Taxonomy of Australian clinical isolates of the genus *Photorhabdus* and proposal of *Photorhabdus asymbiotica* subsp. *asymbiotica* subsp. nov. and *P. asymbiotica* subsp. *australis* subsp. nov.

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The relationship of *Photorhabdus* isolates that were cultured from human clinical specimens in Australia to *Photorhabdus asymbiotica* isolates from human clinical specimens in the USA and to species of the genus *Photorhabdus* that are associated symbiotically with entomopathogenic nematodes was evaluated. A polyphasic approach that involved DNA–DNA hybridization, phylogenetic analyses of 16S rRNA and *gyrB* gene sequences and phenotypic characterization was adopted. These investigations showed that *gyrB* gene sequence data correlated well with DNA–DNA hybridization and phenotypic data, but that 16S rRNA gene sequence data were not suitable for defining species within the genus *Photorhabdus*. Australian clinical isolates proved to be related most closely to clinical isolates from the USA, but the two groups were distinct. A novel subspecies, *Photorhabdus asymbiotica* subsp. *australis* subsp. nov. (type strain, 9802892^T = CIP 108025^T = ACM 5210^T), is proposed, with the concomitant creation of *Photorhabdus asymbiotica* subsp. *asymbiotica* subsp. nov. Analysis of *gyrB* sequences, coupled with previously published data on DNA–DNA hybridization and PCR-RFLP analysis of the 16S rRNA gene, indicated that there are more than the three subspecies of *Photorhabdus luminescens* that have been described and confirmed the validity of the previously proposed subdivision of *Photorhabdus temperata*. Although a non-luminescent, symbiotic isolate clustered consistently with *P. asymbiotica* in *gyrB* phylogenetic analyses, DNA–DNA hybridization indicated that this isolate does not belong to the species *P. asymbiotica* and that there is a clear distinction between symbiotic and clinical species of *Photorhabdus*.

INTRODUCTION

Photorhabdus spp. are generally found in symbiotic association with entomopathogenic nematodes (*Heterorhabditis* spp.) or in the cadavers of insects infected with these nematodes (Poinar *et al.*, 1980; Boemare *et al.*, 1993). The bacteria are transported into the haemocoel of a host insect within the intestinal tract of infective-stage nematodes,

where they multiply, killing the host and producing conditions that are favourable for the nematodes' maturation and reproduction. Two phases are recognized in *Photorhabdus*, with phase I being isolated from wild-type *Heterorhabditis* infective juveniles and phase II appearing in laboratory cultures (Akhurst, 1980; Boemare & Akhurst, 1988). *Photorhabdus*–*Heterorhabditis* associations have become important biopesticides, particularly as the nematodes are mobile and can locate insect pests in cryptic habitats (Bedding & Miller, 1981).

Farmer *et al.* (1989) reported the isolation of *Photorhabdus* from human clinical specimens in the USA. Six isolates were

The GenBank/EMBL/DDBJ accession numbers for the *gyrB* gene sequences determined in this study are AY278492–AY278517 and AY322432 and those for the 16S rDNA sequences are AY216500, AY217761 and AY280572–AY280574.

recovered from several states over the period 1977–1989. The route of infection could not be ascertained and no further isolations have been reported from the USA. These isolates were very homogeneous and were shown to constitute a fifth DNA hybridization group, distinct from nematode-symbiotic *Photorhabdus* (Farmer *et al.*, 1989). Fischer-Le Saux *et al.* (1999) included these isolates in their polyphasic examination of the taxonomy of the genus *Photorhabdus* and concluded that they constituted a separate species from any of the nematode-symbiotic *Photorhabdus* spp. They designated the novel species *Photorhabdus asymbiotica*. No phase I isolates of this species have been reported.

Peel *et al.* (1999) subsequently reported the isolation of *Photorhabdus* sp. from four human infections in two Australian states between 1994 and 1998. PCR-RFLP analysis of the 16S rRNA gene showed that these Australian isolates were also highly homogeneous, but distinct from the American isolates. It was not clear from the analysis whether the American isolates were more similar to the Australian isolates or to some symbiotic *Photorhabdus* isolates (Peel *et al.*, 1999). Two more isolations were performed subsequently (Gerrard *et al.*, 2003).

A polyphasic approach to the taxonomic study of the Australian clinical isolates was conducted, in order to determine their relationship to clinical and symbiotic bacteria of the genus *Photorhabdus*. The isolates were characterized phenotypically by biochemical and morphological tests. DNA–DNA relatedness was assessed by measurement of reassociation and ΔT_m of heterologous DNA. In addition to sequencing the 16S rRNA gene for comparison with sequences in various databases, the *gyrB* gene of the Australian clinical isolates and *Photorhabdus* isolates that are representative of the taxonomic groups described by Fischer-Le Saux *et al.* (1999) was sequenced for phylogenetic analysis. The sequence of the *gyrB* gene has proven to be useful for investigating the intrageneric relationships of disparate groups of bacteria (Venkateswaran *et al.*, 1999; Kasai *et al.*, 2000; Yamamoto *et al.*, 2000). The polyphasic taxonomic study indicated that human clinical isolates of *Photorhabdus* fall into two distinct subspecies of *P. asymbiotica*.

METHODS

Bacteria. Clinical isolates (Table 1) were provided by the Microbiological Diagnostic Unit of the University of Melbourne, the Gold Coast Hospital and the Centers for Disease Control and Prevention (USA). Symbiotic isolates were obtained from the CSIRO Entomology collection.

