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Bacillus popilliae and *Bacillus lentimorbus*, bacteria causing milky disease in Japanese beetles and related scarab larvae

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***Bacillus popilliae* and *Bacillus lentimorbus*, causative agents of milky disease in Japanese beetle and related scarab larvae, have hitherto been differentiated based upon a small number of phenotypic characteristics, but they have not previously been examined at the molecular level. In this study 34 isolates of these bacteria were examined for DNA similarity and by random amplified polymorphic DNA (RAPD) analysis. Two distinct but related similarity groups were identified: the first contained strains of *B. popilliae* and the second contained strains of *B. lentimorbus*. Two strains distinct from but related to *B. popilliae* may represent a subspecies. Some strains received as *B. popilliae* were found to be most closely related to *B. lentimorbus* and some received as *B. lentimorbus* were found to be most closely related to *B. popilliae*. RAPD analysis confirmed the DNA similarity results. Paraspore formation, previously believed to be a characteristic unique to *B. popilliae*, was found to occur among a sub-group of *B. lentimorbus* strains. Growth in media supplemented with 2% NaCl was found to be a somewhat less reliable characteristic in distinguishing the species than vancomycin resistance, the latter being present only in *B. popilliae*.**

Keywords: *Bacillus popilliae*, *Bacillus lentimorbus*, insect pathogens, milky disease

INTRODUCTION

Bacillus popilliae and *Bacillus lentimorbus* are pathogens of Japanese beetles (*Popillia japonica*) and related scarab larvae. Larvae feeding in the soil consume spores of these bacteria and following spore germination in the larval gut, vegetative cells penetrate into the haemocoel. A period of vegetative growth is followed by asynchronous sporulation, and death of the larvae. At the time of larval death, the haemolymph may contain up to 5×10^{10} spores ml^{-1} (1). The milky colour of haemolymph at time of death has given the condition the name 'milky disease' (14). Because of its action against economically important insects, efforts have been made to develop *B. popilliae* as an insecticide. However, the inability to mass-produce spores *in vitro* has prevented large-scale manufacture and utilization (9).

Abbreviations: RAPD, random amplified polymorphic DNA; UPGMA, unweighted pair group method with arithmetic averages.

Dutky (4) reported a difference in colour of the haemolymph in insects infected by either *B. popilliae* (type A disease) or *B. lentimorbus* (type B disease). In addition, Dutky (4), Gordon *et al.* (5) and St Julian & Bulla (15) suggested that a primary distinguishing characteristic between these two species is the production of a parasporal body by *B. popilliae* but the absence of this structure in *B. lentimorbus*. Serological studies prompted Krywienczyk & Luthy (11) to propose a single species, *B. popilliae*, with three varieties, *B. popilliae* var. *popilliae*, *B. popilliae* var. *lentimorbus* and *B. popilliae* var. *melolonthae* (the last variety based on a European isolate also known as *fribourgensis*). This approach was similar to that proposed by Wyss (21), who emphasized physiological and morphological characteristics in his taxonomic arrangement. Milner (12) utilized the position and size of the spore and paraspore in the sporangium to group the bacteria. In this system all milky disease isolates were considered varieties of *B. popilliae*. Group A1 contained strains with small parasporal bodies overlapping the spore. Group A2 had a large parasporal body separated from

the spore. Group B1 had a large central spore and lacked a paraspore, and group B2 had a small spore and no paraspore. The utility of these morphological characteristics in determination is limited because the paraspore is produced at the time of sporulation, which only occurs in living larvae. Therefore, only those laboratories capable of collecting and infecting the larvae are able to identify the species (17). It has been reported that *B. popilliae* will grow in media containing 2% NaCl whereas *B. lentimorbus* will not grow under these conditions (17).

The genetic relationship between *B. popilliae*, *B. lentimorbus*, and less well-known bacteria producing milky disease is unknown. In this study we utilized DNA reassociation and random amplified polymorphic DNA (RAPD) analysis to define relationships between these species. Our results validate the existence of the two species and have identified the presence of sub-groups within the species. Phenotypic characteristics presented by the species and sub-groups were investigated to facilitate identification.

METHODS

Strains and growth conditions. The strains used are listed in Table 1. The initial species assignments were made by the strain contributors based on the presence or absence of parasporal bodies associated with the bacteria in infected larval haemolymph. All isolates were grown in MYPGP broth (3) and were stored at −80 °C in 10% (v/v) glycerol. For DNA isolation, bacteria were grown with shaking (150 r.p.m.) at 30 °C in MYPGP broth, harvested by centrifugation and stored at −20 °C. Field-collected Japanese beetle and masked chafer larvae were infected with vegetative-phase bacteria as described by Klein (10).

