stained by the Leifson method (Fig. 7). Examination of strains 394, 396, and 397 by means of the electron microscope indicated that these cells had tufts of 2-8 sheathed flagella (Fig. 19) having a diameter of 24-30 nm. This type of flagellation has been previously reported for P. fischeri (Allen and Baumann, 1971; Baumann et al., 1971a; Hendrie et al., 1970; Johnson et al., 1943). Motile strains of P. phosphoreum and P. mandapamensis (strains 400-442, 444-455, 457-462, 464-491, 494-498, 500, and 501) when grown in liquid medium had 1-3 polar flagella (Figs. 8-10). A similar type of flagellation was observed when 10 representative strains of each species were grown on solid medium. Examination of 10 representative strains of P. phosphoreum and P. mandapamensis by means of the electron microscope indicated that these strains had 1-3 unsheathed, polar flagella which had a diameter of 14-16 nm (Figs. 20 and 21).

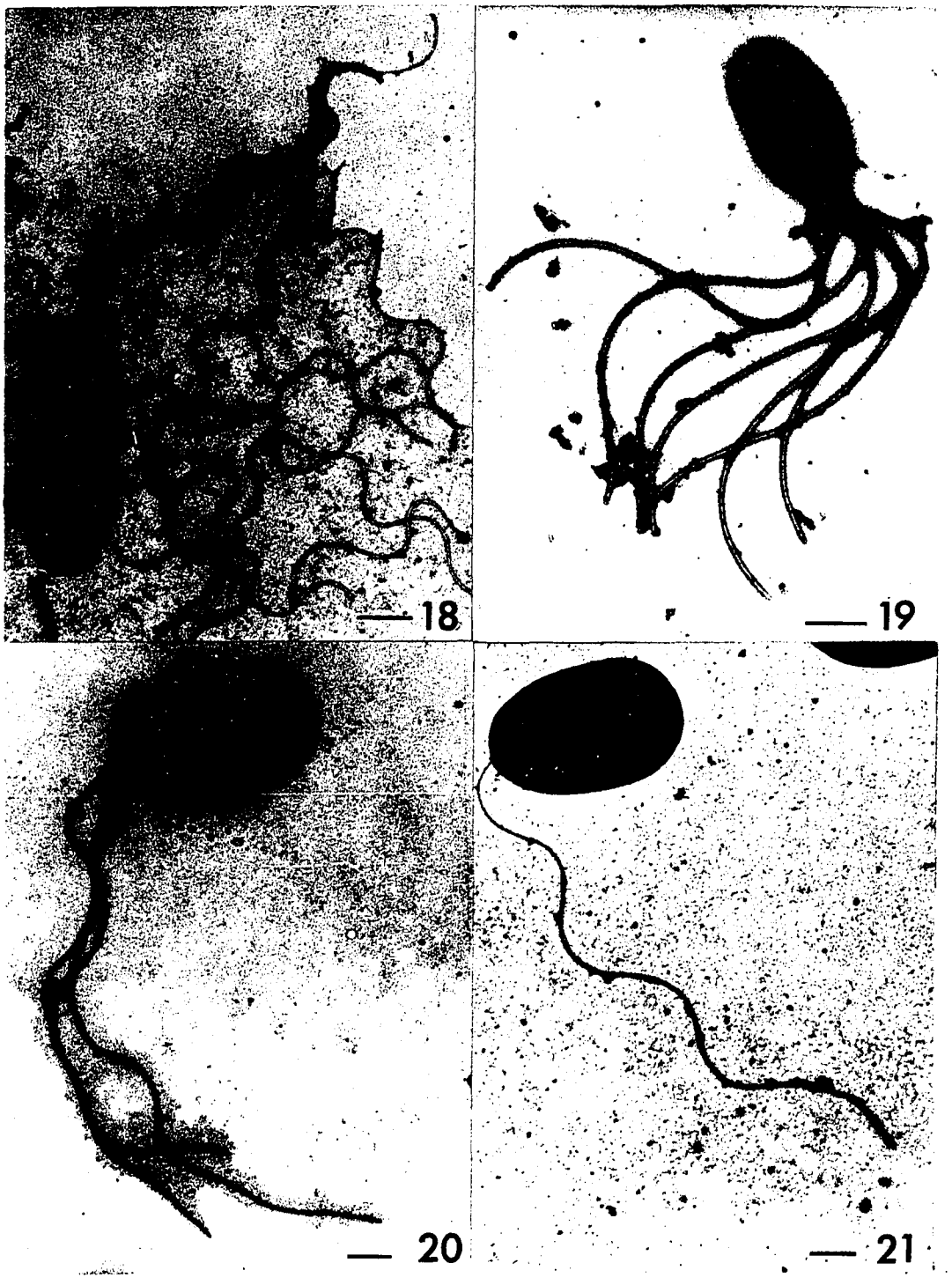
Figs. 18-21. Electron micrographs of species of marine, luminous bacteria. Negatively stained. Marker in each Fig. represents 0.5 μm.

Fig. 18. B. harveyi, strain 383, having a sheathed, polar flagellum and unsheathed, peritrichous flagella. Harvested from solid medium after 14 hours. X16,000.

Fig. 19. P. fischeri, strain 394, having a polar tuft of sheathed flagella. X18,000.

Fig. 20. P. phosphoreum, strain 450, having a polar tuft of unsheathed flagella. X13,000.