Phenotypic characterization. Phenotypic tests were conducted on isolates MB, 9800946 and 9802892^T, as well as on isolates 9802336, 9802397 and 9805888, which were obtained from the same patient as isolate 9802892^T. Most phenotypic tests were conducted by following the methods of Fischer-Le Saux *et al.* (1999). Acid production from and assimilation of carbon sources was tested with API kits (bioMérieux). Luminescence was assessed visually by

observation after 10 min dark-adaptation and by analysis of a just visibly turbid aqueous suspension of cells from a 24 h nutrient broth (Oxoid) culture in a Beckman LS2800 scintillation counter, on the tritium setting. Insect pathogenicity was assessed by the insect pin test of Peel *et al.* (1999). Maximum temperature for growth was determined in nutrient broth that was inoculated from a 24 h nutrient broth culture that had been incubated in a water bath with accuracy of $\pm 0.2^\circ\text{C}$ for 48 h; the five *P. asymbiotica* isolates from the USA (Table 1) were tested simultaneously with the seven Australian clinical isolates.

DNA isolation. DNA was isolated from cells harvested from 24 h nutrient broth (Oxoid) cultures by using a Qiagen DNeasy kit.

PCR amplification and sequencing of the 16S rRNA gene.

The prokaryote-specific primers of Fischer-Le Saux *et al.* (1999) were used in PCR amplification of the 16S rRNA gene. PCR was performed on 0.3 µg target DNA in 100 µl Gibco PCR mix that contained 4 mM MgCl₂, 1 mM each dNTP, 25 pM each primer, 100 µg cresol red, 13% (w/v) sucrose and 2.5 U *Taq* DNA polymerase (Gibco). Reactions were run on a Corbett FTS 320 thermocycler, with 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2.5 min, followed by a final extension at 72 °C for 3 min and a renaturation step at 25 °C for 10 min. The completed reactions were electrophoresed on 1.5% (w/v) agarose gel at 5 V cm⁻¹. The band was excised and the amplicon was purified with a NucleoTrap Gel Extraction kit (Clontech), cloned into a pGEM-T Easy vector (Promega) and transformed into *Escherichia coli* strain DH10β by electroporation. In order to identify and eliminate nucleotide changes that were introduced during PCR, four individual clones recovered from each ligation were sequenced with the primer set described by Fischer-Le Saux *et al.* (1999). Sequencing reactions were performed with an ABI Prism Dye Terminator cycle sequencing ready reaction kit and analysed with an ABI 373A automated sequencer, under the conditions recommended by the manufacturer (Perkin Elmer). The consensus sequence of the four clones was used in subsequent phylogenetic analysis.

PCR amplification and sequencing of the *gyrB* gene.

PCR amplification of *gyrB* genes was performed with the universal primers of Yamamoto & Harayama (1995). PCR was carried out on 50 ng target DNA in 20 µl Dynazyme buffer that contained 2.5 mM each dNTP, 10 µM each primer and 1 U Dynazyme EXT DNA polymerase. Reactions were run on a Corbett FTS 320 thermocycler, with a 2 min denaturation step at 94 °C, followed by 34 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 75 s. A final extension was conducted at 72 °C for 2 min and was followed by a renaturation step at 25 °C for 5 min.

gyrB amplicons were sequenced with primers UP-1S and UP-2Sr (Yamamoto & Harayama, 1995) and UP-3Sr (5'-AGAAGAGAAT-TTTGGATCAG-3'). Sequencing reactions were performed with an ABI Prism Dye Terminator cycle sequencing ready reaction kit and analysed with an ABI 373A automated sequencer, under the conditions recommended by the manufacturer (Perkin Elmer).

Phylogenetic analyses. 16S rRNA and *gyrB* gene sequences were aligned against homologous sequences (see Figs 1 and 2) by using the program CLUSTAL X version 1.81 (Thompson *et al.*, 1997). This alignment was then checked manually and corrected, and regions of uncertain alignment were eliminated, with the software SEAL version 1.d1 (A. Rambaut, Department of Zoology, University of Oxford, UK). Further analyses were restricted to the unambiguously aligned regions, totalling 1400 positions of the 16S rRNA gene and 1129 positions of *gyrB*.

Evolutionary analyses were carried out with the PHYLIP package version 3.573c (J. Felsenstein, Department of Genome Sciences,

