

Historical microbiology: revival and phylogenetic analysis of the luminous bacterial cultures of M. W. Beijerinck

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Introduction

The ability of certain bacteria to produce light has been known since 1875, when Pflüger (1875) made the connection between the luminescence coming from the slime of fish and bacteria present in the slime (Harvey, 1957; Robertson *et al.*, 2011). Following Pflüger's work, several other scientists working in the late 1800s and early 1900s isolated and named luminous bacteria, including 'Bacterium lucens', 'Micrococcus phosphorescens', 'Micrococcus pflügeri', 'Bacillus phosphorescens', and 'Bacterium phosphoreum' (Cohn, 1878; Neush, 1879; Ludwig, 1884; Fischer, 1887; Molisch, 1912; Harvey, 1952, 1957; Robertson *et al.*, 2011). Particularly notable among early researchers of bacterial luminescence was Martinus W. Beijerinck, a founder of general microbiology, who carried out research on the physiology of light-emitting

Abstract

Luminous bacteria isolated by Martinus W. Beijerinck were sealed in glass ampoules in 1924 and 1925 and stored under the names *Photobacterium phosphoreum* and '*Photobacterium splendidum*'. To determine if the stored cultures were viable and to assess their evolutionary relationship with currently recognized bacteria, portions of the ampoule contents were inoculated into culture medium. Growth and luminescence were evident after 13 days of incubation, indicating the presence of viable cells after more than 80 years of storage. The Beijerinck strains are apparently the oldest bacterial cultures to be revived from storage. Multi-locus sequence analysis, based on the 16S rRNA, *gapA*, *gyrB*, *pyrH*, *recA*, *luxA*, and *luxB* genes, revealed that the Beijerinck strains are distant from the type strains of *P. phosphoreum*, ATCC 11040^T, and *Vibrio splendidus*, ATCC 33125^T, and instead form an evolutionarily distinct clade of *Vibrio*. Newly isolated strains from coastal seawater in Norway, France, Uruguay, Mexico, and Japan grouped with the Beijerinck strains, indicating a global distribution for this new clade, designated as the *beijerinckii* clade. Strains of the *beijerinckii* clade exhibited little sequence variation for the seven genes and approximately 6300 nucleotides examined despite the geographic distances and the more than 80 years separating their isolation. Gram-negative bacteria therefore can survive for many decades in liquid storage, and in nature, they do not necessarily diverge rapidly over time.

bacteria as one facet of his work. Beijerinck coined the name *Photobacterium*, a genus within which all luminous bacteria were then grouped, and among other *Photobacterium* species, he named *Photobacterium phosphoreum* and '*Photobacterium splendidum*' (Beijerinck, 1889a, b, 1891, 1916; van Iterson *et al.*, 1940; Robertson, 2003; Robertson *et al.*, 2011). The strains now designated as the types of *P. phosphoreum*, ATCC 11040^T, and '*P. splendidum*' (now classified as *Vibrio splendidus*), ATCC 33125^T, however, were not isolated until 1934 (Ast & Dunlap, 2005) and 1953 (Reichelt & Baumann, 1973; Baumann *et al.*, 1980), respectively, many years after Beijerinck's retirement in 1921.

Presently, over 20 species of luminous bacteria, all members of *Gammaproteobacteria*, are known. Marine luminous species are found in *Aliivibrio*, *Photobacterium*, *Vibrio* (*Vibrionaceae*), and *Shewanella* (*Shewanellaceae*),

and terrestrial light-producing species are members of *Photorhabdus* (*Enterobacteriaceae*) (Dunlap & Kita-Tsukamoto, 2006; Urbanczyk *et al.*, 2007, 2011; Ast *et al.*, 2009; Dunlap, 2009; Yoshizawa *et al.*, 2009). Current understanding of the evolutionary relationships of luminous bacteria, as well as recent descriptions of new species, have utilized phylogenetic analysis of multiple, functionally independent housekeeping genes, including the 16S rRNA gene, *gyrB*, *pyrH*, and *recA*, among others (e.g. Ast & Dunlap, 2005; Thompson *et al.*, 2005; Ast *et al.*, 2007b, 2009; Urbanczyk *et al.*, 2007; Yoshizawa *et al.*, 2009). Particularly useful for resolving closely related luminous bacteria is sequence analysis of genes of the bacterial *lux* operon, *luxCDABE*, due to their relatively rapid sequence divergence compared with most housekeeping genes (Ast & Dunlap, 2004, 2005; Dunlap *et al.*, 2004; Ast *et al.*, 2007a; Urbanczyk *et al.*, 2007).