Fig. 21. P. mandapamensis, strain 485, having a single, unsheathed, polar flagellum. X13,000.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Reserve materials. During the examination of the morphology of luminous bacteria by phase contrast microscopy, it was found that strains of P. phosphoreum (400-465 and 494-501) and P. mandapamensis (466-491, 502, and 503), grown in LB, contained variable amounts of phase-bright inclusions. In any microscopic field of observation, the appearance of these inclusions varied from a few granules in some cells to massive accumulations rendering the entire cell refractile (Fig. 15). Observations of cell morphology throughout the growth cycle were made on strains 474, 477, and 480 (P. mandapamensis) grown in YEB, LB, and BM containing 0.2% D-glucose, glycerol, or succinate. No refractile inclusions were observed in strains grown in YEB during any part of the growth cycle. Cells in exponential phase of growth in the remaining media had no or very few inclusions. In early stationary phase, massive accumulation was observed in D-glucose-grown cells (Fig. 15); the extent of accumulation was considerably less in glycerol-, succinate-, or LB-grown cells. Refractile inclusions were never observed in strains of P. fischeri or B. harveyi when grown under similar conditions. Ten representative strains of both P. phosphoreum and P. mandapamensis were harvested in early stationary phase in BM containing 0.2% D-glucose as well as in exponential and stationary phase in YEB and stained with Sudan black B. D-Glucose-grown cells were stained extensively with this dye, the distribution of the stain closely resembling that of the refractile inclusions seen by means of phase microscopy. Cells grown in YEB did not contain material which was stainable by this dye. An examination by means of the electron microscope of thin sections of strain 409 of P. phosphoreum and strain

480 of P. mandapamensis, in exponential phase in YEB and early stationary phase in BM containing 0.2% D-glucose, showed that D-glucose-grown cells contained accumulations of a relatively electron transparent material which had a tendency to vaporize under the electron beam (Fig. 16). No such inclusions were seen in these strains when grown in YEB. An analysis of the PHB content of D-glucose-grown cells showed that in P. phosphoreum (strain 404) and P. mandapamensis (strain 477) this compound comprised about 37 and 32% of the dry weight, respectively. The high PHB content of the cells as well as the microscopic observation that the refractile granules were not digested during the hypochlorite treatment indicated that these inclusions were composed of PHB. The presence of PHB in strain 494 of P. phosphoreum and its absence in strain 392 of B. harveyi has been reported by Eberhard and Rouser (1971). Unlike species of Pseudomonas (Palleroni and Doudoroff, 1972) and other marine bacteria which accumulate PHB (Baumann et al., 1971a; 1972), P. phosphoreum, and P. mandapamensis were unable to utilize β-hydroxybutyrate as a sole or principal source of carbon and energy.

Oxidase test. All of the strains of B. harveyi gave a vigorous oxidase reaction without the addition of toluene, as did most of the strains of P. fischeri. The sole exception in the latter species was strain 396 which gave a slow oxidase reaction, the rate of which was increased by the addition of toluene prior to the addition of the oxidase reagent. All the strains of P. phosphoreum were oxidase-negative without the addition of toluene; however, five strains became positive when this compound was added. In the case of P. mandapamensis,

13 strains gave a positive oxidase test which varied in the rate of development. Upon the addition of toluene, five additional strains of this species gave a positive reaction.

Differential oxidized/reduced cytochrome spectra were determined for four representative strains of B. harveyi and for four representative strains of P. fischeri. The results were similar to those observed in species of Beneckea (Baumann et al., 1973). The principal bands consisted of a Soret band at 427-430 nm, β-bands at 523 and 530 nm, and α-bands at 552 and 560. Differential cytochrome spectra of eight representative strains of P. phosphoreum and eight representative strains of P. mandapamensis gave results qualitatively similar to B. harveyi and P. fischeri, except that the β-band at 523 nm was generally absent and the total cytochrome content was considerably lower. The tested strains of these two species included strains which were oxidase-negative, oxidase-positive without the addition of toluene, and oxidase-positive only when toluene was added. The results suggested that all four species of luminous bacteria had cytochromes of the b and c types (Stanier et al., 1966), although in P. phosphoreum and P. mandapamensis the c cytochrome was not always detected by the oxidase test. No bands characteristic of cytochromes of the a type were detected in any of the species.

DNA base compositions. The moles % GC contents in the DNAs of representative strains of each species are given in Table 7. Six strains of B. harveyi had GC contents which ranged from 46.0 to 37.2 moles %, eight strains of P. phosphoreum had GC contents of 41.2 to 41.8, and six strains of P. mandapamensis had GC contents of 42.8 to

Table 7

Moles % GC contents of DNAs of species of marine, luminous bacteria as determined by thermal denaturation

Species	Strain	Moles % GC content[a]	Mean moles % GC content ± σ
B. harveyi	331	46.3	46.5 ± 1.3
	334	47.2	
	340	46.5	
	376	46.8	
	384	46.0	
	392	46.0	
P. fischeri	61	39.0	39.8 ± 1.1
	64	40.0	
	394	39.8	
	395	40.1	
	396	40.1	
	397	39.7	
	398	39.7	
P. phosphoreum	409	41.8	41.5 ± 0.7
	427	41.5	
	428	41.5	
	439	41.8	
	444	41.5	
	449	41.7	
	455	41.3	
	465	41.2	
P. mandapamensis	466	43.3	42.9 ± 0.5
	474	42.8	
	477	42.9	
	480	42.9	
	486	42.9	
	490	42.8	

[a]Mean of two determinations; strain 384 mean of five determinations.

