CHAPTER ONE

Review of the Literature

Milky Disease

Paenibacillus popilliae and *P. lentimorbus* (formerly genus *Bacillus*) are spore forming pathogens unique to the insect family *Scarabaeidae* and are responsible for milky disease in Japanese beetle and related scarab larvae. Japanese beetles were first introduced to the United States in 1916 (21), and were probably carried in soil around the roots of Japanese iris plants (52). Scarab larvae cause extensive damage to shrubs, grasses, field and orchard crops, and ornamental trees by feeding on the roots and underground stems of plants. Japanese beetles damage about 275 different plants (54) and by 1962, Japanese beetles had infected more than 100,000 square miles in the eastern United States (21). Hawley and White (23) described three groups of beetle diseases based on the appearance of diseased larvae, the black group, the white group, and the fungus group, and concluded that bacteria were responsible for the diseases of the white group. Dutky (18) recognized two distinct types of diseases in the white group, type A and type B milky diseases. In type A milky disease, caused by *P. popilliae*, the larvae turn a milky white color due to the buildup of spores in the hemolymph. *Paenibacillus lentimorbus* is responsible for type B milky disease and the larvae are described as having a brown color due to blood clots that formed in the hemolymph; however, larvae injected with the hemolymph of type B diseased larvae display the type A milky disease (18).

Paenibacillus popilliae spores have been used in the United States for control of the Japanese beetle for more than 50 years and this bacterium was the first microbial insecticide registered in the United States (58). Between 1939 and 1952, approximately 83,600 kg of spore powder was applied

to more than 42,000 hectare in the eastern United States (39). Spore powders were made *in vivo* as described by Dutky (19). Large numbers of spores were injected into the hemolymph of healthy larvae and the milky diseased larvae were ground into powders and mixed with an inert carrier. The inability to achieve *in vitro* sporulation of *P. popilliae* and *P. lentimorbus* and the rapid loss of vegetative cell viability in soil has prevented large-scale production and use of the organisms as biopesticides. Commercial spore powders are made *in vivo* on a small scale from ground milky diseased larvae. Based on a report written by Hanula and Andreadis, Klein (28) noted that very little *P. lentimorbus* was found in field populations and commercial spore powders. Identification and differentiation of *P. popilliae* and *P. lentimorbus* has typically been based on phenotypic characteristics, but recent DNA similarity and RAPD analyses have found that some strains of *P. lentimorbus* have been incorrectly identified as *P. popilliae* (49). Until recently, *P. popilliae* and *P. lentimorbus* were classified within the *Bacillus* genus. Pettersson *et al*. (44) moved the two species to the *Paenibacillus* genus based on 16S rRNA gene sequences.

Pathology of *Paenibacillus popilliae* and *P. lentimorbus* infections

Scarab larvae ingest bacterial spores during feeding on plant roots. Spore germination occurs in the larval gut followed by penetration of the hemocoel by vegetative cells. Germination has not been observed in the hemolymph (18). After a period of growth in the larval hemolymph, the bacteria sporulate and spores are liberated into the soil following death and disintegration of the cadaver. Within 7 to 10 days, following spore ingestion, spore density can be as high as 5×10^9 ml^{-1} in the hemolymph (55). St. Julian *et al*. (55) described a four phase infection process: phase one is an initial incubation phase where no bacteria are present in the hemolymph (days 1-2); during phase

two, vegetative cells enter the hemolymph, grow, and replicate rapidly (days 3-5); phase three is an intermediate phase in which vegetative growth and sporulation occur simultaneously in the hemolymph (days 5-10); during phase four massive sporulation and larval death occur (days 14-21).

During sporulation, proteinaceous parasporal bodies are formed. Both the spore and the parasporal body remain enclosed within the sporangium and the sporangium is very resistant to lysis (52, 66). Mitruka *et al*. (38) reported that the 'refractile' bodies were distinctly different from vegetative cells and spores of *P. popilliae*. Dutky (20) demonstrated that cell-free filtrates of *P. popilliae* cultures were lethal when injected into larvae, and suggested that the bacteria may produce toxic substances. Pridham *et al*. (45) found that *P. popilliae* and *P. lentimorbus* vegetative cells were capable of infecting larvae when injected, but 'typical' milky disease symptoms were not always seen. Weiner (66) found that larvae were killed when solubilized parasporal protein was injected into the larval hemocoel, whereas no toxic effects were seen when parasporal protein was fed to larvae. Zhang *et al*. (69) cloned and sequenced the gene (*cry18Aa1*) encoding the parasporal protein and found that the gene shares significant sequence similarity to *cry* genes of *Bacillus thuringiensis*, suggesting that the paraspore may be an important factor in the disease process. Zhang *et al.* (69) suggested that enzymes, either bacterial or larval, activate the parasporal protein which can then bind to and damage the larvae gut wall to allow vegetative cells to enter the hemolymph. However, the exact cause of larval death is not clearly understood and there is no direct evidence for a role of the parasporal protein in the pathogenic process.