The recent growth in knowledge of the species diversity and ecological importance of light-emitting bacteria (Dunlap & Kita-Tsukamoto, 2006; Dunlap, 2009; Urbanczyk *et al.*, 2011) leads to questions about the earliest studies of these bacteria. What species did early microbiologists actually study, and how do they compare evolutionarily with present-day bacteria? Direct connections to studies of luminous bacteria carried out during the late 1800s and early 1900s, however, are difficult to make. Standards for species identification and description have changed many times in the past century (Sneath, 1992), and in 1980, many older names lost nomenclatural standing (Skerman *et al.*, 1980; Sneath, 1986). Furthermore, as early species descriptions typically were based on very few phenotypic traits, meaningful comparisons between currently available strains and bacteria studied a century ago often are difficult to make. A further and very significant limitation in linking the present to the past in microbiology is that most strains isolated at the end of the 19th and the beginning of the 20th century apparently have been lost due to the lack of effective long-term storage methods at that time. It is therefore often highly problematic to know with certainty what species of bacteria early microbiologists actually studied.

While tracing the origin and history of the type strain of *P. phosphoreum* (Ast & Dunlap, 2005), we learned that glass ampoules containing cultures of luminous bacteria isolated by Beijerinck and stored in the mid-1920s had been retained as part of the historic collection from the Laboratory for Microbiology of the Technische Hogeschool at Delft (LMD). The availability of these ampoules presented an opportunity to attempt to revive these strains and thereby test the possibility that direct connections could be made between the origins of general microbiology and 21st century practice of the science.

Materials and methods

Revival of strains

Glass ampoules containing approximately 15 mL broth each, sealed in 1924 and 1925, and containing strains of luminous bacteria isolated by M. W. Beijerinck (Fig. 1), were obtained from the Netherlands Culture Collection of Bacteria (NCCB). The ampoules, which contained fish extract water [prepared by boiling fish in natural, clean seawater, and adding 10% w/v chalk (CaCO_3 powder)] (Robertson *et al.*, 2011), had been inoculated with luminous bacteria that were then allowed to grow for 2 days; the ampoules were then sealed and stored in the collection at Delft, which became the LMD collection. One of the original ampoules of '*P. splendidum*' (designated here as strain MWB 29, Table 1) had been opened in 1979 by J. van der Toorn (pers. commun.), the last curator of the LMD collection, who revived the culture and re-stored it in multiple ampoules as LMD 22.30 (=NCCB 22030). Three original ampoules sealed in 1924 and 1925 and containing bacterial cultures designated here as strains MWB 21 ('*P. phosphoreum*'), MWB 28 ('*P. splendidum*'), and MWB 30 ('*P. splendidum*') and one of the re-stored ampoules of MWB 29 (Table 1) were opened in this study. Dates of storage of the original cultures, from the labels on the original ampoules, are: MWB 21, September 1, 1924; MWB 28, September 4, 1924; MWB 29, March 19, 1925; MWB 30, December 12, 1925. Approximately

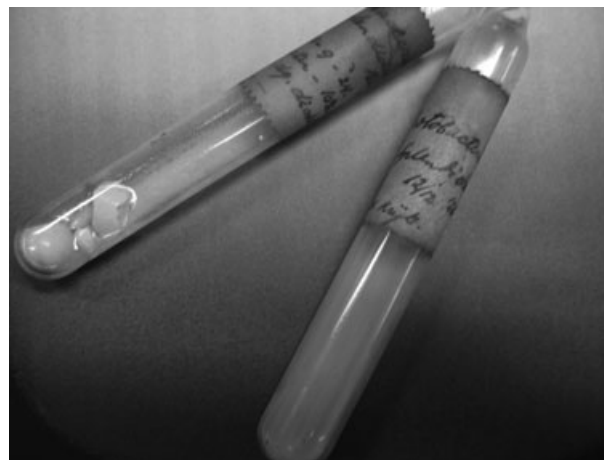


Fig. 1. The original glass ampoules containing cultures of luminous bacteria isolated by and possibly stored by M. W. Beijerinck. The cultures were grown up on fish extract water (prepared by boiling fish in seawater) containing 10% w/v chalk (the white material in the ampoules). The ampoule labels indicate: *Photobacterium splendidum* September 4, 1924 (designated here as MWB 28) (upper ampoule); and, *P. splendidum* December 12, 1925 (designated here as MWB 30) (lower ampoule).

