# CHAPTER THREE

## DNA Similarity Analysis and Phenotypic Traits of *Paenibacillus popilliae* and *Paenibacillus lentimorbus*


### Abstract

*Paenibacillus popilliae* and *P. lentimorbus* have been differentiated based on phenotypic traits and, more recently, at the molecular level using DNA similarity and random amplified polymorphic DNA (RAPD) analyses. The bacteria used in this study were isolated in Mexico, Costa Rica, Argentina, Colombia, Honduras, and Nicaragua. All strains were received as *P. popilliae* based on the appearance of milky disease in infected larvae. The species of the bacteria were determined using DNA similarity studies and strains were tested for phenotypic characteristics. DNA similarity results revealed that one group of strains was *P. popilliae* and a second group contained strains more closely related to *P. lentimorbus*. Additionally, two European strains (NRRL B-4081 and H1) isolated from larvae of *Melolontha melolonthae* were included in this study. The European strains were more closely related to *P. popilliae* than to *P. lentimorbus*. Strain H1 is of particular interest because it is the strain from which the only published parasporal gene sequence was obtained.

Although the presence of a parasporal body in *P. popilliae* and the absence of a paraspore in *P. lentimorbus* previously appeared to be a reliable phenotypic trait in differentiating the two species, microscopic examination of infected hemolymph revealed that parasporal bodies were present in both species. *Paenibacillus popilliae* has also been distinguished from *P. lentimorbus* by the ability of *P. popilliae* to grow in medium supplemented with 2% sodium chloride. However, I found strains of *P. lentimorbus* capable of growth in 2% sodium chloride and strains

of *P. popilliae* incapable of growth in 2% sodium chloride. Only one of the *P. popilliae* strains (from Mexico) was found to be resistant to the antibiotic vancomycin and all strains of *P. lentimorbus* were vancomycin sensitive. It was surprising to find strains of *P. popilliae* that were sensitive to vancomycin because all strains of *P. popilliae* from North America and Europe that have been characterized are vancomycin resistant. Using PCR, part of the ligase gene necessary for vancomycin resistance was amplified and sequenced in the vancomycin resistant Mexican strain. The sequence is identical to a North American *P. popilliae* strain previously described.

## Results

**DNA similarity.** Percent DNA similarity values are shown in Table 1. Percent similarity data were analyzed using the distance and Q-correlation coefficient algorithms and subjected to clustering by the unweighted pair group method with arithmetic averages (UPGMA). The cophenetic correlations for the distance and correlation clusters were r=0.97 and r=0.99, respectively, indicating a strong correlation between the DNA similarity matrices and the phenograms. The distance-based phenogram is used because it showed better resolution among the groups (Fig. 1).

Table 1. Levels of DNA similarity among strains of *Paenibacillus popilliae* and *P. lentimorbus*