Vancomycin and teicoplanin resistance. Plates containing MYPGP supplemented with 150 μg vancomycin ml^{-1} (Sigma) were streaked from 24 h MYPGP broth cultures of *B. popilliae* or *B. lentimorbus*. The plates were incubated at 30 °C for 48 h and growth determined by visual examination. MIC values for vancomycin and teicoplanin (Marion Merril Dow) were determined using a 96-well microtitre plate. Each antibiotic concentration was tested in triplicate. Each well contained 300 μl MYPGP broth supplemented with antibiotic and was inoculated with 1 μl of a 24 h culture. Vancomycin was used in concentrations of 50, 100, 200, 400, 800 and 1600 μg ml^{-1} while teicoplanin was used in concentrations of 25, 50, 100, 200, 400 and 800 μg ml^{-1}. Lower concentrations of vancomycin (1–16 μg ml^{-1}) were also tested for *B. lentimorbus*. Following incubation at 30 °C for 48 h, the plates were read in an ELISA plate reader at 600 nm.

Sodium chloride tolerance. Each strain was tested at 30 °C in MYPGP broth containing 2% (w/v) NaCl. Growth was defined as being more than a doubling in absorbance. The strains were also incubated at 30 °C on MYPGP plates containing 2% NaCl and growth was determined by visual examination of the plates.

DNA isolation. Bacteria from 1 litre of MYPGP were resuspended in 20 ml buffer (10 mM Tris/HCl pH 8·0, 1 mM EDTA, 0·35 M sucrose, 0·1 mg lysozyme ml^{-1}) and incubated for 2 h at 37 °C, followed by the addition of 10 ml 5 M $NaClO_4$ and 20 ml lysing solution [100 mM Tris/HCl pH 8·0, 20 mM EDTA, 0·3 M NaCl, 2% (w/v) SDS, 2% (v/v) β-mercaptoethanol, 100 μg proteinase K ml^{-1}] with further incubation for 3 h at 55 °C. DNA was purified by multiple phenol/chloroform extractions, precipitated with 0·6 vols 2-propanol, air-dried, and resuspended in 20 ml TE (10 mM Tris/HCl pH 8·0, 1 mM EDTA). RNase (250 μl of a stock solution containing 1 mg RNase A $ml^{-1}$and 4000 U T1 RNase ml^{-1}) was added to the sample and incubated for 1 h at 37 °C, followed by a chloroform extraction and precipitation of the DNA by ethanol (8). DNA was resuspended in 3 ml TE and quantified by the absorbance at 260 nm or by fluorometry.

DNA–DNA similarity. DNA samples were fragmented and radioactively labelled as previously described (7, 8). Hybridization of sheared fragments of ^{125}I-labelled DNA with unlabelled DNA was carried out by the S1 nuclease method (2). DNA samples were heated for 5 min at 65 °C before use. Reaction tubes containing 10 μl labelled DNA (0·4 mg ml^{-1}), 50 μl unlabelled DNA (0·4 mg ml^{-1}) and 50 μl buffer (13·2 × SSC, 5 mM HEPES pH 7·0) were incubated for 24 h at 65 °C to allow reassociation. Following reassociation, 1 ml buffer (0·3 M NaCl, 0·05 M acetic acid, 0·5 mM $ZnCl_2$), 100 U S1 nuclease and 50 μl denatured salmon sperm DNA (0·4 mg ml^{-1}) were added to each reaction and incubated for 1 h at 50 °C. Five hundred microlitres of HCl buffer (1 M HCl, 1% $Na_4P_2O_7$, 1% NaH_2PO_4) and 50 μl native salmon sperm (1·2 mg ml^{-1}) were added to the reactions and incubated for 1 h at 4 °C to precipitate the DNA. The precipitated DNA was collected on Whatman glass filters and its radioactivity measured with a gamma counter. All determinations were carried out in duplicate.