Table 1. Strains of the *beijerinckii* clade examined in this study

Strain	Year of isolation	Geographic origin*	Source or reference
MWB 21	Before 1924	Scheveningen, The Netherlands	This study
MWB 28	Before 1924	Scheveningen, The Netherlands	This study
MWB 29	Before 1925	Scheveningen, The Netherlands	This study
MWB 30	Before 1925	Scheveningen, The Netherlands	This study
B-356	Before 1973	Bermuda	Reichelt & Baumann (1973), this study
fram1	2006	Oslo, Norway	This study
GB-215	1992	Guaymas Basin, Mexico	This study
idb.1.4	2004	Île de Bendor, France	This study
SB-84	2004	Suruga Bay, Japan	This study
seaur.1.1	2005	José Ignacio, Uruguay	This study

*All strains were isolated from surface coastal seawater except B-356, which was isolated from the surface of a fish (Reichelt & Baumann, 1973), and GB-215, which was isolated from offshore water at 250 depth.

5 mL of the liquid from each ampoule was added to a Kluver flask containing 250 mL of MWB broth, which contained 400 mM NaCl, 9 mM KCl, 27.5 mM Na₂SO₄, 2.25 mM NaHCO₃, 28.5 mM MgCl₆·6H₂O, and 5 g peptone and 1.0 g L(-)-asparagine per liter of demineralized water. Kluver flask cultures were incubated at room temperature and sparged with air. Control cultures were made in Kluver flasks using the same procedures, but with sterile medium in place of the contents of the ampoules. Ampoule contents were also streaked onto MWB agar plates, which contained 15 g L⁻¹ agar. One milliliter portions of the ampoule contents also were inoculated into Erlenmeyer flasks containing 25 mL of MWB and incubated at room temperature with shaking. The revived strains were restored as NCCB 100079 (MWB 21), NCCB 100087 (MWB 28), NCCB 100088 (MWB 29), and NCCB 100089 (MWB 30).

Isolation of bacteria from coastal seawater and growth conditions

Other luminous bacteria newly reported in this study were isolated from coastal seawater collected from various locations (Table 1). Aliquots, 100–300 µL, of aseptically collected seawater samples were spread on agar plates of LSW-70 (Luria 70% seawater) medium (Dunlap *et al.*, 2004), which contained 10 g tryptone, 5 g yeast extract, 350 mL double-strength artificial seawater (Nealson, 1978), 650 mL de-ionized water, and 40 g L⁻¹ agar (Baumann *et al.*, 1984). Plates were incubated overnight at 22–25 °C and examined for luminous colonies that were then picked and purified on the same medium. The newly isolated strains and the four revived Beijerinck strains were stored in cryoprotective medium at –75 °C (Dunlap & Kita-Tsukamoto, 2006) in the Genomic Diversity Collection at the University of Michigan (see <http://www.umich.edu/~pvdunlap>). For routine culture, bacterial

strains were grown at room temperature (22 °C) with aeration in LSW-70 broth or on LSW-70 plates made with 15 g L⁻¹ agar.

DNA amplification and sequencing

Genomic DNA was purified from 1 mL of overnight broth cultures using a Qiagen (Valencia, CA) DNeasy kit. For DNA amplification by PCR, MasterTaq polymerase (Eppendorf, Hauppauge, NY) and the following protocol were used: 95 °C initial denaturing step for 2 min; 35 cycles with a 94 °C denaturing step for 20 s, a variable temperature annealing step for 15 s, and an extension step at 68 °C for 1 min; a 7 min final extension step at 68 °C; snap cooling to 4 °C. PCR primer sequences for the 16S rRNA gene, glyceraldehyde-3-phosphate dehydrogenase (*gapA*) gene, DNA gyrase B subunit (*gyrB*) gene, uridylylase kinase (*pyrH*) gene, recombinase A (*recA*) gene, and luciferase α and β subunit (*luxA* and *luxB*) genes, and annealing temperatures and exceptions to the protocol above are listed in Table S1 (Supporting Information). PCR products were visualized using electrophoresis on 1% agarose gels stained with ethidium bromide, and were purified using Montage PCR Filter kits (Millipore, Hayward, CA). PCR products were sequenced using the respective PCR primers. Sequencing was carried out by the staff of the University of Michigan Sequencing Core using dye terminator cycle sequencing on a Perkin-Elmer (Waltham, MA) ABI 3730 or 3700 DNA Analyzer. The GenBank accession numbers for sequences analyzed in this study are listed in Table S2.

Evolutionary analysis

16S rRNA gene sequences were aligned by eye, and sequences of protein coding genes (*gapA*, *gyrB*, *pyrH*, *recA*, *luxA*, and *luxB*) were aligned by inferred amino acid

sequences. For the Bayesian analysis, the genes were partitioned into non-coding and coding regions. Model parameters for each region were set to the general time-reversible with gamma-distributed rate variation across sites, including invariable sites, with rates allowed to vary between the partitions. Two independent chains were run for 200 000 generations, with sampling every hundred generations. The consensus tree was based on the 1500 trees remaining after initial burn-in trees were discarded. For the parsimony analysis, uninformative characters were inactivated, and gaps were considered informative. Genes were analyzed simultaneously using the program TNT (Goloboff *et al.*, 2006), with 10 000 replicates of tree bisection-regrafting (TBR). The resulting shortest hypoth-

eses were checked with 10 000 iterations of the parsimony ratchet. Jackknife resampling values were based on 10 000 replicates (34% chance of deletion per replicate).