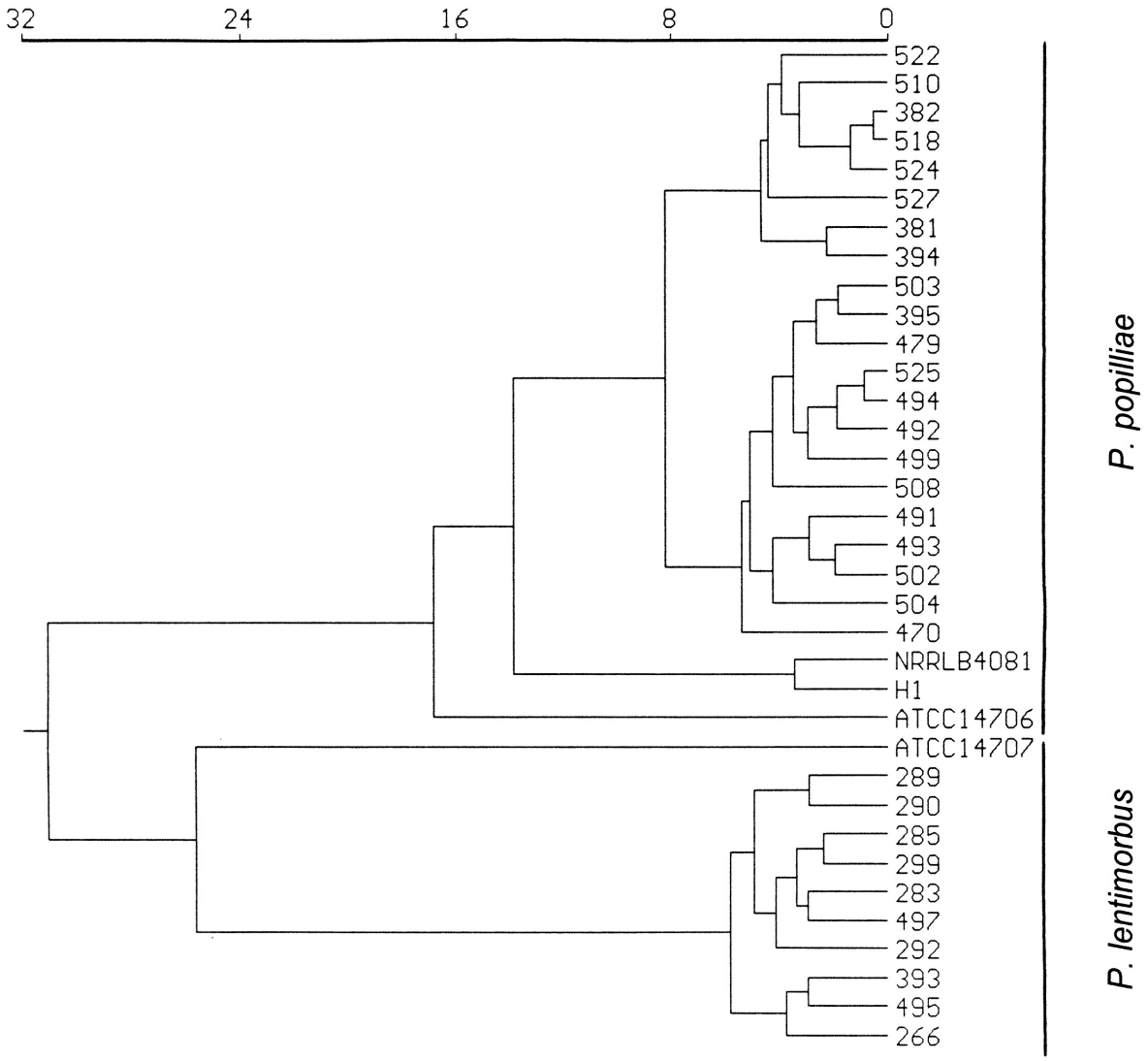
| Strain | % similarity with: *P. popilliae* | | *P. lentimorbus* | |
|---|---|---|---|---|
| | ATCC 14706$^T$ | 522 | ATCC 14707$^T$ | 289 |
| *P. popilliae* | | | | |
| ATCC 14706$^T$ | 100 | 69 | 49 | 54 |
| NRRL B-4081 | 61 | 62 | 47 | 50 |
| H1 | 58 | 61 | 48 | 56 |
| 522 | 73 | 100 | 47 | 55 |
| 381 | 74 | 92 | 47 | 57 |
| 382 | 70 | 96 | 51 | 57 |
| 394 | 77 | 89 | 46 | 58 |
| 395 | 70 | 84 | 49 | 55 |
| 470 | 65 | 76 | 49 | 53 |
| 479 | 75 | 85 | 51 | 58 |
| 491 | 77 | 76 | 47 | 59 |
| 492 | 71 | 78 | 48 | 57 |
| 493 | 73 | 75 | 52 | 57 |
| 494 | 71 | 80 | 49 | 54 |
| 499 | 76 | 79 | 48 | 53 |
| 502 | 74 | 74 | 50 | 60 |
| 503 | 73 | 86 | 49 | 55 |
| 504 | 72 | 72 | 46 | 53 |
| 508 | 76 | 84 | 48 | 49 |
| 510 | 71 | 97 | 53 | 62 |
| 518 | 70 | 95 | 51 | 57 |
| 524 | 70 | 94 | 49 | 56 |
| 525 | 71 | 81 | 48 | 55 |