RAPD. Oligonucleotide primers were obtained from Operon Technologies and were resuspended to a concentration of 0·125 μg ml^{-1}. The sequences of the eight primers used in this study are given in Table 2. The contents of RAPD reaction mixtures were as previously described by Woodburn *et al.* (20). A control reaction was run in each experiment that contained all the RAPD components except template DNA. Amplification reactions were performed in a PTC-100 thermocycler (MJ Research). The temperature profile was as follows: initial denaturation at 95 °C for 5 min, followed by 75 cycles consisting of 94 °C for 20 s, 36 °C for 20 s and 72 °C for 2 min. Following amplification, 12 μl of product was electrophoresed in a 1·7% gel containing 1·0% (w/v) Synergel (Diversified Biotech) and 0·7% (w/v) agarose in TBE buffer (0·09 M Tris base, 0·09 M boric acid, 0·002 M EDTA, pH 8·0). Electrophoresis was carried out at 3·2 V cm^{-1} in TBE buffer; the gel was stained in a solution containing 0·5 μg ethidium bromide ml^{-1} and photographed in UV light.

Data analysis. The percentage DNA hybridization data were analysed with the distance and *Q*-correlation coefficient algorithms. The matrices obtained were subjected to clustering by the unweighted pair group method with arithmetic averages (UPGMA). The cophenetic coefficients for the clusters were computed and the correlation between these coefficients and the similarity matrix data was computed by using normalized Mantel statistics *z* (13). The presence of RAPD bands was determined for the 34 strains for each of the eight primers tested. The data were treated as discrete bistate characters. The data obtained were combined in one rectangular matrix of 34 operational taxonomic units and were analysed using the Jaccard similarity coefficient algorithm. The matrix of coefficients obtained was subjected

Table 1. *B. popilliae* and *B. lentimorbus* strains and their sources

Strain	Host insect	Source*
ATCC 14706^T	*Popillia japonica*	USA[1]
ATCC 14707^T	*Popillia japonica*	USA[1]
A8	*Anomola orientalis*	USA[2]
BlPj1†	*Popillia japonica*	USA[2]
Bp1‡	*Papuana woodlarkiana*	Papua New Guinea[3]
Bp3	*Cyclocephala hirta*	USA[3]
Bp6	*Popillia japonica*	USA[3]
Bp7‡	*Cyclocephala borealis*	USA[3]
BpCb1‡	*Cyclocephala borealis*	USA[2]
BpCb2‡	*Cyclocephala borealis*	USA[2]
BpCh1	*Cyclocephala hirta*	USA[2]
BpCp1‡	*Cyclocephala parallela*	USA[2]
BpPa1‡	*Phyllophaga anxia*	USA[2]
BpPj1	*Popillia japonica*	USA[2]
BpPj2	*Popillia japonica*	USA[2]
BpPj3	*Popillia japonica*	USA[2]
BpPj4	*Popillia japonica*	USA[2]
BpPj5	*Popillia japonica*	USA[2]
DGB1‡	*Cyclocephala parallela*	USA[2]
DNG1	*Anomala orientalis*	USA[2]
DNG2	*Popillia japonica*	USA[2]
DNG4	*Anomala orientalis*	USA[2]
DNG10	*Popillia japonica*	USA[2]
DNG11	*Anomala orientalis*	USA[2]
DNG12	*Anomala orientalis*	USA[2]
KLN1	*Popillia japonica*	USA[2]
KLN2	*Popillia japonica*	USA[2]
KLN3	*Popillia japonica*	USA[2]
NRRL B-2309	*Popillia japonica*	USA[4]
NRRL B-2522†		USA[4]
NRRL B-2524	*Popillia japonica*	USA[4]
NRRL B-4081	*Melolontha melolonthae*	Europe[4]
NRRL B-4145		USA[4]
NRRL-B-4154	*Odontria* (strain *Odontria*)	USA[4]

* 1, ATCC; 2, D. P. Stahly, University of Iowa, USA; 3, M. G. Klein, USDA, Wooster, OH, USA; 4, L. Nakamura, Northern Regional Research Laboratory, Peoria, IL, USA.

† These strains were received as *B. lentimorbus* but have been shown to be *B. popilliae* by DNA similarity.

‡ These strains were received as *B. popilliae* but have been shown to be *B. lentimorbus* by DNA similarity.

Table 2. Primers used in RAPD analysis

Primer	Sequence
OPA-03	5′-AGT CAG CCA C-3′
OPA-04	5′-AAT CGG GCT G-3′
OPA-05	5′-AGG GGT CTT G-3′
OPA-07	5′-GAA CGG GGT G-3′
OPA-08	5′-GTG ACG TAG G-3′
OPA-10	5′-GTG ATC GCA G-3′
OPA-11	5′-CAA TCG CCG T-3′
OPA-15	5′-TTC CCG ACC C-3′

to clustering by UPGMA. The NTSYS-pc program (version 1.8) was used in the analysis of the data.