Results

Revival of Beijerinck's luminous bacterial cultures

Three ampoules containing luminous bacteria isolated by M. W. Beijerinck and sealed in 1924 and 1925 (Table 1) were opened, and the contents were inoculated into MWB broth in Kluver and Erlenmeyer flasks or streaked onto MWB agar plates. Kluver flasks inoculated with

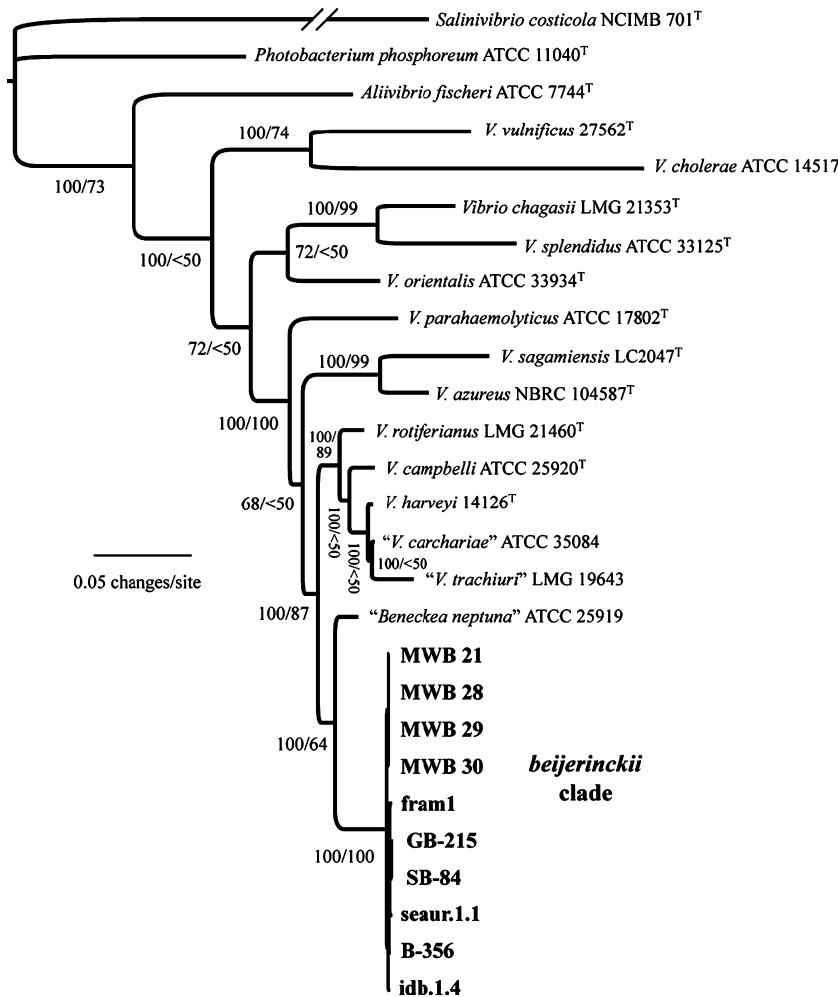


Fig. 2. Phylogenetic placement of the strains of the *beijerinckii* clade based on housekeeping gene sequences. Numbers at nodes are Bayesian posterior probabilities and parsimony jackknife resampling values. Shown here is the Bayesian consensus tree resulting from simultaneous analysis of the concatenated housekeeping genes (16S rRNA gene, *gapA*, *gyrB*, *pyrH*, and *recA*). In both Bayesian and parsimony analyses, the Beijerinck strains, together with several recently isolated strains, form a strongly supported group that is distinct from other *Vibrio* species, designated here as the *beijerinckii* clade.

5 mL of ampoule contents (MWB 21, MWB 28, and MWB 30) yielded growth and luminescence after 13 days of incubation. A fourth ampoule, containing a culture isolated by M. W. Beijerinck, stored in 1925 (Table 1) and then revived and re-stored in 1979 (MWB 29), was also opened and handled in the same manner; growth and luminescence were evident after 5 days of incubation. Uninoculated control cultures neither grew nor produced light. Thus, the Beijerinck strains were viable after more than 80 years of storage (MWB 21, MWB 28, and MWB 30) and 25 years of storage (MWB 29). Subsequent cultures of all four strains grew and produced light overnight on MWB agar plates and broth. Plates streaked with ampoule contents and Erlenmeyer flasks inoculated with 1 mL of ampoule contents, however, did not yield growth or luminescence. These results and the long lags in growth of the cultures suggest that the ampoules contained only a small number of viable cells at the time they were opened.