| | | | | |
|---|---|---|---|---|
| 527 | 67 | 90 | 49 | 54 |
| *P. lentimorbus* | | | | |
| ATCC 14707$^{T}$ | 41 | 49 | 100 | 64 |
| 289 | 47 | 55 | 61 | 100 |
| 266 | 55 | 43 | 66 | 100 |
| 283 | 53 | 52 | 62 | 95 |
| 285 | 46 | 52 | 63 | 97 |
| 290 | 43 | 55 | 65 | 100 |
| 292 | 49 | 45 | 65 | 93 |
| 299 | 48 | 52 | 60 | 94 |
| 393 | 52 | 51 | 66 | 100 |
| 495 | 55 | 48 | 66 | 100 |
| 497 | 51 | 47 | 61 | 97 |

Analysis revealed two distinct groups for the Central and South American strains: one group contained strains of *P. popilliae* and the second contained strains more closely related to *P. lentimorbus* (Fig. 1). The European strains (NRRL B-4081 and H1) were distinct from the Central and South American strains but related to *P. popilliae*. Central American strain 522 was determined to be *P. popilliae* based on 73% similarity to the *P. popilliae* type strain, ATCC 14706. Strain 289 was more closely related to the *P. lentimorbus* type strain ATCC 14707 (61%) than to the *P. popilliae* type strain (47%). Twenty of the strains showed 65% to 77% similarity to the *P. popilliae* type strain. These same strains showed 72% to 97% similarity to strain 522, indicating a high degree of relatedness within the group. Nine of the strains had 60% to 66% similarity to the *P. lentimorbus* type strain and 93% to 100% similarity to strain 289, showing the high degree of relatedness among this group of strains. Strains NRRL B-4081 and H1 were more closely related to *P. popilliae* (61% and 58%, respectively) than to *P. lentimorbus*

(47% and 48%, respectively) and appear to represent a subgroup of *P. popilliae* distinct from the Mexican and Central and South American strains of *P. popilliae*.

Figure 1. UPGMA phenogram showing relationships among strains of *P. popilliae* and *P. lentimorbus* as determined by DNA similarity data. The X-axis represents UPGMA percent dissimilarity based on arithmetic distances.



**Phenotypic characterization**. The results of the phenotypic testing are shown in Table 2. Parasporal body formation was once thought to be unique to *P. popilliae,* but I found that both *P. popilliae* and *P. lentimorbus* strains from Mexico and Central and South America were capable of producing a paraspore. Our data support a report by Rippere *et al*. (4) in which they identified a subgroup of North American *P. lentimorbus* strains that produced paraspores. A phenotypic test utilized by Gordon *et al*. (1) was the ability of *P. popilliae* and the inability of *P. lentimorbus* to grow in media supplemented with 2% sodium chloride. Eighteen of the 21 (86%) *P. popilliae* strains were able to grow in medium supplemented with 2% NaCl whereas only four of the 10 (40%) *P. lentimorbus* strains were able to grow in this medium. Rippere *et al*. (4) also reported strains of *P. lentimorbus* capable of growth in 2% sodium chloride and strains of *P. popilliae* that were incapable of growth in this medium. Stahly *et al.* (5) found that the antibiotic vancomycin could be used in media for the selection and quantitation of *P. popilliae* spores in soils and in commercial spore powders. All North American and European strains of *P. popilliae* that were examined by Rippere *et al*. (4) were found to be resistant to vancomycin, whereas all *P. lentimorbus* isolates were sensitive. However, only one of the Mexican and Central and South American strains of *P. popilliae*, Mexican strain 508, was found to be resistant to vancomycin at concentrations ranging from 150 to 1000 μg/ml. Vancomycin sensitive strains of *P. popilliae* were also tested on media containing vancomycin concentrations ranging from 1.5 to 100 μg/ml. No growth was observed at any of the vancomycin concentrations. All strains of *P. lentimorbus,* like their North American and European counterparts, were sensitive to vancomycin. PCR was used to amplify a portion of the *vanE* ligase gene in the vancomycin resistant Mexican strain 508 (Fig. 2) with primers described by Rippere *et al.* (3). Vancomycin sensitive strain 289 from South American was also tested for the ligase gene, and, as expected, the gene was not detected

(Fig. 2). Sequencing of the PCR product from strain 508 revealed a gene sequence identical to *P. popilliae* strain Bp17 that was described by Rippere *et al*. (3).