RESULTS

DNA similarity

DNA was prepared from 34 strains of bacteria that had been originally isolated from scarab larvae suffering from milky disease. This DNA was compared to labelled reference DNA from the type strains of both *B. popilliae* and *B. lentimorbus* as well as to DNA from three additional strains, one of which was a

Table 3. Levels of DNA–DNA homology for *B. popilliae* and *B. lentimorbus* strains as determined by the S1 nuclease method

Strain	Percentage homology with:				
	B. popilliae			*B. lentimorbus*	
	ATCC 14706^T	BpPj5	NRRL B-4081	ATCC 14707^T	Bp7
B. popilliae					
ATCC 14706^T*	100	99	66	64	57
NRRL B-4081	77	73	100	61	59
BpPj5	97	100	66	63	53
Bp6	97	94	60	60	47
DNG4	88	87	65	60	55
NRRL B-2309	90	90	60	60	51
A8	84	83	59	61	50
NRRL B-2522	86	88	67	62	52
NRRL B-2524	89	87	60	60	59
NRRL B-4145	95	96	66	63	46
NRRL B-4154	92	90	67	65	55
Bp3	73	73	82	62	54
BpCh1	91	95	64	62	51
BpPj1	91	95	65	62	56
BpPj2	97	95	62	62	48
BpPj3	92	96	62	62	49
BpPj4	98	95	64	66	51
DNG1	87	88	63	60	55
DNG2	89	86	54	60	45
DNG10	84	86	63	63	53
DNG11	88	88	66	64	53
DNG12	87	88	62	64	52
KLN1	93	93	59	66	55
KLN3	85	87	64	62	55
BlPj1	95	93	62	59	47
B. lentimorbus					
ATCC 14707^T†	62	63	50	100	68
KLN2	59	60	45	90	65
Bp7	60	58	49	73	100
DGB1	58	60	48	76	78
Bp1	59	58	50	75	82
BpCb1	60	58	51	77	84
BpCb2	62	59	50	78	91
BpPa1	58	58	52	75	90
BpCp1	58	58	51	74	89

* *B. popilliae* type strain.

† *B. lentimorbus* type strain.

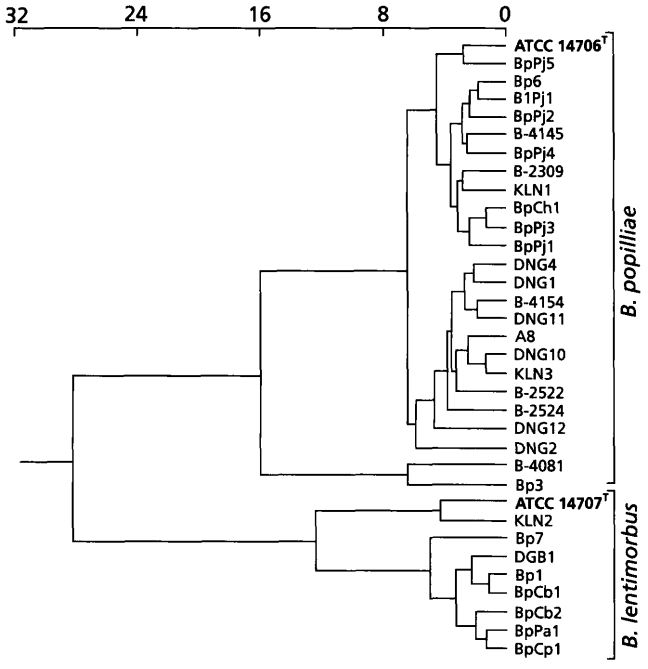

Fig. 1. UPGMA dendogram showing relationships among strains of *B. popilliae* and *B. lentimorbus* as determined by DNA reassociation. The *x*-axis represents UPGMA percentage dissimilarity based on arithmetic distances.