43.3. These values are in general agreement with the results of Hendrie et al. (1970); these authors did not, however, detect the slight difference in the moles % GC contents of P. phosphoreum and P. mandapamensis. There is a considerable discrepancy in the GC range of P. fischeri as determined in these two studies. Hendrie et al. (1970) found the GC content of strain 395 to be 39.0 moles %, in agreement with the value obtained here (40.1 moles %). Their determinations for two additional strains of this species (43.5 and 45.5) were, however, considerably higher than the values obtained in this study for six strains of P. fischeri which ranged from 39.0 to 40.1 moles %. It should be noted that the GC content of P. phosphoreum, strain 439 (ATCC 11040, NCMB 1282), was 41.8 moles % and not 65.1 as has been stated by Sakazaki et al. (1970).

Enzymes of glucose and gluconate catabolism. As seen from Table 8, cell-free extracts of either D-glucose- or D-gluconate-grown cells (succinate-grown in the case of P. fischeri, the only species which was not able to utilize D-gluconate) contained enzyme activities necessary for the catabolism of D-glucose via the Embden-Meyerhof pathway. Cell-free extracts of D-glucose-grown cells contained no gluconokinase activity and low activities of 6-P-gluconate dehydrase and 2-keto-3-deoxy-6-P-gluconate aldolase while D-gluconate-grown cells had gluconokinase activity and increased activities of 6-P-gluconate dehydrase and 2-keto-3-deoxy-6-P-gluconate aldolase, the enzymes necessary for the catabolism of this compound via the Entner-Doudoroff pathway. In some cases a considerably higher activity of 6-P-fructokinase and glucokinase was observed in D-gluconate-grown cells than in D-glucose-grown cells.

Table 8

Specific activities of selected enzymes of D-glucose and D-gluconate catabolism in cell-free extracts of species of marine, luminous bacteria[a]

Enzyme	B. harveyi 384		P. fischeri 397		P. phosphoreum 446		P. mandapamensis 474	
	D-Glucose[b]	D-Gluconate	D-Glucose	Succinate	D-Glucose	D-Gluconate	D-Glucose	D-Gluconate
Glucokinase	57	84	76	97	82	143	75	40
P-Glucose isomerase	1700	N.T.[c]	483	N.T.	514	N.T.	1600	N.T.
6-P-Fructo-kinase	309	500	150	219	115	151	119	137
Fructose-1,6-P_2 aldolase	222	N.T.	164	N.T.	137	N.T.	249	N.T.
Glucose-6-P dehydrogenase	87	N.T.	6	N.T.	130	N.T.	96	N.T.
6-P-Gluconate dehydrogenase	72	54	14	13	89	108	96	98
Glucokinase	<1	85	<1	N.T.	<1	63	<1	184

Table 8. (Continued) Specific activities of selected enzymes of D-glucose and D-gluconate catabolism in cell-free extracts of species of marine, luminous bacteria[a]

	B. harveyi 384		*P. fischeri* 397		*P. phosphoreum* 446		*P. mandapamensis* 474	
Enzyme	D-Glucose[b]	D-Gluconate	D-Glucose	Succinate	D-Glucose	D-Gluconate	D-Glucose	D-Gluconate
6-P-Gluconate dehydrase and 2-keto-3-deoxy-6-P-gluconate aldolase[d]	12	129	<1	N.T.	27	79	24	54

[a]Specific activities are expressed as nmoles substrate utilized/min/mg protein.

[b]Substrate supporting growth.

[c]N.T. = not tested.

[d]Assayed jointly with 6-P-gluconate as the substrate.

The low activities of glucose-6-P dehydrogenase and 6-P-gluconate dehydrogenase in cell-free extracts of P. fischeri was reproducible; equally low activities of these two enzymes were detected in strain 61 of this species. The results presented in Table 8 are similar to those found in species of Beneckea (Baumann et al., 1973) and suggest that the four luminous species of marine bacteria catabolize D-glucose via the Embden-Meyerhof pathway and that B. harveyi, P. phosphoreum, and P. mandapamensis catabolize D-gluconate via the Entner-Doudoroff pathway. The former pathway is constitutive while some enzymes of the latter pathway are inducible.

DISCUSSION

Taxonomic considerations

Hendrie et al. (1970), in their study of the taxonomy of luminous bacteria, showed that marine, luminous strains could be assigned to four species which they placed into three genera. These species were designated Lucibacterium harveyi, Photobacterium phosphoreum, P. mandapamensis, and Vibrio fischeri. The present study supports their conclusions by showing that many additional strains can be assigned to these species and strengthens their characterization by establishing a considerable number of new diagnostic traits. The specific designations assigned in this study are in accord with the recommendations of Hendrie et al. (1970). The following strains are common to both studies: L. harveyi (384, 492, 493), P. phosphoreum (439, 497-501), P. mandapamensis (477), and Vibrio fischeri (395, 398). Although the general conclusions reached in this study are similar to those of Hendrie et al. (1970), they differ with regard to the assignment of the species "harveyi" to the genus Lucibacterium and "fischeri" to the genus Vibrio.