Table 1. *Photorhabdus* isolates used in this study

Species/isolate no.	Host	Geographical origin	Reference
<i>P. luminescens</i> subsp. <i>luminescens</i> :			
Hb ^T	<i>Heterorhabditis bacteriophora</i> Brecon*	Australia	Poinar <i>et al.</i> (1977)
Hm	<i>Heterorhabditis</i> sp.†	USA	Bleakley & Nealson (1988)
<i>P. luminescens</i> subsp. <i>akhurstii</i> :			
D1	<i>Heterorhabditis indica</i>	Australia	Akhurst (1983)
Tetuan	<i>Heterorhabditis</i> sp.	Cuba	Akhurst <i>et al.</i> (1996)
K81	<i>Heterorhabditis</i> sp.	Guadeloupe	Akhurst <i>et al.</i> (1996)
<i>P. luminescens</i> subsp. <i>laumondii</i> :			
K80	<i>Heterorhabditis</i> sp.	Argentina	Akhurst <i>et al.</i> (1996)
HP88	<i>Heterorhabditis bacteriophora</i> HP88*	USA	Akhurst <i>et al.</i> (1996)
<i>P. luminescens</i> :			
C8404	<i>Heterorhabditis</i> sp. (<i>indica</i> ?)‡	China	Akhurst (1987)
C8406	<i>Heterorhabditis</i> sp. (<i>bacteriophora</i> ?)‡	China	Akhurst (1987)
HV16	<i>Heterorhabditis bacteriophora</i> HV16*	Australia	Akhurst <i>et al.</i> (1996)
<i>P. temperata</i> :			
C1	<i>Heterorhabditis bacteriophora</i> NC1*	USA	Akhurst (1983)
NZH3	<i>Heterorhabditis zealandica</i>	New Zealand	Akhurst (1987)
Meg	<i>Heterorhabditis megidis</i>	USA	Poinar <i>et al.</i> (1987)
Habana	<i>Heterorhabditis</i> sp.	Cuba	Akhurst <i>et al.</i> (1996)
HF85	<i>Heterorhabditis megidis</i>	The Netherlands	Smits & Ehlers (1991)
HL81	<i>Heterorhabditis megidis</i>	The Netherlands	Smits & Ehlers (1991)
HW79	<i>Heterorhabditis megidis</i>	The Netherlands	Smits & Ehlers (1991)
XINach ^T	<i>Heterorhabditis megidis</i>	Russia	Akhurst (1987)
XILit	<i>Heterorhabditis megidis</i>	Lithuania	Akhurst <i>et al.</i> (1996)
<i>Photorhabdus</i> sp. Q614(A)	<i>Heterorhabditis</i> sp.	Australia	Akhurst <i>et al.</i> (1992)
<i>P. asymbiotica</i> :			
1216-79	Blood, 73-year-old female	USA	Farmer <i>et al.</i> (1989)
2407-88	Abdomen and submandible, 36-year-old female	USA	Farmer <i>et al.</i> (1989)
2617-87	Lesion cavity, 45-year-old male	USA	Farmer <i>et al.</i> (1989)
3105-77	Blood, 80-year-old female	USA	Farmer <i>et al.</i> (1989)
3265-86 ^T	Pre-tibial wound, 78-year-old male	USA	Farmer <i>et al.</i> (1989)
<i>Photorhabdus</i> sp.:			
MB	Skin biopsy abscess, 55-year-old male	Australia	Peel <i>et al.</i> (1999)
9800946	Blood, 90-year-old male	Australia	Peel <i>et al.</i> (1999)
9802336§	Abscess, 50-year-old male	Australia	Peel <i>et al.</i> (1999)
9802397§	Abscess, 50-year-old male	Australia	Peel <i>et al.</i> (1999)
9802892 ^T §	Abscess, 50-year-old male	Australia	Peel <i>et al.</i> (1999)
9805888§	Abscess (granulomatous tissue), 50-year-old male	Australia	Peel <i>et al.</i> (1999)
GCH001	Pus, 78-year-old male	Australia	Gerrard <i>et al.</i> (2003)

*As there is some uncertainty about the homogeneity of the species *H. bacteriophora*, we have adopted the convention of Fischer-Le Saux *et al.* (1999) and included isolate names to distinguish the different designations.

†The nematode host of isolate Hm has never been positively identified, though it is often referred to as *H. bacteriophora*.

‡The tentative identifications are based on the isozyme analysis of Akhurst (1987).

§These four isolates were obtained from the same patient, albeit at different times and from different sites (e.g. upper left arm, upper left leg).

University of Washington, Seattle, USA; Felsenstein, 1989). Evolutionary distances between pairs of micro-organisms were determined with the correction of Jukes & Cantor (1969), implemented in the DNADIST program, and phylograms were derived with the FITCH and NEIGHBOR programs, employing least-squares (Fitch & Margoliash, 1967) and neighbour-joining (Saitou & Nei, 1987) algorithms, respectively. Significance of nodes was tested by bootstrap analysis that

generated evolutionary distances and used neighbour-joining to produce 1000 trees, and then by compiling a consensus tree, using the programs SEQBOOT, DNADIST, NEIGHBOR or FITCH and CONSENSE. Maximum-likelihood and maximum-parsimony analyses of the sequence data employed the programs DNAML and DNAPARS, respectively. Sequences used as outgroups were the 16S rRNA gene sequences of *E. coli* (GenBank accession no. J01695) and *Proteus vulgaris* (X07652)

Fig. 1. Evolutionary-distance dendrogram, based on comparative analysis of 16S rRNA gene sequences, showing the relationships of four Australian clinical isolates and isolate Q614(A) (shown in bold) to members of the genus *Photorhabdus*. Recognized groups within the genus are indicated on the right of the figure. The tree was constructed from Jukes & Cantor (1969) distances by the least-squares algorithm (Fitch & Margoliash, 1967). The 16S rRNA gene sequences of *E. coli* (GenBank accession no. J01695) and *Proteus vulgaris* (X07652) were used as outgroups (not shown). Numbers at branch-points indicate the number of times (expressed as a percentage) that the sequences to the right of the branch-point were recovered as a monophyletic group in a parallel bootstrap analysis of 1000 data-sets. GenBank accession numbers for the gene sequences are indicated in parentheses. Bar, 0.01 substitutions per nucleotide position.

and the *gyrB* sequences of *E. coli* (X04341) and *Xenorhabdus nematophila* isolate AN6 (AY322431). Trees were represented graphically with the software TREEVIEW version 1.4 (R. D. M. Page, Division of Environmental and Evolutionary Biology, University of Glasgow, Glasgow, UK).