Phenotypically, the revived strains were luminous, Gram-negative, asporogenous, motile rods that grew and produced light up to 35 °C and grew, but did not produce light at 40 °C. After a few to several days of incubation, cultures exhibited a diffusible brown pigment and a strong odor. In these traits, the Beijerinck strains appeared similar to *Vibrio harveyi* (Baumann *et al.*, 1980, 1984; Dunlap *et al.*, 2008). Consistent with that possibility, sequence analysis of the *gyrB* gene placed the Beijerinck strains within genus *Vibrio* and suggested a close relationship with members of the *V. harveyi* group. This group includes *V. harveyi*, *Vibrio campbellii*, *Vibrio rotiferianus*, and strains now classified as *V. harveyi*, but originally described as separate species, i.e. '*Vibrio carchariae*', '*Vibrio trachuri*', and '*Beneckeia neptuna*' (Baumann *et al.*, 1971; Reichelt & Baumann, 1973; Pedersen *et al.*, 1998; Thompson *et al.*, 2002). This preliminary taxonomic placement, however, differed from the names assigned to these strains by M.W. Beijerinck, *P. phosphoreum* (MWB 21), and '*P. splendidum*' (MWB 28, MWB 29, and MWB 30).

Multi-locus evolutionary analysis

Therefore, to determine the evolutionary relationships of the Beijerinck strains with currently known luminous bacteria, we carried out a multi-locus analysis based on sequences of the 16S rRNA, *gapA*, *gyrB*, *pyrH*, *recA*, *luxA*, and *luxB* genes. Included in the analysis were representatives of each of the previously identified members of the *V. harveyi* group, the type strains of *P. phosphoreum* and *V. splendidus*, and representatives of other luminous and non-luminous *Vibrionaceae* species, including the recently identified luminous species, *Vibrio azureus* and *Vibrio sagamiensis* (Yoshizawa *et al.*, 2009, 2010).

The analysis based on housekeeping gene sequences demonstrated that the Beijerinck strains are evolutionarily distant from the type strains of *P. phosphoreum* and *V. splendidus* (Fig. 2). Furthermore, the four Beijerinck strains were resolved as a clade within *Vibrio* that is evolutionarily distinct from other *Vibrio* species, including members of the *V. harveyi* group (Fig. 2). Most closely related to the Beijerinck strains is ATCC 25919, previously classified as the type strain of '*B. neptuna*' and now considered, apparently incorrectly based on the data presented here, to be a member of *V. harveyi*, followed by members of the *V. harveyi* group. Analysis of the sequences of the *luxA* and *luxB* genes confirmed the separation between the Beijerinck strains and other members of *Vibrionaceae*, with substantial evolutionary separation from *V. splendidus* and *V. harveyi*, the most closely related luminous species for which *luxA* and *luxB* gene sequences were available (Fig. 3).

Geographic distribution and phylogenetic coherence

The Beijerinck strains were isolated over 80 years ago from the same general location, coastal seawater at

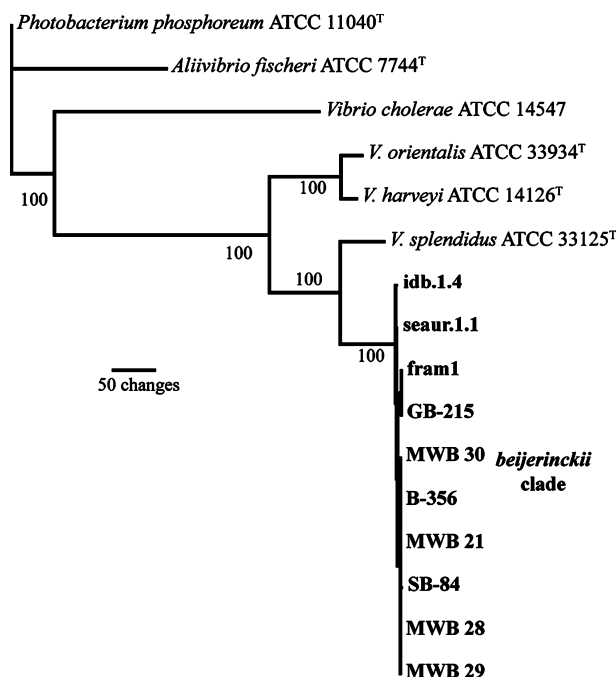


Fig. 3. Phylogenetic placement of the *beijerinckii* clade based on *lux* gene sequences. Shown is one of 10 most parsimonious trees resulting from analysis of the concatenated dataset comprising *luxA* and *luxB* (tree length = 1008, consistency index = 0.78, retention index = 0.76). The trees differed in only minor ways within the *beijerinckii* clade. Numbers at nodes are jackknife support values.