Two species were retained in the genus Photobacterium by Hendrie et al. (1970), P. phosphoreum, the type species of this genus, and P. mandapamensis, a species created to accommodate four of their isolates. The results of this study confirm the existence of the latter species and are in agreement with the decision of these investigators to assign P. phosphoreum and P. mandapamensis to a single genus, distinct from the other species of luminous bacteria. On the basis of these two

species, the genus Photobacterium can be described as consisting of non-pigmented, non-sporeforming, gram-negative, straight or curved rods of marine origin which accumulate PHB as an intracellular reserve product and are motile by means of 1-3 unsheathed, polar flagella. The GC content in the DNA ranges from 41 to 44 moles %. All are chemo-organotrophic, facultative anaerobes which ferment D-glucose with the production of acid; some strains produce gas. All strains luminesce; most strains reduce nitrate to nitrite and grow at 25°C. None denitrifies or has a constitutive arginine dihydrolase. Many strains are able to grow on a mineral medium containing ASW and glycerol as the sole source of carbon and energy and ammonium ion as the sole source of nitrogen. Some strains, however, have growth factor requirements which usually include L-methionine alone or in combination with other amino acids. Sodium ion is required for growth. Most strains produce a chitinase and utilize a restricted number of organic compounds as sole sources of carbon and energy, including D-glucose, D-mannose, D-galactose, D-fructose, D-gluconate, N-acetylglucosamine, succinate, fumarate, glycerol, and L-aspartate. None of the strains has an amylase, gelatinase, alginase, or cellulase; hydrolyzes agar; or utilizes D-xylose, L-arabinose, sucrose, trehalose, cellobiose, formate, C_3-C_{10} monocarboxylic acids, C_6-C_{10} dicarboxylic acids, sugar alcohols, alcohols, aromatic compunds, amines, purines and pyrimidines, and most amino acids. The strains which comprise this genus are common inhabitants of the marine environment, being isolated from the open ocean, surfaces of fish and cephalopods, intestines of fish, and the specialized luminous organs of fish and cephalopods.

Some of the properties of the genus Photobacterium are found in a group of five non-luminous strains which have been characterized by Baumann et al. (1971a) and designated group B-2. When grown in liquid medium they have 1-3 unsheathed, polar flagella (Allen and Baumann, 1971); two representative strains have GC contents in their DNAs of 40.8 and 41.3 moles %; and all the strains have refractile inclusions similar to those found in P. phosphoreum and P. mandapamensis. Members of this group (which has some internal phenotypic heterogeneity) differ from both of these luminous species by a number of phenotypic traits (Baumann et al., 1971a). Additional studies with strains similar to group B-2 are necessary before the relation of this group to Photobacterium can be elucidated and its taxonomy formalized.

Strains 331-393, 492, and 493 of the species "harveyi" differ from the members of the genus Photobacterium in having single, sheathed, polar flagella when grown in liquid medium, GC contents in their DNAs of 46-48 moles % as well as by their inability to accumulate PHB as an intracellular reserve product. Seventy-seven percent of these strains have unsheathed, peritrichous flagella in addition to the sheathed, polar flagellum when grown on solid medium. These properties are sufficiently distinctive to justify a generic separation of these strains from the genus Photobacterium, a conclusion previously indicated by Henrie et al. (1970) who placed similar strains into the genus Lucibacterium. The creation of this genus was primarily based on the recognition by these authors that the strains designated by the specific epithet "harveyi" have a sheathed, polar flagellum and unsheathed, peritrichous flagella. Since these authors harvested their

strains for electron microscopic observation from solid medium, they did not observe the shift in flagellation which occurs in most strains of this species (including strain 384, the type strain of "harveyi") when grown in liquid and solid medium. The moles % GC content in the DNAs of these strains (46-48), their flagellation, as well as their general nutritional properties indicate that they have characteristics of the genus Beneckea (Baumann et al., 1971a). Since this genus has priority over Lucibacterium, "harveyi" should be transferred to Beneckea and the definition of this genus altered to include luminous strains.

Strains 61-66 and 394-399 of the species "fischeri" differ from the other luminous species in their flagellation (tufts of sheathed, polar flagella) and the GC contents of their DNAs (39.8 ± 1.1 moles %). Strains 395 and 398 were included in the study of Hendrie et al. (1970) who removed these and other phenotypically similar organisms from the genus Photobacterium (Breed et al., 1957) and assigned them to the genus Vibrio. This study also indicates that "fischeri" deserves a generic status distinct from B. harveyi, P. phosphoreum, and P. mandapamensis but it does not appear possible at present to make a valid generic choice for this species. The genus Vibrio has undergone a drastic redefinition since the 7th edition of BERGEY'S MANUAL OF DETERMINATIVE BACTERIOLOGY (Breed et al., 1957) in which it was primarily defined by cell curvature and polar flagellation and included strict aerobes, strict anaerobes, and facultative anaerobes. The most recent provisional definition (Hugh and Feeley, 1972a, b; Hugh and Sakazaki, 1972) describes the genus Vibrio as consisting of straight or curved rods, motile by means of a single polar flagellum, oxidase

and catalase positive, fermenting glucose with the production of acid but not gas, and having a GC content in the DNA of 40-50 moles %. Other diagnostic traits include acid from mannitol, presence of an L-lysine and L-ornithine decarboxylase, and absence of an arginine dihydrolase. The genus was stated to include V. cholerae (the type species of the genus Vibrio), V. albensis, V. parahaemolyticus, V. alginolyticus, and V. costicolus, species which have moles % GC contents in their DNAs of 46-50 (Baumann et al., 1971a; Hendrie et al., 1970; Hill, 1966). Recent work has shown that V. parahaemolyticus and V. alginolyticus have the type of flagellation characteristic of a number of gram-negative, facultatively anaerobic marine bacteria. In liquid medium these species have a single, sheathed, polar flagellum, while on solid medium they have unsheathed, peritrichous flagella in addition to the single, sheathed, polar flagellum (Allen and Baumann, 1971; Baumann and Baumann, 1973c; Baumann et al., 1971a, 1973). Hendrie et al. (1970) found V. albensis (ATCC 14546) to be motile by usually one but sometimes as many as four polar flagella. Examination of this strain in this study showed that most cells had polar tufts of 2-3 sheathed flagella. Adherence to the current definition of Vibrio would exclude these three species as well as "fischeri" (which has tufts of 2-8 polar flagella) from this genus since it is restricted to organisms having polar, monotrichous flagellation.