DNA labelling. High-molecular-mass genomic DNA was sheared by sonication with a Branson sonifier 250 on 50% duty cycle at power level 5 for a total of 5 min. This produced DNA fragments with an average size of 0.5 kb. The sheared DNA was labelled with ^{32}P by random-primed synthesis using an NEBlot kit (New England Biolabs). Labelled DNA was separated from unincorporated label by gel filtration using Probe-Quant G-50 Micro columns (Amersham Pharmacia Biotech).

DNA-DNA hybridization. Measurements of reassociation between genomes were performed by a modified version of the competitive hybridization method of De Ley *et al.* (1970). Nitrocellulose membranes (Nitropure, MSI), to which 2 μg denatured, high-molecular-mass genomic DNA was bound, were incubated with 0.2 μg sheared, denatured [^{32}P]DNA of the same isolate, in the presence or absence of a 250-fold excess of competitor DNA. Competitor DNA was 50 μg unlabelled, sheared, denatured DNA of either the same isolate or another isolate. Hybridization was performed in a buffer of $2 \times \text{SSC}$ (Sambrook *et al.*, 1989) + 30% (v/v) formamide for 18 h at 50 °C. After five brief rinses in $2 \times \text{SSC}$ + 0.1% (w/v) SDS at room temperature, filters were washed twice in the same solution at 50 °C for 30 min. Filter-bound radioactivity was measured by using a Beckman LS2800 scintillation counter. Reassociation (%) was

calculated as $100 \times (\text{counts in the absence of competing DNA} - \text{counts with heterologous competing DNA}) / (\text{counts in the absence of competing DNA} - \text{counts with homologous competing DNA})$.

Thermal stability of DNA-DNA hybrids was determined after hybridizing, as described above, filter-bound DNA and [^{32}P]DNA of either the same isolate or another isolate. After hybridization, filters were washed five times in $2 \times \text{SSC}$ + 0.1% SDS at room temperature and then incubated in successive 2 ml volumes of $2 \times \text{SSC}$ + 30% (v/v) DMSO at a range of temperatures between 50 and 95 °C. The amount of radioactivity released from the filters at each temperature was measured by Cerenkov radiation, with tritium discriminators on a Beckman LS2800 scintillation counter.

T_m , defined as the temperature at which 50% of the radioactive probe remains bound to the filter, was calculated for each hybridization, and the difference in melting temperature between homologous and heterologous duplexes (ΔT_m) was used as a measure of the similarity between DNA sequences.

RESULTS AND DISCUSSION

This polyphasic approach to investigating taxonomic relationships within the genus *Photorhabdus* shows a clear distinction at the species level between those isolates that were obtained from entomopathogenic nematodes of the genus *Heterorhabditis* and those obtained from human

Fig. 2. Evolutionary-distance dendrogram, based on comparative analysis of *gyrB* sequences, showing the relationships of four Australian clinical isolates and isolate Q614(A) (shown in bold) to members of the genus *Photorhabdus*. Recognized groups within the genus are indicated on the right of the figure. The tree was constructed from Jukes & Cantor (1969) distances by the neighbour-joining algorithm (Saitou & Nei, 1987). The *gyrB* sequences of *E. coli* (GenBank accession no. X04341) and *Xenorhabdus nematophila* (AY322431) were used as outgroups (not shown). Numbers at branch-points indicate the number of times (expressed as a percentage) that the sequences to the right of the branch-point were recovered as a monophyletic group in a parallel bootstrap analysis of 1000 data-sets. GenBank accession numbers for the gene sequences are indicated in parentheses. Bar, 0.05 substitutions per nucleotide position.

clinical specimens. It also shows that there are two subgroups of isolates from human clinical specimens.

Phenotypic characterization

Each of the six Australian clinical isolates was Gram-negative, rod-shaped and motile with peritrichous flagella. All were phase II variants; they were not noticeably pigmented on nutrient agar, but were either yellow (9800946), off-white (GCH001) or cream on egg-yolk agar. None adsorbed dye from MacConkey agar or showed antibiotic activity against *Micrococcus luteus*. Isolate GCH001 showed a weak lecithinase reaction on egg-yolk agar; all others were negative. Few cells produced inclusions. Luminescence was detected in all isolates by scintillation counting, but not usually by the dark-adapted eye, whether the bacteria were in liquid or on solid media. All isolates were positive for catalase, lipase (Tween 20) and gelatin hydrolysis (Kohn's) and were negative for nitrate reduction, oxidase, Voges-Proskauer reaction, hydrogen sulfide production, β -galactosidase activity with *o*-nitrophenyl β -D-galactopyranoside, urease, tryptophan deaminase, indole, lysine and ornithine decarboxylases and arginine dihydrolase. All isolates were totally or partially haemolytic on sheep and horse blood agar; annular haemolysis (Akhurst *et al.*, 1996) was observed on sheep blood agar for isolates MB, 9800946 and GCH001, but not for the four isolates that were obtained from one patient (9802336, etc.; see Table 1); none

of the isolates displayed annular haemolysis on horse blood agar. Isolate 9800946 exhibited total haemolysis on sheep blood agar and partial haemolysis on horse blood agar, whereas the four isolates from the same patient exhibited partial and total haemolysis on sheep and horse blood agar, respectively. Total haemolysis was recorded for the MB isolate on both sheep and horse blood agar. Maximum temperature for growth of all Australian clinical isolates was 40 °C, whereas the maximum growth temperature for USA isolates of *P. asymbiotica* was 38 °C.