European isolate sometimes referred to as *B. popilliae* var. *melolonthae* (NRRL B-4081). The clusters obtained from the distance and correlation matrices were almost identical in their topology. The cophenetic correlations for both clusters were $r = 0{\cdot}98$–$0{\cdot}99$, underscoring the extremely high fit between the original matrices and the phenograms. The distance-based phenogram will be used here because it showed higher resolution within the groups. The similarity study revealed the existence of two groups of strains (Table 3, Fig. 1). The first group showed 84–97 % similarity to the type strain of *B. popilliae*, ATCC 14706^T, and a high similarity to BpPj5, another *B. popilliae* isolate. The strains in this group were primarily North American in origin and most were isolated from diseased *Popillia japonica* except for a few from *Anomola orientalis* (Northern masked chafer). Two strains, NRRL B-4081 and Bp3, showed markedly lower similarity (77 % and 73 %, respectively) to the ATCC 14706^T reference strain than did the other strains of *B. popilliae*. Bp3 displayed 82 % similarity to NRRL B-4081 whereas the other strains of the *B. popilliae* group showed only 59–67 % similarity to that reference strain. Two strains, BlPj1 and NRRL B-2522, were received as *B. lentimorbus* but showed 95 % and 86 % similarity to the *B. popilliae* reference strain and 59 % and 62 % similarity, respectively, to the *B. lentimorbus* type strain. Following growth and sporulation in Japanese beetle larvae, paraspores were detected in NRRL B-2522 but not in BlPj1.

Eight strains showed higher similarity to the *B. lentimorbus* reference strain, ATCC 14707^T, than to *B. popilliae*. Only one of these, KLN2, was received as *B. lentimorbus*; the other seven were received as *B. popilliae*. However, these latter seven strains had lower similarity (73–78 %) to *B. lentimorbus* than did strain KLN2 (90 %) (Table 3). Microscopic examination of haemolymph from Japanese beetles or masked chafers infected with six of these strains, Bp7, DGB1, BpCb1,

BpCb2, BpPa1 and BpCp1, revealed the presence of parasporal bodies in the sporangia. Strain Bp1 has not yet been retested.

Growth in the presence of 2% NaCl or of vancomycin

Growth in media supplemented with 2% NaCl has been used as a characteristic to separate *B. popilliae* from *B. lentimorbus*. Although we found this to be an accurate indicator of species for most strains tested (Table 4), there were a few exceptions on both solid and liquid media.

Stahly *et al.* (18) described a selective medium designed for the recovery of *B. popilliae* spores from soil. This medium utilized vancomycin to select for *B. popilliae* while suppressing growth of *B. lentimorbus* and many other soil micro-organisms. Although Stahly *et al.* (18) reported that *B. popilliae* was generally resistant to vancomycin, there were several isolates that appeared to be susceptible. When we examined the response to vancomycin of the strains studied by DNA similarity, it was found that strains identified as *B. popilliae* were resistant to vancomycin and all strains identified as *B. lentimorbus* were sensitive (Table 4). The strains of *B. popilliae* that Stahly *et al.* (18) reported as being sensitive to the antibiotic were found to be *B. lentimorbus* by DNA similarity; and one *B. lentimorbus* strain, BlPj1, that Stahly *et al.* (18) reported to be resistant, we have found to be *B. popilliae*. When the MICs were determined for the type strains, *B. popilliae* ATCC 14706^T was found to be highly resistant (MIC 800 μg ml^{-1}), whereas *B. lentimorbus* ATCC 14707^T was sensitive (MIC < 1 μg ml^{-1}). The MIC for *B. popilliae* NRRL B4081 was 400 μg ml^{-1}. All three strains were sensitive to the related glycopeptide antibiotic teicoplanin (MIC < 1 μg ml^{-1}).

RAPD analysis

DNA templates prepared from 34 strains of milky disease bacteria were used with eight decamer primers to generate DNA fragments by the PCR. Controls run without DNA templates either produced no bands or occasionally produced a band that did not match any of those produced with templates. Figs 2 and 3 show the patterns obtained with primer OPA-03. Lane 1 in Fig. 2 contains DNA from the type strain of *B. popilliae*, and lanes 2–19 in Fig. 2 and lanes 1–7 in Fig. 3 contain DNA from other isolates of this species. There were two main patterns within this species and a third pattern containing strains NRRL B-4081, Bp3 and Bp6 that were more divergent. Lane 9 in Fig. 3 contains DNA from the type strain of *B. lentimorbus* and lanes 10–18 contain DNA from the other isolates of this species. Again there were two patterns: lanes 10 and 11 contain DNA from 14707 (obtained from a non-ATCC source) and KLN2, and lanes 12–18 (Fig. 3) contain DNA from the group of strains received as *B. popilliae* but shown by DNA similarity to be more closely related to *B. lentimorbus*. Although the primer used to generate the pattern shown in Fig. 3 did not yield common bands between the *B. lentimorbus* type strain (lane 9) and the sub-group of strains in lanes 12–18, other primers used in the study did yield common bands. These results and those from seven additional primers were analysed and are presented as a dendrogram in Fig. 4.