The species included in Vibrio (Hugh and Sakazaki, 1972) comprise at least three ecologically distinct groups: human and fresh water isolates (V. cholerae, V. albensis), marine strains (V. parahaemolyticus, V. alginolyticus), and the moderate halophile (Larsen, 1962) V.

costicolus. As a consequence of their distinct habitats, these organisms have different ionic requirements for optimal growth (Larsen, 1962). A considerable body of work (Baumann et al., 1971a, 1972; reviews by MacLeod, 1965, 1968) has indicated that gram-negative, marine bacteria require sodium ion for growth, a requirement which is greatly reduced or absent in gram-negative terrestrial organisms. Studies of marine bacteria suggest that this requirement may reflect differences between the cell walls, cell membranes, and permease systems of gram-negative marine and terrestrial strains (Forsberg et al., 1970; MacLeod, 1965, 1968; Thompson and MacLeod, 1971). These differences have led Rüger (1972) to propose the exclusion of marine organisms having properties of Vibrio from this genus, a suggestion previously considered by Tubiash et al. (1970). A similar proposal was made by Baumann et al. (1971a) who placed marine species which were peritrichously flagellated when grown on solid medium (V. parahaemolyticus, V. alginolyticus, and two other species) into the genus Beneckea which was redefined to include phenotypically related, polarly flagellated, marine strains. Although these proposals require further substantiation from biochemical and physiological studies on the nature of the salt requirement by marine strains and comparative studies involving gram-negative, terrestrial strains, the redefined genus Beneckea (Baumann et al., 1971a) does have the merit of describing a large and ecologically restricted group of phenotypically related organisms which are readily isolated from the marine environment. Since a great deal of future work will be necessary before adequate generic assignments of the facultatively anaerobic, marine and terrestrial

organisms having moles % GC contents of 40-50 can be made and since the present definition of Vibrio is unsatisfactory, it seems prudent that "fischeri" should provisionally revert to its previous genus, Photobacterium, which contains only marine strains. As in the case of P. phosphoreum and P. mandapamensis, no systematic study of non-luminous strains phenotypically similar to P. fischeri has been performed. One non-luminous marine isolate, however, has been shown to be similar to this species (to be discussed) suggesting that before P. fischeri is assigned to a new genus such strains should be studied. A summary of the taxonomic conclusions is presented in Table 9.

The following discussion will consider the properties and taxonomy of the four species of luminous bacteria. The complete phenotypes of these species are listed in Tables 1-3 and their nutritional versatility is shown in Fig. 2. The traits of use in differentiating these species are presented in Table 4.

Beneckea harveyi. The luminous strains comprising this species consist of 62 straight (Fig. 11) and three curved rods which have a single, sheathed, polar flagellum when grown in liquid medium (Figs. 4 and 17). When grown on solid medium, 50 strains have unsheathed, peritrichous flagella in addition to the sheathed, polar flagellum (Figs. 5, 6, and 18). The GC content of six representative strains is 46.5 ± 1.3 moles %. All strains are oxidase-positive, ferment D-glucose with the production of acid but no gas, grow at 35°C but not at 4°C, and produce an extracellular amylase, lipase, chitinase, and, with the exception of one strain, a gelatinase. B. harveyi is the most nutritionally versatile species of luminous bacteria (Fig. 2). All or

Table 9

Summary of taxonomic conclusions

Genus	Moles % GC Content of DNAs	Flagellation	Luminous species	Similar non-luminous organisms
Beneckea	45.4-47.4	In liquid medium single polar, sheathed flagellum; on solid medium may have additional unsheathed, peritrichous flagella	B. harveyi	Groups C-1, 2, and 3[a] and seven species of Beneckea
Photobacterium	41.2-43.3	1-3 unsheathed, polar flagella	P. phosphoreum P. mandapamensis	Group B-2
Photobacterium fischeri[b]	39.0-40.1	2-8 sheathed, polar flagella	P. fischeri	ATCC 15382[a]

[a]Organism(s) phenotypically similar to the luminous strains suggesting their inclusion in one species.

[b]Species deserving a new generic status; provisionally assigned to Photobacterium.

most of the strains are able to utilize a variety of carbohydrates, fatty acids, amino acids, and tricarboxylic acid cycle intermediates as sole or principal sources of carbon and energy (Table 2). Strain 384 (ATCC 14126) is the type strain of this species (Johnson and Shunk, 1936). It should be noted that some of these strains are able to grow at 40°C but do not utilize sucrose, traits which have been used by some authors for the identification of the pathogenic species, B. parahaemolytica (discussed by Baumann et al., 1973). In view of the fact that strains are not usually tested for their ability to luminesce, it is possible that some of the organisms which have been identified as B. parahaemolytica primarily on the basis of these two diagnostic traits are luminous bacteria.