Acid, but not gas, was produced from glucose by all isolates. Other carbon sources from which acid was produced by all isolates were: fructose, glycerol, *N*-acetyl-D-glucosamine, mannose, maltose and ribose. No acid was produced by any isolate from adonitol, aesculin, amygdalin, L-arabinose, D- or L-arabitol, arbutin, cellobiose, dulcitol, erythritol, D-fucose, galactose, gentiobiose, glycogen, inulin, 2-ketogluconate, lactose, D-lyxose, mannitol, melezitose, melibiose, methyl D-glucoside, methyl D-mannoside, raffinose, rhamnose, sorbitol, sorbose, starch, sucrose, D-tagatose, trehalose, D-turanose, xylitol, β -D-xyloside or D- or L-xylose. All isolates assimilated the following: glucose, mannose, maltotriose, maltose, ribose, glycerol, L-malate, citrate, *N*-acetyl-D-glucosamine, D-gluconate, succinate, fumarate, L-aspartate, L-glutamate, L-proline, L-alanine and L-serine. None of the following compounds was assimilated by any isolate: DL- α -amino-*n*-butyrate,

adonitol, aesculin, DL- α -amino-*n*-valerate, L-arabinose, D- or L-arabitol, benzoate, betaine, caprate, caprylate, D-cellobiose, *m*-coumarate, dulcitol, *i*-erythritol, ethanolamine, D-(+)-galactose, D-galacturonate, β -gentiobiose, glutarate, DL-glycerate, histamine, L-histidine, *m*-hydroxybenzoate, DL- β -hydroxybutyrate, hydroxyquinoline- β -glucuronide, itaconate, 2-keto-D-gluconate, 5-keto-D-gluconate, DL-lactate, α -lactose, lactulose, D-lyxose, malonate, maltitol, D-mannitol, D-melezitose, α -D-(+)-melibiose, methyl α -D-glucopyranoside, methyl α -galactopyranoside, methyl β -D-glucopyranoside, methyl β -galactopyranoside, methyl D-glucopyranose, mucate, 2-oxoglutarate, palatinose, phenyl 3-propionate, phenylacetate, *p*-hydroxybenzoate, protocatechuate, putrescine, quinate, D-(+)-raffinose, α -L-rhamnose, D-saccharate, D-sorbitol, L-(+)-sorbitol, sucrose, D-tagatose, D-(−)-tartrate, L-(+)-tartrate, *meso*-tartrate, *trans*-aconitate, tricarballoylate, trigonelline, tryptamine, L-tryptophan, D-turanose, xylitol or D-xylose.

Each of the six Australian clinical isolates killed *Galleria mellonella* larvae when injected.

Characters that varied between isolates are presented in Table 2.

Table 2. Phenotypic characters that varied between the Australian clinical isolates of *Phototrhhabdus*

Strains: 1, 9800946; 2, 9802336; 3, 9802397; 4, 9802892^T; 5, 9805888; 6, MB. +, Positive; −, negative; +w, weakly positive; NT, not tested.

Character	1	2	3	4	5	6
Hydrolysis of:						
Tween 40	+	−	−	−	−	−
Tween 60	+	−	−	−	−	−
Tween 80	+	−	−	−	−	−
Tween 85	−	+	+	+	+	+
DNase	−	+	+	+	+	+
Simmons' citrate	+	+	−	+	+	−
Acidification of:						
Inositol	+	+w†	−	−	+	−
Aesculin	+	+w	−	−	−	−
Salicin	+	−	−	−	−	−
Gluconate	+w	+	+	+	+	−
5-Ketogluconate	+w	−	−	−	−	+
Assimilation of:						
Fructose	+	−	−	−	+	NT
Trehalose	+	−	−	−	−	NT
<i>myo</i> -Inositol	+	+w	−	+w	+	NT
α -L-(−)-Fucose	+w	−	−	−	−	NT
D-Glucuronate	+	+	−	−	+	NT
D-Alanine	+	−	−	−	−	NT
Propionate	+	−	−	−	−	NT
L-Tyrosine	+	−	+	−	−	NT

There were differences between the data reported here and those reported by Peel *et al.* (1999), who characterized the same isolates, with the exception of GCH001. For example, Peel *et al.* (1999) reported that the isolates were all positive for urease and negative for DNase and acidification of maltose, whereas this study found no urease activity, that all Australian clinical isolates acidified maltose and that only isolate 9800946 was negative for DNase. Different protocols, involving different temperatures and times of incubation, were employed for many tests in these two studies. Peel *et al.* (1999) conducted almost all their biochemical tests at 35 °C, whereas the corresponding tests reported here were conducted at 28 °C. The choice of protocols and conditions in the study reported here was made to remain consistent with those used previously to characterize isolates of the genus *Phototrhhabdus* (Boemare & Akhurst, 1988; Fischer-Le Saux *et al.*, 1999). This allowed comparison of the data gathered in this study with those obtained in earlier studies, to test whether the novel taxon suggested by sequence and DNA–DNA hybridization analyses could be differentiated from existing taxa in the genus *Phototrhhabdus* on the basis of phenotypic characters.