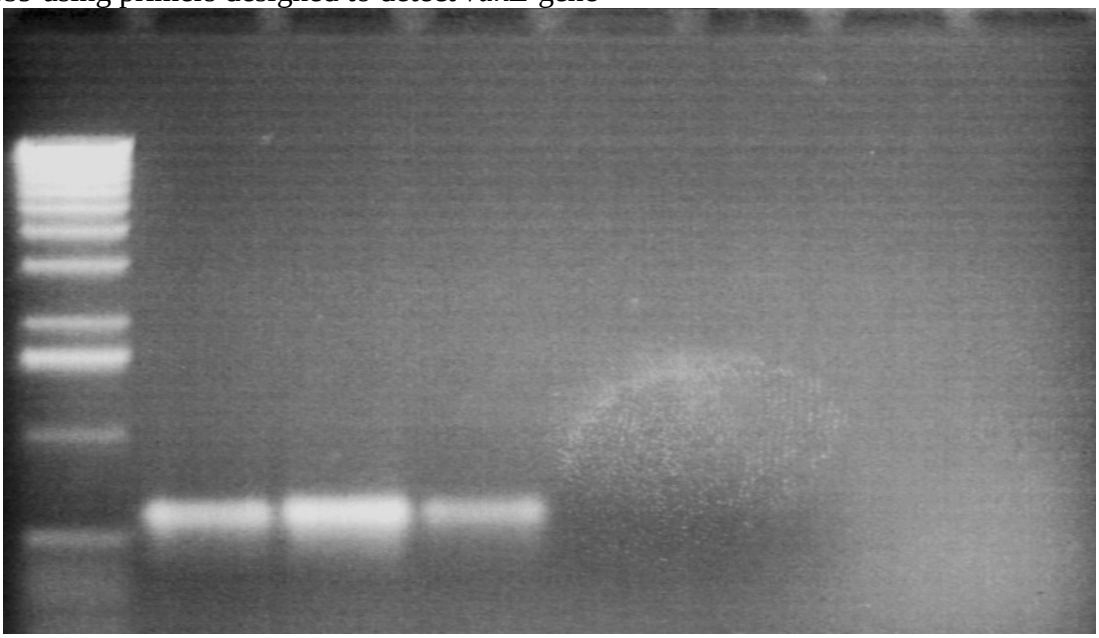
Table 2. Phenotypic characteristics of *Paenibacillus popilliae* and *P. lentimorbus*

| Strain | Paraspore[1] | Vancomycin Resistance[2] | NaCl[3] |
|---|---|---|---|
| *P. popilliae* | | | |
| ATCC 14706$^T$ | + | + | + |
| NRRL B-4081 | + | + | - |
| H1 | + | + | - |
| 381 | + | - | + |
| 382 | + | - | + |
| 394 | + | - | - |
| 395 | + | - | + |
| 470 | + | - | + |
| 479 | + | - | - |
| 491 | + | - | + |
| 492 | + | - | + |
| 493 | + | - | + |
| 494 | + | - | + |
| 499 | + | - | + |
| 502 | + | - | + |
| 503 | + | - | - |
| 504 | + | - | + |
| 508 | + | + | + |
| 510 | + | - | + |
| 518 | + | - | + |
| 522 | + | - | + |
| 524 | + | - | + |
| 525 | + | - | + |

| | | | |
|---|---|---|---|
| 527 | + | - | + |
| *P. lentimorbus* | | | |
| ATCC 14707$^{T}$ | - | - | - |
| 266 | + | - | + |
| 283 | + | - | - |
| 285 | + | - | - |
| 289 | + | - | + |
| 290 | + | - | + |
| 292 | + | - | - |
| 299 | + | - | + |
| 393 | + | - | - |
| 495 | + | - | - |
| 497 | + | - | - |