The cluster of luminous strains which has been designated B. harveyi (Fig. 1) contains three subclusters which link at an S-value of 78% and are not separable from one another by any universally positive or negative traits. A phenotypic comparison of B. harveyi and the previously characterized species of Beneckea indicates that this species is indistinguishable from B. neptuna. The latter species consists of three groups (C-1, 2, and 3) which link at an S-value of 80% and are separable by a number of phenotypic traits (Baumann et al., 1973). Strains comprising groups C-1 and C-3 have been isolated from sea water and sea fish (Baumann et al., 1971a) while strains of group C-2 have been isolated from localized tissue infections (Baumann et al., 1973). A numerical analysis of the phenotypic data for strains of B. harveyi and groups C-1, 2, and 3 as well as all the species of Beneckea previously characterized, has shown that the luminous strains and

groups C-1, 2, and 3 are linked at an S-value of 75%. A dendrogram of this cluster, which links to other species of Beneckea at an S-value of 66% or less, is presented in Fig. 3. As seen from this figure, the strains of groups C-1, 2, and 3 tend to remain in distinct subclusters which are interspersed among the luminous strains. These results are consistent with the previous numerical analyses (which considered the luminous and non-luminous strains separately) and indicate that there is considerable phenotypic heterogeneity within both. Luminous and non-luminous strains having the greatest phenotypic similarity are found in the subcluster consisting of luminous strains 340-342, 353-355, and non-luminous strains 139-141 which are linked at an S-value of 94%. The next highest linkage of luminous and non-luminous strains is found in the subcluster consisting of C-2 and luminous strains 333, 350-352, 386, and 387 which are linked at an S-value of 86%. The various subclusters which form at S-values of 78 and 82% cannot be adequately differentiated from one another. On the other hand, the composite cluster, containing both luminous and non-luminous strains, is a readily identifiable unit which can be distinguished from other species of the genus Beneckea by a number of unrelated, phenotypic traits (Table 6). The similarity of these strains is also suggested from a study of the allosteric regulation of aspartokinase activity in marine, luminous bacteria and Beneckea species (Baumann and Baumann, 1973b). Species of Beneckea have three isofunctional aspartokinases, one of which (aspartokinase III) is inhibited by L-lysine. The concentration of L-lysine necessary to inhibit 50% of the activity of aspartokinase III in representative strains of B. harveyi, groups C-1 and C-2, is

similar and, with the exception of B. campbellii, distinct from other species of Beneckea. B. campbellii can, however, be distinguished from these strains since it has different relative amounts of the three isofunctional aspartokinases. These results as well as the evidence obtained from the phenotypic analysis suggest that, on the basis of their overall phenotypic similarity, luminous strains having the properties of the genus Beneckea and groups C-1, 2, and 3 could be assigned to a single species, B. harveyi. The data, however, indicate the possible existence of biotypes of both luminous and non-luminous strains. The formal taxonomic assignment of groups C-1, 2, and 3 to B. harveyi should, therefore, await the study of the relationship of these strains by means other than an extensive nutritional characterization.

Photobacterium fischeri. The 12 strains which comprise this species consist of one curved and 11 straight rods (Fig. 12). Eleven strains are motile by means of tufts of 2-8 sheathed, polar flagella (Figs. 7 and 19); one strain is non-motile. The GC content in the DNAs of seven representative strains is 39.8 ± 1.1 moles %. All strains are oxidase-positive and produce a light yellow, cell-associated pigment. None of the strains produces gas during the fermentation of D-glucose. Most or all strains grow at 25°C, produce an extracellular lipase, and are able to utilize D-ribose, D-glucose, D-mannose, D-galactose, D-fructose, maltose, cellobiose, N-acetylglucosamine, succinate, fumarate, mannitol, glycerol, and L-proline as sole or principal sources of carbon and energy. Strain 398 (ATCC 7744) has been designated the neotype strain of this species by Hendrie et al. (1971).

Colwell and Morita (1964) have isolated a number of marine strains to which they assigned the designation Vibrio marinus. The bases of these assignments are questionable since the initial description by Russell (1891) and its emendation by Ford (1927) are not adequate to permit the recognition of this species and since the type strain was not available for comparison. Two strains of this species have been deposited in culture collection, ATCC 15381 the proposed neotype, and ATCC 15382 an atypical strain of this species (Colwell, 1965). Hendrie et al. (1971) have synonymized these two strains with Photobacterium fischeri. An examination of their data indicates that although strain ATCC 15382 is similar to P. fischeri, strain ATCC 15381 differs from both ATCC 15382 and P. fischeri in a number of traits making their synonymization dubious. This question has been re-examined by performing a phenotypic characterization of both strains. The results show that strains ATCC 15381 and 15382 ferment D-glucose with the production of acid but not gas, grow at 4°C, give a negative Voges-Proskauer reaction, produce an extracellular lipase, reduce nitrate to nitrite, and do not denitrify. Both strains utilize D-ribose, D-galactose, D-glucose, D-fructose, maltose, N-acetylglucosamine, succinate, fumarate, glycerol, L-aspartate, L-glutamate, and L-proline as sources of carbon and energy. Strain ATCC 15381 differs from 15382 by its ability to produce a light yellow, cell-associated pigment and grow at 25°C, as well as by its inability to utilize D-mannose, sucrose, trehalose, and cellobiose as sources of carbon and energy. Strain ATCC 15381, unlike ATCC 15382, produces an extracellular gelatinase and chitinase and is able to utilize D-gluconate, acetate, caprate,

DL-malate, DL-lactate, α-ketoglutarate, D-α-alanine, L-serine, and L-threonine. In addition, strain ATCC 15381 has a single, sheathed, polar flagellum while strain ATCC 15382 has tufts of 2-8 sheathed, polar flagella. The moles % GC contents in the DNAs of strains ATCC 15381 and 15382, determined in the course of this study, are 42.2 and 40.5, respectively. These results indicate that strains ATCC 15381 and 15382 differ in a total of 18 phenotypic traits and cannot be considered members of the same species. Strain ATCC 15382 is, however, very similar to P. fischeri in both phenotypic properties and in the moles % GC content of its DNA, differing from the 12 characterized strains of this species by its ability to grow at 4°C and by its inability to luminesce. Unlike 11 strains of P. fischeri, it is also not able to grow at 30°C and utilize mannitol. Strain ATCC 14382 therefore appears to be a non-luminous strain of P. fischeri while strain 15381 is distinct from this species as well as from other species previously studied (Baumann et al., 1971a, b, 1973).