DNA–DNA hybridization

DNA of isolate MB reassociated most strongly with that of the *P. asymbiotica* isolate 3265-86^T (Table 3) and its reassociation with isolates from other *Phototrhhabdus* spp. was always <55 %. ΔT_m for isolate MB was >6 °C for all isolates, except for 3265-86^T (4.1 °C) and Q614(A) (4.9 °C). DNA of isolate Q614(A) had 52 % reassociation with that of isolate MB and only 31 % with that of isolate 3265-86^T of *P. asymbiotica*.

Phylogenetic analyses

Analysis of 16S rRNA gene sequences (by four different methods) supported the subspecies-level groupings of isolates of Fischer-Le Saux *et al.* (1999). These groupings were the same in all analyses. The relationship of these subspecies-level groupings with each other, i.e. their organization into recognized species, was not supported by these analyses. Each different analysis gave different groupings of the subspecies into higher-level clades, and few of these higher-level groupings were given any statistical support (Fig. 1). The 16S rRNA gene sequences of the four Australian clinical isolates were slightly more similar to those from isolates of *P. asymbiotica* (mean Jukes–Cantor-corrected distance, 2.95%; range, 2.91–2.99%) than to sequences from members of other clusters (mean Jukes–Cantor-corrected distance, 3.66%; range, 3.29–4.19%).

Phylogenies based on 16S rRNA gene sequences have previously been used to distinguish several groups within the genus *Phototrhhabdus* that were consistent with DNA–DNA hybridization and phenotypic data (Szallas *et al.*, 1997; Fischer-Le Saux *et al.*, 1999). However, 16S rRNA gene

Table 3. DNA–DNA hybridization of isolate MB with isolates representing each species and subspecies of the genus *Photorhabdus*

For each pair, reassociation values (%) are the means of duplicate experiments with ^{32}P -labelled MB DNA. ΔT_m values are means of reverse hybridizations.

Species	Strain/isolate	Reassociation (%)	ΔT_m (°C)
<i>P. asymbiotica</i>	3265-86 ^T	76	4.1
<i>P. luminescens</i>	C8406	47	6.5
<i>P. luminescens</i> subsp. <i>luminescens</i>	Hb ^T	29	7.5
<i>P. luminescens</i> subsp. <i>akhurstii</i>	D1	35	8.1
<i>P. luminescens</i> subsp. <i>laumondii</i>	K80	23*	7.6†
<i>P. temperata</i>	C1	37	7.6
<i>P. temperata</i>	XINach ^T	20*	8.5
<i>Photorhabdus</i> sp.	Q614(A)	52	4.9†

*Not replicated.

†Determined only with bound DNA from isolate MB.

sequence analysis proved less reliable with the additional set of sequences used in this study. Although phylogenetic analysis of 16S rRNA gene sequences supported the grouping of isolates into the subspecies groupings identified by Fischer-Le Saux *et al.* (1999), these subspecies groupings did not form consistent groups at the species level. Isolates of *Photorhabdus luminescens* subsp. *luminescens* did not cluster closely with the other two subspecies of *P. luminescens*, and *P. asymbiotica* could not be distinguished as a separate species (Fig. 1). The inconsistency of the species-level groupings in these analyses suggests that 16S rRNA gene sequence data are not suitable for the definition of species within the genus *Photorhabdus*, although they are useful at the subspecies level. This is because sequence differences are restricted to a few variable regions, and so the information content is not sufficient to provide reliable species-level groupings. Members of each subspecies displayed sequences that were very similar, even in variable regions, thus drawing the members of the subspecies together.

In contrast to 16S rDNA data, the *gyrB* gene provides useful data for investigating bacterial intragenetic phylogenies (Venkateswaran *et al.*, 1999; Kasai *et al.*, 2000; Yamamoto *et al.*, 2000). The *gyrB* sequence data for *Photorhabdus* provided a good match with DNA–DNA hybridization and phenotypic data at both subspecies and species levels (Fig. 2). This is because the proportion of invariant nucleotide positions is lower than in the 16S rRNA gene. Each of the four clustering methods tested in the analysis of the *gyrB* data grouped isolates into the subspecies recognized by Fischer-Le Saux *et al.* (1999), and then the subspecies into recognized species in a highly reproducible (all four methods) and statistically significant (bootstrap values of 99 % or greater) manner. It is clear from these analyses that the species and subspecies identified by Fischer-Le Saux *et al.* (1999) are valid.

There may be more than the three subspecies of *P. luminescens* that were proposed by Fischer-Le Saux *et al.* (1999). In analysis of *gyrB* sequences, although the two Chinese isolates C8404 and C8406 clustered consistently with *P. luminescens*, the level of sequence divergence suggests that they may not fall into any of the recognized subspecies. Akhurst *et al.* (1996) showed that these isolates had 64–82 % DNA–DNA reassociation with *P. luminescens* subsp. *luminescens* isolate Hb and *P. luminescens* subsp. *akhurstii* isolate D1. Isolate C8406 forms a discrete PCR-RFLP profile, with greatest similarity to those of the *P. luminescens* subsp. *luminescens* group (Brunel *et al.*, 1997; Fischer-Le Saux *et al.*, 1999) and isolate C8404 produces a novel 16S rRNA gene PCR-RFLP profile that is most similar to that of the *P. luminescens* subsp. *akhurstii* group (S. Pages, personal communication). The *gyrB*, DNA relatedness and PCR-RFLP data suggest that, whilst they are members of the species *P. luminescens*, isolates C8404 and C8406 do not fall into any recognized subspecies. The *gyrB* analysis also suggests that they may not belong to a common subspecies.