Table 2. Nucleotide sequence divergence from MWB 21 (NCCB 100079)* of the *beijerinckii* clade

Species and strain	Gene					
	16S rRNA	<i>gapA</i>	<i>gyrB</i>	<i>pyrH</i>	<i>recA</i>	<i>luxAB</i>
<i>beijerinckii</i> clade						
MWB 28 (NCCB 100087)	0	0	0	0	0	0
MWB 29 (NCCB 100088)	0	0	0	0	0	0
MWB 30 (NCCB 100089)	0	0	0	0	0	0
B-356	1	0	7	2	0	0
fram1	0	0	8	1	3	5
GB-215	0	0	10	1	0	2
idb.1.4	0	0	9	1	0	6
SB-84	0	0	10	0	0	3
seaur.1.1	1	1	9	1	0	2
' <i>Beneckea neptuna</i> ' ATCC 25919	5	4	53	36	66	– [†]
<i>Vibrio rotiferianus</i> LMG 21460 ^T	12	14	81	27	63	–
<i>Vibrio campbellii</i> ATCC 25920 ^T	13	28	97	35	67	–
' <i>Vibrio trachiuri</i> ' LMG 19643	21	28	65	39	58	–
<i>Vibrio azureus</i> NBRC 104587 ^T	10	66	57	52	na [‡]	na
<i>Vibrio harveyi</i> ATCC 14126 ^T	18	28	87	43	60	234
' <i>Vibrio carchariae</i> ' ATCC 35084	20	29	87	40	61	–
<i>Vibrio parahaemolyticus</i> ATCC 17802 ^T	14	32	141	59	73	–
<i>Vibrio sagamiensis</i> LC2047 ^T	12	74	112	57	57	na
<i>Vibrio chagasii</i> LMG 21353 ^T	41	56	165	81	121	–
<i>Vibrio orientalis</i> ATCC 33924 ^T	36	40	116	66	110	246
<i>Vibrio vulnificus</i> ATCC 27562 ^T	49	43	205	90	126	–
<i>Vibrio splendidus</i> ATCC 33125 ^T	48	69	205	79	134	144
<i>Vibrio cholerae</i> ATCC 14547	99	113	236	113	127	369
<i>Aliivibrio fischeri</i> ATCC 7744 ^T	61	74	221	92	135	473
<i>Photobacterium phosphoreum</i> ATCC 11040 ^T	87	49	255	120	165	520
<i>Salinivibrio costicola</i> NCIMB 701 ^T	130	133	235	na	208	–

*Total number of aligned sites: 16S rRNA gene, 1538 (1316 in *V. azureus* and *V. sagamiensis*); *gapA*, 877; *gyrB*, 1218 (846 in *S. costicola*; 801 in *V. azureus* and *V. sagamiensis*); *pyrH*, 604 (495 in *V. azureus* and *V. sagamiensis*); *recA*, 842 (498 in *V. sagamiensis*); *luxAB*, 1313 (excludes the *luxAB* spacer region). Due to natural variation and differences in primers used, not all genes for all taxa have exactly the same length.

[†]–, non-luminous, *luxAB* genes not known to be present.

[‡]na, nucleotide sequence not available.

Scheveningen, the Netherlands (Table 1; Robertson *et al.*, 2011). To determine if phylogenetically similar strains currently exist, we isolated luminous bacteria from coastal seawater at various locations (Table 1) and tested them for phylogenetic placement based on sequence analysis of the 16S rRNA, *gapA*, *gyrB*, *pyrH*, and *recA* genes. Several newly isolated strains, from coastal seawater of Norway, France, Mexico, Uruguay, and Japan, were found that tightly grouped with the Beijerinck strains (Fig. 2). In addition, strain B-356, isolated over 35 years ago from the surface of a fish in Bermuda and previously classified as *V. harveyi* (Reichelt & Baumann, 1973), was found to be a member of this group. Analysis of the *luxA* and *luxB* genes confirmed the grouping of these strains with the Beijerinck strains (Fig. 3). Members of this previously unrecognized group therefore occur in coastal seawater with an apparently worldwide geographic distribution. On the basis of these results and pending detailed

taxonomic study, we provisionally designate this group of strains as the *beijerinckii* clade, in recognition of M. W. Beijerinck.