Table 4. Growth of *B. popilliae* and *B. lentimorbus* in MYPGP broth and on plates supplemented with 2% NaCl or 150 μg vancomycin ml^{-1}

Strain	2% NaCl plates	2% NaCl broth*	Vancomycin plates
B. popilliae			
ATCC 14706^T	+	+	+
NRRL B-2309	+	+	+
BpPj5	+	+	+
Bp6	+	+	+
DNG4	+	+/−	+
NRRL B-4081	−	ND	+
A8	+	+	+
NRRL B-2522†	−	+	+
NRRL B-2524	+	+	+/−
NRRL B-4145	−	+	+
NRRL B-4154	+	−	+
Bp3	+	+/−	+
BpCh1	+	+/−	+
BpPj1	+	+	+
BpPj2	+	+	+
BpPj3	+	−	+
BpPj4	+	+	+
DNG1	+	+	+
DNG2	+	+	+
DNG10	+	+	+
DNG11	+	+	+
DNG12	+	+	+
KLN 1	+	+	+
KLN 3	+	+	+
BlPj1†	+	+	+
B. lentimorbus			
ATCC 14707^T	−	−	−
KLN2	+	+	−
Bp7‡	−	−	−
DGB1‡	−	−	−
Bp1‡	−	−	−
BpCb1‡	−	−	+/−
BpCb2‡	−	+/−	−
BpPa1‡	+	−	−
BpCp1‡	−	−	−

ND, Not determined.

*Growth was determined as more than a doubling in optical density.

†These strains were received as *B. lentimorbus* but have been shown to be *B. popilliae* by DNA homology.

‡These strains were received as *B. popilliae* but have been shown to be *B. lentimorbus* by DNA homology.

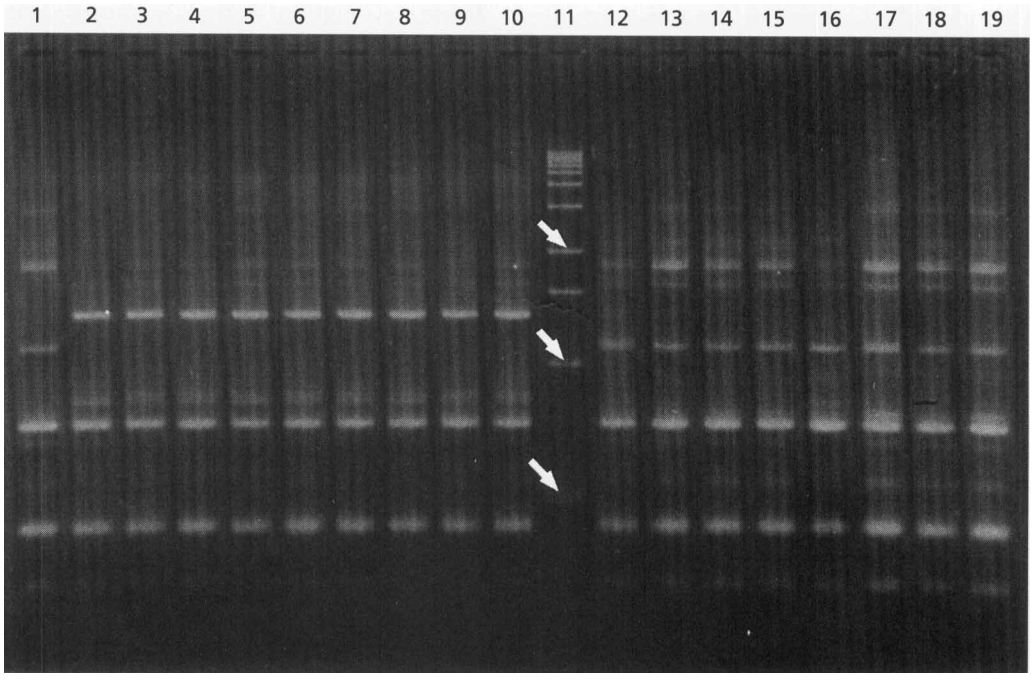

Fig. 2. RAPD band patterns generated using primer OPA-03 and template DNAs from strains of *B. popilliae*. DNA ladder (Gibco-BRL) is in lane 11 [arrows indicate bands of 2·0, 1·0 and 0·5 kb (top to bottom)]. Lanes: 1, ATCC 14706^T; 2, DNG1; 3, DNG2; 4, DNG4; 5, DNG10; 6, DNG11; 7, DNG12; 8, A8; 9, KLN3; 10, NRRL B-2524; 11, ladder; 12, BpPj1; 13, BpPj2; 14, BpPj3; 15, BpPj4; 16, BpPj5; 17, BpCh1; 18, BlPj1; 19, KLN1.