Photobacterium phosphoreum. The 74 strains belonging to this species are straight rods (Fig. 13) of which 69 are motile by 1-3 unsheathed, polar flagella (Figs. 8 and 20); the remaining five strains are non-motile. All strains accumulate PHB as an intracellular reserve product (Figs. 15 and 16). The GC content in the DNAs of eight representative strains is 41.5 ± 0.7 moles %. Most of the strains make gas during the fermentation of D-glucose, produce an extracellular chitinase, and are capable of growth at 4°C but not at 35°C. Twenty-five strains require L-methionine, either alone or in combination with one or two other amino acids. P. phosphoreum is nutritionally the least

versatile species of luminous bacteria (Fig. 2). All or most strains are able to utilize D-glucose, D-mannose, D-galactose, D-fructose, maltose, D-gluconate, N-acetylglucosamine, succinate, fumarate, DL-glycerate, and glycerol as sole or principal sources of carbon and energy. Since the type strain of P. phosphoreum is not available and no neotype strain has been designated, strain 439 (ATCC 11040) is proposed as the neotype strain of this species.

Photobacterium mandapamensis. The 28 strains which belong to this species consist of 27 straight rods (Fig. 14) and one curved rod which are motile by means of 1-3 unsheathed, polar flagella (Figs. 9, 10, and 21). All strains accumulate PHB as an intracellular reserve product (Figs. 15 and 16). The GC content of six representative strains is 42.9 ± 0.5 moles %. Most of the strains produce an extracellular lipase and chitinase and are capable of growth at 35°C but not at 4°C. All or most of the strains utilize D-ribose, D-glucose, D-mannose, D-galactose, D-fructose, D-gluconate, N-acetylglucosamine, acetate, succinate, fumarate, DL-lactate, pyruvate, glycerol, L-aspartate, and L-proline as sole or principal sources of carbon and energy. The cluster of strains designated P. mandapamensis includes strain 477 which was previously given this species designation by Hendrie et al. (1970). Since these authors did not designate a type strain, and since strain 477 is not typical of this species in that it makes gas during the fermentation of D-glucose, strain 480 (ATCC 27561) is designated the type strain of P. mandapamensis.

Hendrie et al. (1970) have stated that the oxidase reaction distinguishes P. phosphoreum from P. mandapamensis and that gas

production during the fermentation of D-glucose is characteristic of the latter species. The results of this study with strain 477 of P. mandapamensis and strains 439 and 497-501 of P. phosphoreum, which are common to both studies, are in agreement with those of Hendrie et al. (1970). It has been found, however, that only 2 out of 28 strains of P. mandapamensis produced gas during the fermentation of D-glucose, suggesting that the four strains characterized by these investigators are not typical of the species. The results of the present study indicate that 54% of the strains of P. mandapamensis and 7% of the strains of P. phosphoreum were oxidase positive. Since the oxidase test appears to detect the presence of cytochrome c and is usually uniform within a species (Baumann et al., 1968; Stanier et al., 1966), differential oxidized/reduced cytochrome spectra were determined for representative oxidase-positive and oxidase-netaive strains of both species. In all cases, bands characteristic of cytochrome c were detected indicating that the oxidase test is of no value for the differentiation of P. mandapamensis and P. phosphoreum. It is probable that Graham et al. (1972) and Wilson and Wilson (1972) were unable to place their marine, luminous isolates into one of the four species described by Hendrie et al. (1970) due to the use of the oxidase reaction and gas production as diagnostic traits in the manner proposed by these authors.

Support for speciation from comparative enzymology. Strains from each of the four species characterized in this study have been the subjects of biochemical investigations into the mechanism of luminescence [for a review see Hastings (1969)] as well as the control

of aspartokinase activity (Baumann and Baumann, 1973). From the studies on luminescence it became apparent that although the mechanism is similar in all luminous marine bacteria, the detailed properties of the system vary. The most extensive comparisons of the luminous system can be made on the basis of data obtained by Hastings and his collaborators for B. harveyi (strain 392) [designated MAV (Hastings et al., 1969) or, incorrectly P. fischeri MAV (Nealson et al., 1970)] and P. fischeri (strain 398) the neotype of this species. A summary of the comparative data for the luciferase and aspartokinase reactions is presented in Table 10. As seen from this table, the differences in the properties of these two reactions support the taxonomic groupings obtained in the present study.