The tight phylogenetic clustering of the strains of the *beijerinckii* clade, which were isolated many years apart and from widely different locations (Figs 2 and 3), was unexpected, as it suggested that members of this clade have not diverged substantially in nature at the nucleotide sequence level over several decades. To assess this clustering in greater detail, we quantified the individual nucleotide differences between strains for the several genes sequenced (Table 2). In comparison with the Beijerinck strains, the other strains of the *beijerinckii* clade had no or few nucleotide differences over the seven genes and the approximately 6300 nucleotides examined. Most variable in sequence among strains of the *beijerinckii* clade was the *gyrB* gene, followed by a small number of differences in the *luxA* and *luxB* genes. The limited sequence variation

within the *beijerinckii* clade, however, contrasted with the many nucleotide sequence differences between MWB 21 and strains classified as other species (Table 2), for example, '*B. neptuna*' ATCC 25919 and *V. rotiferianus* LMG 21460^T, two taxa most closely related to the *beijerinckii* clade. These results indicate that although the *beijerinckii* clade is evolutionarily distinct from other species of *Vibrio*, the different strains of the *beijerinckii* clade have not diverged substantially from each other in DNA sequence despite the geographic distances and more than 80 years separating their isolation.

Discussion

Using large inoculums and an extended incubation, we succeeded in reviving bacteria isolated by M. W. Beijerinck, a founder of general microbiology, and stored over 80 years ago. The Beijerinck strains are apparently the oldest bacterial cultures to have been revived. They represent a novel bacterial lineage within *Vibrio* (*Gammaproteobacteria: Vibrionaceae*), designated here as the *beijerinckii* clade. The revival of these strains provides a direct and tangible connection to early studies of luminous bacteria (Beijerinck, 1889a, b, 1891, 1916; van Iterson *et al.*, 1940; Harvey, 1952), and they serve as a reference point for assessing the extent of DNA sequence divergence over time in members of this previously unrecognized, but widely distributed coastal marine bacterial clade.

To trace the provenance of the Beijerinck strains, we examined Beijerinck's laboratory notebooks and other historic records (Robertson *et al.*, 2011). The available information indicates that Beijerinck isolated most of the luminous strains he studied from Dutch coastal seawater, especially at Scheveningen, using direct plating on fish extract agar. The species epithet, '*P. splendidum*', was first used in Beijerinck's notebooks in the summer of 1898. Beijerinck had been working with a number of luminous strains, including one designated '*phosphorescens*' (= *P. phosphoreum*) since 1890. According to J. van der Toorn (pers. commun.), the strain of '*P. splendidum*' revived in 1979 had been deposited in the Delft Collection in 1922, and he speculated that the '*P. splendidum*' ampoules prepared in 1924 and 1925 may have been sub-cultures of this strain. Consistent with this possibility, we found the four Beijerinck strains to be essentially identical despite the different species names and dates of storage; the sequences of the examined genes, including the 12 ambiguous bases in the 16S rRNA gene, are identical, and the strains appear to exhibit only a few biochemical differences (data not shown). Alternatively, Beijerinck might have isolated essentially identical strains at different times from seawater along the Dutch coast. As Beijerinck continued to work on luminous bacteria from his home

laboratory at Gorssel following his retirement (Robertson *et al.*, 2011), he could have isolated the strains either before his retirement in 1921, or afterwards, between 1921 and 1924 or 1925. The lack of strain designations and Beijerinck's common practice of isolating new strains of luminous bacteria from nature to replace strains that had gone dark in laboratory culture (Robertson *et al.*, 2011), however, preclude determining exactly how long before 1924 and 1925 the strains were isolated. With regard to who stored the strains, the handwriting on the ampoule labels closely resembles that of Beijerinck on other labels and in correspondence, and it differs from the handwriting of others who may have been involved in storing strains at the time, specifically C. B. van Niel and L. E. van Dooren de Jong, who were the assistants of A. J. Kluyver, Beijerinck's successor at Delft, during the early to mid 1920s (Kamp *et al.*, 1959; Battley, 1987; Robertson *et al.*, 2011). We therefore believe that the strains may have been stored by Beijerinck. The ampoules later were moved to the NCCB after the national collections, LMD and Phabagen, merged in 1998 (Robertson *et al.*, 2011).