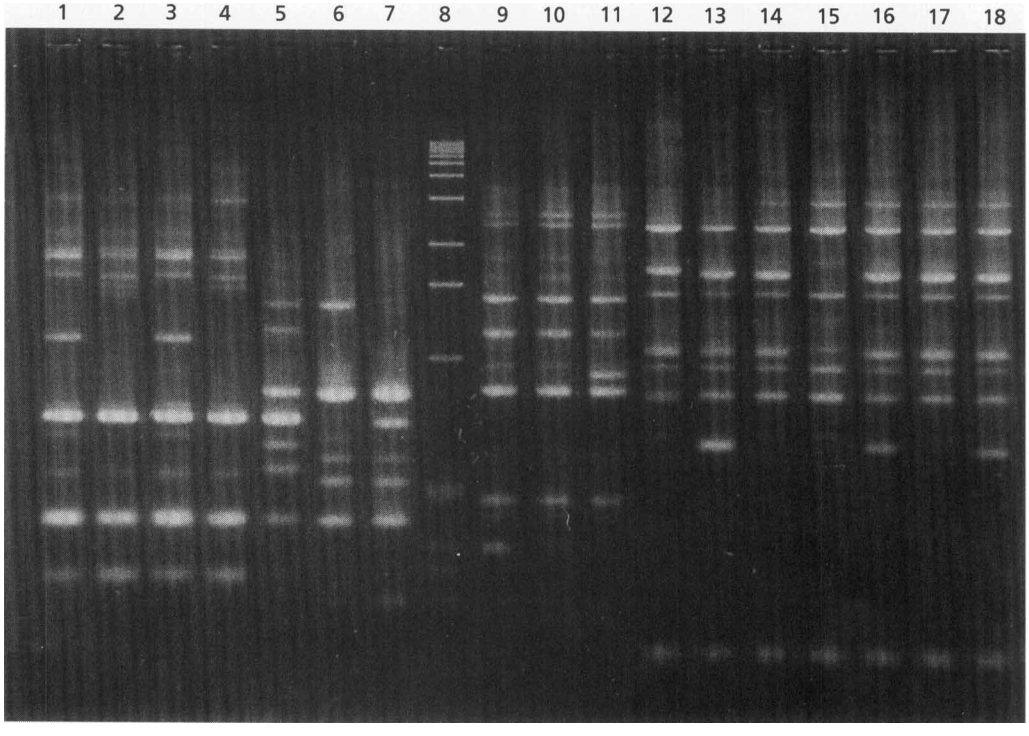

Fig. 3. RAPD band patterns generated using primer OPA-03 and template DNAs from strains of *B. popilliae* and *B. lentimorbus*. DNA ladder is in lane 8 (sizes as in Fig. 2). Lanes: 1, NRRL B-2309; 2, NRRL B-2522; 3, NRRL B-4145; 4, NRRL B-4154; 5, Bp6; 6, NRRL B-4081; 7, Bp3; 8, ladder; 9, ATCC 14707^T; 10, 14707; 11, KLN2; 12, Bp1; 13, Bp7; 14, DGB1; 15, BpCb1; 16, BpCb2; 17, BpPa1; 18, BpCp1.