Ecological considerations

Only a few ecological generalizations can be made concerning the distribution of the luminous species in different habitats since this problem has not been adequately studied. The collection of strains of B. harveyi has been obtained by direct isolation from surfaces of marine animals and from sea water. Attempts to isolate luminous bacteria off the coast of Oahu, Hawaii, indicate that in these waters B. harveyi is the sole or predominant luminous species. In a limited number of attempts to isolate luminous bacteria from the open ocean (five miles off Pokoii Bay, Oahu, at depths of 50-500 m) strains of B. harveyi, P. phosphoreum, and P. mandapamensis were found in approximately equivalent numbers. The latter two species have also been obtained by direct isolation from the surfaces as well as the

Table 10

Comparative enzymology of strains of marine, luminous bacteria characte

Property	B. harveyi	P. fischeri	P. phosphoreum
Luciferase			
Luciferases differ in antigenic properties, amino acid compositions, electrophoretic mobilities, and pH optima. Active hybrid luciferases are not formed with subunits from different species.	392[a]	398	
Dissociation constant (k_d) for the luciferase-$FMNH_2$ complex	392: 0.8 μM	398: 0.97 μM	496: 0.1 μM
Chain length of the aldehyde giving optimal light emission	392: C_{10}	398: C_{14}	496: C_{13} or longer
Decay rate of the long-lived intermediate of the luciferase reaction (a) in the presence of a C_{10} aldehyde	392: 0.25 sec^{-1} 333: 0.28 sec^{-1}	398: 0.14 sec^{-1}	496: 2.0 sec^{-1}

Table 10

ins of marine, luminous bacteria characterized in this study

	P. fischeri	P. phosphoreum	P. mandapamensis	References
	398			Hastings et al., 1969; Gunsalus-Miguel et al., 1972; Meighen et al., 1970.
	398: 0.97 μM	496: 0.1 μM		Meighen and Hastings, 1971; Watanabe and Nakamura, 1972.
	398: C_{14}	496: C_{13} or longer		Hastings et al., 1963, 1969; Watanabe and Nakamura, 1972.
$^{-1}$ $^{-1}$	398: 0.14 sec^{-1}	496: 2.0 sec^{-1}	491: 0.24 sec^{-1}	Cline and Hastings, 1971; Hastings

Table 10. (Continued) Comparative enzymology of strains of marine, in this study

Property	B. harveyi	P. fischeri	P. phosphoreum
(b) in the presence of a C_{12} aldehyde	392: 0.04 sec^{-1} 333: 0.04 sec^{-1}	398: 050 sec^{-1}	
Purified luciferases differ in absorption spectra		398	496
Distinct "activator" for induction of luciferase	392	398	
Aspartokinase[b]			
Concentration of L-lysine necessary for inhibition of 50% of the activity of aspartokinase III	384(L66[c]): 6 μM	61: 103 μM 397(L54): 104 μM	
Predominant isofunctional enzyme	384,392(L123): III	61,397: III	404(L196), 447(L76): I

[a]Strain designation assigned in the present study.

[b]I = L-Threonine sensitive aspartokinase; III = L-Lysine sensitive aspartokinase.

[c]Strain designation used by Baumann and Baumann, 1973b.

parative enzymology of strains of marine, luminous bacteria characterized in this study

	P. fischeri	P. phosphoreum	P. mandapamensis	References
$^{-1}$ $^{-1}$	398: 050 sec^{-1}		491: 0.40 sec^{-1}	and Mitchell, 1971; Nakamura and Matsuda, 1971.
	398	496		Hastings et al., 1965; Nakamura and Matsuda, 1971.
	398		482	Eberhard, 1972.
μM	61: 103 μM 397(L54): 104 μM		480(L165): 6 μM	Baumann and Baumann, 1973b.
):	61,397: III	404(L196), 447(L76): I	474(L29), 480: I	Baumann and Baumann, 1973b.

nt study.

III = L-Lysine sensitive aspartokinase.

aumann, 1973b.

intestines of marine animals. Since 37 out of the 40 strains isolated from fish, squid, or octopus incubated overnight at 10-15°C were P. phosphoreum, this method of enrichment appears to be relatively specific for this species. Of the 18 strains received as symbionts of the luminous organs of fish and squid, six were P. phosphoreum and 12 were P. mandapamensis. These strains were phenotypically similar to strains of P. phosphoreum and P. mandapamensis isolated from sea water and the surfaces and intestines of sea animals, suggesting that light organs of fish and squid which luminesce due to the presence of luminous bacteria are colonized by free-living strains. Furthermore, the isolation of only two species from luminous organs suggests that the ability to colonize these organs is restricted to the two phenotypically related species, P. phosphoreum and P. mandapamensis. Haneda and Tsuji (1971) observed large numbers of bacteria in electron micrographs of thin sections through the light organs of Photoblepharon palpebratus and Anomalops kaloptra but were unable to cultivate these organisms. An examination of their electron micrographs indicates that these bacteria have inclusions similar in appearance to the PHB granules found in P. phosphoreum and P. mandapamensis. The remaining species, P. fischeri, appears to be rarely encountered in the marine environment; most of the strains of this species in this collection were isolated from sea water.

The strains characterized in this study have been isolated from many different sources and geographical locations (restricted, however, to temperate and tropical regions) and are probably a fairly representative sampling of luminous, marine bacteria. In assembling this

collection of strains, a single property, the ability to luminesce, was used as the criterion for admission. It is therefore possible that strains which are non-luminous but phenotypically similar to luminous isolates may be found in nature. In the case of luminous strains having properties of the genus Beneckea, a comparison with previously characterized isolates of this genus has indicated that a number of non-luminous strains have a great deal of phenotypic similarity to the luminous isolates suggesting their assignment to a single species (B. harveyi). The question of the occurrence of non-luminous strains having a high phenotypic similarity to P. fischeri, P. phosphoreum, or P. mandapamensis remains unanswered.

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