As far as we have been able to ascertain, the Beijerinck strains are the oldest cultures of bacteria, luminous or non-luminous, to have been revived from storage. Despite being Gram-negative non-spore-forming bacteria, they survived for over 80 years. The use of sealed glass ampoules that were kept in the dark was presumably important for their survival, by preventing desiccation and avoiding the possible effects of exposure to light. The use of large inoculums, extended incubation, and sparging with air may have been important for their revival. Many studies of long-term survival of bacterial cultures have been carried out using a variety of different storage methods and experimental conditions; survival typically has been assessed over months to several years (Morita, 1997). There are also several reports and some studies of survival of Gram-negative non-spore-forming bacteria, e.g. *Agrobacterium*, *Proteus*, *Pseudomonas*, and *Salmonella*, in sealed agar stabs, under paraffin oil, or in liquid, for longer periods, up to 45 years (Hartsell, 1956; Liu, 1984; Calhoun, 1985; Cash, 1985; English & McManus, 1985; Iacobellis & DeVay, 1986; Sutton *et al.*, 2000). For example, Haneda (1981) used a method very similar to that of Beijerinck, storing cultures of luminous *Photobacterium*, grown in a medium of fresh squid muscle tissue boiled in seawater, in sealed glass ampoules; the bacteria were viable after 28 years of storage. Furthermore, survival of various non-spore-forming Gram-negative bacteria, e.g. *Shigella*, for over 60 years has been reported (Murray, 1985). These studies and the survival of the Beijerinck strains for over 80 years indicate that spore formation is not essential for very long-term survival of bacteria in liquid or agar storage.

Evolutionary analysis based on multiple, unlinked loci demonstrates that the *beijerinckii* clade is phylogenetically distinct from other species of *Vibrio* (Figs 2 and 3). Nonetheless, much additional work will be necessary to determine if the *beijerinckii* clade warrants status as a new species, including DNA-hybridization analysis and comparison of biochemical traits. As revealed here (Table 2) and elsewhere (e.g. Ast *et al.*, 2007b), the sequence of the 16S rRNA gene may differ very little between otherwise well-delimited species of *Vibrionaceae*, and so a 16S rRNA gene sequence identity of 97% or greater, a trait commonly used in bacterial taxonomy in defining a species, is not always effective for fine-scale, i.e. species level, resolution. In contrast, DNA hybridization analysis can provide support for species resolved using phylogenetic analysis. For example, in a recent study of closely related *Photobacterium* species that differed <1% in 16S rRNA gene sequence identity, multi-locus evolutionary analysis demonstrated robust phylogenetic separation, and that separation was supported by DNA hybridization analysis, values for which were well below 70% (Ast *et al.*, 2007b). The identification of a new and geographically widely distributed clade of *Vibrio* indicates that much as-yet undiscovered phylogenetic diversity is likely to be present in this well-studied genus.

Regardless of the eventual taxonomic placement of the *beijerinckii* clade, the revival of bacteria isolated by M. W. Beijerinck and stored over 80 years ago provides a direct and tangible link to the origins of general microbiology at the end of the 19th century and the beginning of the 20th century. As reported here for the Beijerinck strains, the revived strains can be studied using molecular phylogenetic methods to determine their evolutionary relationships with more recently isolated and currently known bacteria. We demonstrate with this approach that the Beijerinck strains represent a new bacterial clade, the *beijerinckii* clade, and that members of this clade currently exist in coastal seawater at many locations worldwide. The analysis also clarifies the relationship between the original names given to these bacteria and their current nomenclature; bacteria phenotypically similar, but evolutionarily distinct from those isolated and studied by Beijerinck now carry the names he first used, *P. phosphoreum* and *V. splendidus* ('*P. splendidum*') (Reichelt *et al.*, 1976; Baumann *et al.*, 1980; Ast & Dunlap, 2005). Revival of the Beijerinck strains also allowed a detailed nucleotide sequence comparison to be made with recent isolates of the *beijerinckii* clade. Despite the geographic distances and the more than 80 years separating the isolations of some of these strains, there were few differences in the sequences of the several different genes and approximately 6300 nucleotides examined. The limited variation indi-

cates that bacteria do not necessarily diverge rapidly in nature over time.

Links to the early days of microbiology of the kind described here are rare, due to the loss of cultures prior to the advent and widespread use of methods for long-term storage of bacteria, i.e. lyophilization in the 1940s and ultra-low temperature in the 1970s. Furthermore, bacterial nomenclature and methods for the description and identification of bacteria have changed various times and substantially over the past century (Skerman *et al.*, 1980; Sneath, 1986, 1992); as a consequence, it often is not possible to know with certainty what species of bacteria early microbiologists studied. Nonetheless, other bacterial cultures stored at the end of the 19th century and the beginning of the 20th century are likely to exist in some national and private collections (Robertson *et al.*, 2011), and, based on the results presented here, some of the stored cultures might contain viable cells. Revival and analysis of these cultures, an area of science for which we propose the name *historical microbiology*, would be of great value for establishing additional links between the origins of general microbiology and present-day practice of the science.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. PCR primers, primer sequences, and reaction conditions used in this study.

Table S2. Genbank accession numbers for gene sequences analyzed in this study.

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