In all analyses of *gyrB* sequences, two main clusters of *Photorhabdus temperata* isolates were formed and isolate NZH3 always branched more deeply, but coherently, with these. The mean Jukes–Cantor-corrected distance between members of different clusters was 4.95 % (range, 4.10–6.57 %). This suggests that there are three genetically distinct lineages of *P. temperata*, given that levels of sequence divergence between these clusters were similar to those for recognized subspecies of *P. luminescens* (mean Jukes–Cantor-corrected distance between members of different clusters was 5.06 %, with a range of 4.56–5.70 %).

Isolates MB, GCH001, 9800946 and 9802892^T always grouped together and formed a sister group to the cluster of *P. asymbiotica* isolates. Isolate Q614(A) always branched

more deeply, but reproducibly, with isolates of *P. asymbiotica* and the Australian clinical isolates. The mean Jukes–Cantor-corrected distance between any of the American isolates of *P. asymbiotica* and any of the four Australian clinical isolates was 5.57 % (range, 5.23–6.18 %). Coherence of the branching pattern in all analyses, statistical support for the groupings and the level of sequence divergence between any of the American or Australian clinical isolates and isolate Q614(A) (5.51–7.54 %) all suggested strongly that there are at least three genetically distinct lineages of *P. asymbiotica*. In analyses of *gyrB* sequences, isolate Q614(A) always grouped with the four Australian clinical isolates and the *P. asymbiotica* isolates; the bootstrap value for this grouping was 100 %.

Non-luminescent isolate Q614

The unexpected grouping of the non-luminescent isolate Q614 (Akhurst & Boemare, 1986) with a subgroup of *P. luminescens* based on comparative 16S rRNA gene sequence analysis (Szallás *et al.*, 1997) was investigated. An earlier DNA–DNA hybridization study had shown that isolate Q614, recovered from a nematode host and characterized by two of the authors (R. J. A. and N. E. B), had <50 % reassociation with the DNA of two *P. luminescens* isolates (Boemare *et al.*, 1993). The correct identity of the original isolate from the CSIRO Culture Collection was demonstrated by its lack of bioluminescence when tested by scintillation counter, as it is the only non-luminescent isolate of *Photorhabdus* in the CSIRO collection. The 16S rRNA gene sequence of that isolate, labelled Q614(A), had only 95.5 % sequence similarity to the sequence submitted by Szallás *et al.* (1997), labelled Q614(S). It is evident that the isolate used by Szallás *et al.* (1997) was identified incorrectly as the non-luminescent isolate Q614 and that the sequence deposited with GenBank accession no. Z76749 is annotated incorrectly. Consequently, the phylogenetic positioning of isolate Q614 in subcluster IV (Szallás *et al.*, 1997) is incorrect.

The verified isolate Q614(A) did not branch consistently with any subspecies grouping in different analyses of 16S rRNA gene sequences, which is consistent with our observation that comparative analysis of this gene is not able to group isolates into recognized species. In contrast, analyses of *gyrB* sequences always grouped Q614(A) with the four Australian clinical isolates and with isolates of *P. asymbiotica*; the bootstrap value for this grouping was 100 % (Fig. 2). This suggests that Q614(A) may represent a novel subspecies of *P. asymbiotica*. However, DNA–DNA hybridization data suggest that Q614(A) is not conspecific with *P. asymbiotica*, as it had only 52 and 31 % reassociation with isolates MB and 3265-86^T, respectively, and a ΔT_m of nearly 5 °C with isolate MB (Table 2). As Q614(A) is the only representative of a putative subgroup, it would be prudent to wait until more isolates are available for investigation before a formal decision on its taxonomic position can be made.

Clinical isolates of *Photorhabdus*

Both the *gyrB* phylogeny and DNA–DNA hybridization data show that the Australian and USA clinical isolates are closely related (Fig. 2, Table 2). As isolate MB had 76 % DNA–DNA reassociation and a ΔT_m of 4.1 °C with *P. asymbiotica* isolate 3265-86^T, they meet the definition of Wayne *et al.* (1987) for being regarded as conspecific. In the phylogenetic analysis conducted with *gyrB* sequence data, the USA clinical isolates, identified previously as *P. asymbiotica*, and the Australian clinical isolates formed a discrete group, albeit with Q614(A). Further, with each of the four clustering methods employed in the *gyrB* analysis, the Australian clinical isolates and the USA clinical isolates formed discrete subgroups. Moreover, DNA–DNA reassociation and ΔT_m values for the MB/3265-86^T

Table 4. Phenotypic characters differentiating the Australian clinical isolates from described species of *Photorhabdus*

Species: 1, *P. asymbiotica* (Australian clinical isolates); 2, *P. asymbiotica* (USA clinical isolates); 3, *P. luminescens*; 4, *P. temperata*. Data are from Fischer-Le Saux *et al.* (1999) and this study. All tests were conducted at 28 °C, unless otherwise noted. Results are indicated as: +, >90 % isolates positive; [+], 76–89 % positive; D, 26–75 % positive; [–], 11–25 % positive; –, <10 % positive; w, weak reaction. Results for isolate 9802892^T are presented in parentheses.