[1]Presence of paraspore was determined microscopically by examining hemolymph smears
[2](+) indicates growth on MYPGP plates supplemented with 150 μg/ml vancomycin
[3](+) indicates growth on MYPGP plates supplemented with 2% NaCl

Figure 2. PCR products for *P. popilliae* strains ATCC 14706, H1, and 508 and *P. lentimorbus* strain 289 using primers designed to detect *vanE* gene



Lane 1, 1 kb DNA ladder; Lane 2, ATCC 14706; Lane 3, H1; Lane 4, 508; Lane 5, 289; Lane 6, negative control (no DNA)

## Discussion

DNA similarity studies were used to determine the species of thirty-one strains of bacteria isolated from larvae infected with milky disease. The Mexican and Central and South American strains separated into two groups based on similarity to the *P. popilliae* type strain ATCC 14706 or to the *P. lentimorbus* type strain ATCC 14707. Although some investigators (2, 4) have used a similarity value of 70% or greater when placing strains into a species, Ursing *et al*. (6) considered strains with a 50 to 70% similarity to be within a species. Vandamme *et al*. (7) also noted that the 70% rule should not be considered absolute. Using these criteria, twenty-one of the strains (strains 522 through 470) with 65 to 77% similarity to the *P. popilliae* type strain were classified as *P. popilliae* (Fig. 1). Ten strains (strains 289 through 266) showed 60 to 66% similarity to the *P. lentimorbus* type strain and have been classified as *P. lentimorbus* (Fig. 1). European strains NRRL B-4081 and H1 appear to be a distinct subgroup within the *P. popilliae* group of strains (Fig. 1). Strain NRRL B-4081 was also included in a study by Rippere *et al*. (4) and they suggested that this strain represented a subspecies of *P. popilliae*. Speciation of the Mexican and Central and South American strains would not be possible if based entirely on phenotypic characteristics because there were exceptions to each trait: parasporal bodies were present in all strains of both species, only one *P. popilliae* strain was vancomycin resistant, and the ability to grow in medium supplemented with 2% sodium chloride was variable. Both NRRL B-4081 and H1 are vancomycin resistant and are incapable of growth in media supplemented with 2% sodium chloride. It is clear that a simple phenotypic test is required for the differentiation of these genetically distinct species.

Vancomycin resistance was found in a strain of *P. popilliae* preserved as a hemolymph smear since 1945, before vancomycin resistance became a clinical problem in enterococci (3).

Because of the critical importance of vancomycin in treatment of human pathogens that have become resistant to other antibiotics, the mechanism of resistance to this antibiotic has become the subject of intensive investigation. Rippere *et al*. (3) have suggested that the genes of the enterococci and *P. popilliae* may have a common ancestor or that possibly the genes of *P. popilliae* have been transferred to the enterococci because of the presence of a gene in *P. popilliae* homologous to the *van*A and *van*B ligase genes of the enterococci. The absence of vancomycin resistance in the Central and South American strains of *P. popilliae* indicates a unique geographic distribution of the resistance genes. The absence of vancomycin resistance in any of the *P. lentimorbus* strains suggests an inability of these bacteria to exchange genetic material with *P. popilliae*, despite living in apparently similar ecological niches. Based on the available data, it appears safe to conclude that vancomycin-resistant milky disease isolates obtained from infected larvae in North America and probably Europe are *P. popilliae*, whereas vancomycin sensitive isolates from those areas are probably *P. lentimorbus*. However, this characteristic is of no help in identifying isolates from Central or South America.

Correlations between species, host insect, geographical or environmental source could not be determined based on results of phenotypic tests. However, it should be noted that the vancomycin resistant Mexican strain (508) was the only Mexican and Central and South American strain isolated from *Phyllophaga crinita*. Sequencing data revealed that the gene sequence was identical to another *P. popilliae* strain (Bp17) also isolated from *Phyllophaga crinita*. The sequence of the ligase gene in *P. popilliae* strain Bp17 was reported by Rippere *et al*. (3). Comparisons of phenotypic traits and DNA similarity studies are needed on strains from more diverse geographic areas in order to gain a better understanding of the diversity among the strains within these two species.

## References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