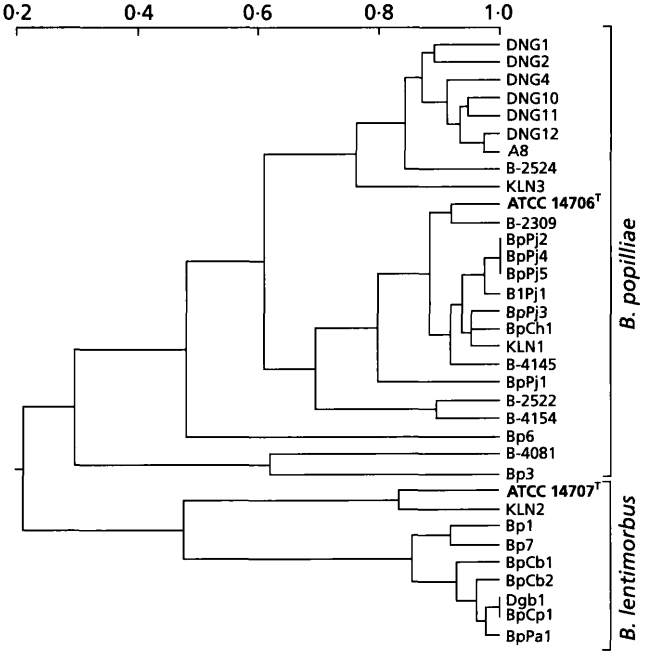

Fig. 4. UPGMA dendogram derived from similarity coefficients calculated by the Jaccard algorithm showing relationships between strains of *B. popilliae* and *B. lentimorbus*. The *x*-axis represents UPGMA similarities based on a *Q*-correlation matrix.

DISCUSSION

DNA similarity analysis was used to elucidate the genetic relationship between 34 isolates of bacteria causing milky disease in scarab larvae. The strains separated into two species based on greater than 70% similarity to the type strains of the species (6, 16, 19). Twenty-four were shown to be *B. popilliae* by their relatedness to the type strain, ATCC 14706^T, and eight were shown to be *B. lentimorbus* by their relatedness to ATCC 14707^T. Strains NRRL B-2522 and BlPj1 were received as *B. lentimorbus* but were found to be most closely related to *B. popilliae*. Both these strains grew with 2% NaCl in the medium (NRRL B-2522 only in broth) and both were resistant to vancomycin, traits that are associated with *B. popilliae*. Both DNA reassociation and RAPD analysis revealed the existence of sub-groups within the main body of *B. popilliae* strains. One closely related group contains strains DNG1 through KLN3 and a second group contains strains ATCC 14706^T through NRRL B-4154 (Fig. 4). The European isolate referred to as *B. popilliae* var. *melolonthae* (NRRL B-4081) and the North American isolate Bp3 had lower DNA similarity to the *B. popilliae* type strain than did the remaining isolates of this species, and the main body of *B. popilliae* isolates showed less than 70% similarity to NRRL B-4081, suggesting that these strains may constitute a subspecies of *B. popilliae*. The vancomycin resistance of these two strains points to their relationship to *B. popilliae*; however, both the DNA similarity and RAPD analysis clearly indicated their uniqueness.

Of the eight strains showing greater than 70% similarity to the *B. lentimorbus* type strain, seven had been received as *B. popilliae*. Only one of these, BpPa1, grew with 2% NaCl in the medium (and then only on plates), and all of them were sensitive to 150 μg vancomycin ml^{-1}. Six of these strains displayed parasporal bodies when retested by infecting Japanese beetle or masked chafer larvae. Although the presence of a parasporal body has been used as a distinguishing characteristic of *B. popilliae*, we have shown that paraspores may also be formed by *B. lentimorbus*. It appears that the paraspore-forming isolates may constitute a distinct sub-group of this species. The strains that were received as *B. popilliae* but that are now known to be *B. lentimorbus* had lower similarity to the type strain (73–78%) than did KLN2 (90%), received as *B. lentimorbus*. The RAPD analysis also supports the existence of two groups within the species *B. lentimorbus*, one that is similar to the type strain and fails to produce paraspores and a second whose strains produce paraspores. It is noteworthy that all of the isolates of the second sub-group were isolated from insects other than *Popillia japonica*.

The observation that vancomycin resistance is a uniform trait among strains of *B. popilliae*, as that species is defined by the DNA similarity, offers a simple phenotypic test for identifying the species. This test appears to be somewhat more reliable than growth in the presence of 2% NaCl.

This study focused mainly on North American isolates that were available in pure culture or that we were able to recover from larval material supplied to us. We have not examined A_2 or B_2 isolates, and these may reveal further diversity among the milky disease bacteria. There are also some strains that have been described in the literature solely on their appearance in infected larval haemolymph but which have not been grown *in vitro*. It would be of value to be able to examine their relationships to the better-known strains. An understanding of the genetic relationships among these bacteria and the discovery of sub-groups within the species may provide insight into the specificity which these bacteria exhibit in their infection of various species of scarab larvae.

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