Character	1	2	3	4
Maximum growth temperature (°C)	40 (40)	38	39	34
Hydrolysis of:				
Tween 60	[–] (–)	+	+	+
Tween 80	[–] (–)	+	+	+
DNase	[+] (+)	–	D	D
Urease	– (–)	+	D	D
Aesculin hydrolysis	[–] (–)	+	+	[+]
Fermentation of:				
Aesculin	D (–)	[+] (+)	[+] (+)	+
5-Ketogluconate	D (+)	DW	–	[–]
Mannitol	– (–)	–	D	[–]
Trehalose	– (–)	+	+	[+]
Assimilation of:				
L-Fucose	[–] (–)	–	DW	D
DL-Glycerate	– (–)	D	–	+
L-Histidine	– (–)	D	D	[+]
myo-Inositol	[+] (+w)	–	[+] (+)	D
DL-Lactate	– (–)	–	DW	–
D-Mannitol	– (–)	–	[+] (+)	–
Indole	– (–)	–	+	[–]
Tryptophan deaminase	– (–)	–	–	[+]
Simmons' citrate	D (+)	+w	D	D
Annular haemolysis at 25 °C:				
Sheep blood agar	D (+)	+	D	D
Horse blood agar	– (–)	+	D	[+]

combination were near the species definition limits of Wayne *et al.* (1987) of 70 % and 5 °C, respectively, and the two subgroups could be differentiated on the basis of several phenotypic characters (Table 4). On the basis of these differences, we propose that the Australian and USA clinical isolates of the genus *Photorhabdus* should be assigned to different subspecies of *P. asymbiotica*.

Emended description of *Photorhabdus asymbiotica*

Photorhabdus asymbiotica (a.sym.bi.o'ti.ca. Gr. pref *a* not; N.L. fem. adj. *sympiotica* living together; N.L. fem. adj. *asymbiotica* not symbiotic).

Cells are Gram-negative rods of 2–3 × 0.5–1.0 µm that are motile by peritrichous flagella. Maximum temperature for growth in nutrient broth is 40 °C. Yellow, brown or no pigment. Catalase-positive; oxidase-negative; nitrate is not reduced. Negative for *o*-nitrophenyl β-D-galactopyranoside activity and the Voges–Proskauer test. Bioluminescent. No phase I isolates have been detected; isolates do not absorb dyes, are negative for lecithinase on egg-yolk agar and are, at most, weakly antibiotic. Positive for Simmons' citrate. Negative for H₂S from triple-sugar iron agar, tryptophan deaminase, lysine and ornithine decarboxylases, arginine dihydrolase and indole. Acid is produced from fructose, *N*-acetylglucosamine, glucose, glycerol, maltose, mannose and ribose. No gas is produced from glucose. Proteinaceous inclusions are produced poorly. Annular haemolysis is usually evident on sheep blood agar at 25 °C. Does not use L-fucose, DL-lactate or mannitol.

The type strain is 3265-86^T (=ATCC 43950^T=CIP 106331^T=DSM 15149^T). Natural habitat is uncertain; all isolates were obtained from human clinical specimens. The GenBank accession numbers for the 16S rRNA and *gyrB* gene sequences of the type strain are Z76755 (Szállás *et al.*, 1997) and AY278494, respectively.

Description of *Photorhabdus asymbiotica* subsp. *asymbiotica* subsp. nov.

Photorhabdus asymbiotica subsp. *asymbiotica* (a.sym.bi.o'ti.ca. Gr. pref *a* not; N.L. fem. adj. *sympiotica* living together; N.L. fem. adj. *asymbiotica* not symbiotic).

Maximum temperature for growth in nutrient broth is 37–38 °C. Yellow or brown pigment. Positive for urease and aesculin hydrolysis; weakly positive for Simmons' citrate. Negative for DNase. Acid is produced from trehalose and usually from aesculin; variable for gluconate. Proteinaceous inclusions are produced poorly. Annular haemolysis occurs on sheep blood and horse blood agars. Positive for Tween 60 and Tween 80 esterases; Tween 40 esterase activity is variable.

The type strain is 3265-86^T (=ATCC 43950^T=CIP 106331^T=DSM 15149^T). Natural habitat is uncertain; all isolates were obtained from human clinical specimens in

the USA. The GenBank accession numbers of the 16S rRNA and *gyrB* gene sequences of the type strain are Z76755 (Szállás *et al.*, 1997) and AY278494, respectively.

Description of *Photorhabdus asymbiotica* subsp. *australis* subsp. nov.

Photorhabdus asymbiotica subsp. *australis* (aus.tra'lis. L. fem. adj. *australis* southern; this subspecies was detected in the southern hemisphere).

Maximum temperature for growth is 40 °C. Yellow or no pigment; weakly pigmented. Most isolates are positive for DNase and most are negative for aesculin hydrolysis. Negative for urease. Most isolates produce acid from gluconate; variable for acid production from aesculin and negative for trehalose. Proteinaceous inclusions are rare. Annular haemolysis is variable on sheep blood and horse blood agars. Most isolates are negative for Tween 60 and Tween 80 esterases and most grow on *myo*-inositol.

The type strain is 9802892^T (=CIP 108025^T=ACM 5210^T). Natural habitat is uncertain; all isolates were obtained from human clinical specimens in Australia. The GenBank accession numbers of the 16S rRNA and *gyrB* gene sequences of the type strain are AY280572 and AY278496, respectively.

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