Physiology of *Paenibacillus popilliae* and *P. lentimorbus*

Dutky (18) described *Paenibacillus popilliae* and *P. lentimorbus* vegetative cells as gram-

positive, non-motile rods. Gordon *et al*. (22) reported that the sporangia and prespores were gram-positive, but vegetative cells were gram-negative. Black (7,8) used electron microscopy to describe vegetative cells and structural changes during sporulation and found a cell wall structure typical of gram-positive bacteria. Gordon *et al*. (22) reported that some strains of *P. popilliae* were motile with peritrichous flagella, whereas all strains of *P. lentimorbus* observed were non-motile. Luthy *et al*. (32) and Splittstoesser *et al*. (51) also reported that *P. popilliae* cells were motile in tissue cultures and during germination and outgrowth in hemolymph slide mounts, respectively.

Both *P. popilliae* and *P. lentimorbus* are facultative anaerobes, but grow better in the presence of oxygen (57). Glucose, galactose, mannose, maltose, and trehalose can be utilized as energy sources (36). Bulla *et al*. (10) and Pepper and Costilow (41) found that the Embden-Myerhof-Parnas (EMP) and pentose-phosphate pathways are responsible for glucose metabolism *in vitro*. The EMP pathway is the primary pathway when cells are grown under aerobic conditions and acetate, lactate, and carbon dioxide are the primary products formed from the breakdown of glucose (41). Glucose is not catabolized under anaerobic conditions (41). *In vivo*, the pentose-phosphate pathway is the primary pathway for glucose catabolism (53). St. Julian *et al*. (53) proposed that compounds in the larval hemolymph repress EMP enzymes. Taylor and Costilow (61) reported that *P. popilliae* most likely transports glucose into cells by the phosphoenolpyruvate (PEP):sugar phosphotransferase system and that maltose enters as free maltose which can be hydrolyzed to glucose once inside the cells. Bhumiratana *et al*. (5) data suggested that trehalose was also transported into cells by a PEP:sugar phosphotransferase system as trehalose 6-phosphate and subsequently hydrolyzed to glucose and glucose 6-phosphate. Trehalose, not glucose, is found in high concentrations in the larval hemolymph (47). St. Julian *et al*. (53) found that *P. popilliae* does

not have a fully operational tricarboxylic acid cycle and that cells lacked citrate synthase and had low levels of aconitase, isocitric dehydrogenase, and succinic dehydrogenase. St. Julian *et al*. (53) stated that the lack of a complete tricarboxylic acid cycle did not affect sporulation *in vivo*, indicating that sporulation within the larvae was host dependent.

Paenibacillus popilliae and *P. lentimorbus* are highly fastidious and complex, nutritionally rich media is required to maintain cultures *in vitro*. Aromatic amino acids, serine, histidine, asparagine, and methionine must be supplied for growth (11). Thiamine is also required for growth (20, 60). Sylvester and Costilow (60) studied the nutritional requirements of *P. popilliae* and found that biotin, myoinositol, and niacin stimulate growth. They also reported that barbituric acid was necessary, but could not find the function of the barbituric acid. Under aerobic conditions, *P. popilliae* cultures reach a maximum population of 1.2×10^9 cells ml^{-1} within 16-20 hours, while under anaerobic conditions populations reach a maximum of 5×10^8 cells ml^{-1} (11). The number of viable cells declines immediately after exponential growth *in vitro* (11). Pepper and Costilow (42) found the *P. popilliae* cells lack a hydrogen peroxide scavenging system and catalase and peroxidase were not found. Bulla *et al*. (11) and Pepper and Costilow (42) indicated that a buildup of hydrogen peroxide during stationary-phase growth could be one reason for cell death *in vitro*. Pepper and Costilow (42) suggested that *in vivo* the larvae could provide a peroxide scavenging system or an alternative electron acceptor. St. Julian and Bulla (52) speculated that hydrogen peroxide poisoning doesn't occur because formation in vegetative cells was insignificant. St. Julian and Bulla (52) also reported that death due to exposure to superoxide radicals was unlikely because of the high levels of superoxide dismutase found in *P. popilliae*. Rhodes (47) suggested that the rapid loss of cell viability was due to the inability of cells to complete the sporulation process, which starts at a certain

point during vegetative proliferation.

Paenibacillus popilliae and *P. lentimorbus* sporulate *in vivo*. Bulla *et al*. (12) used scanning electron microscopy to characterize spore coats and found that the spore coats of both organisms possess roughly textured surfaces consisting of interconnected longitudinal and perpendicular ridges. Mitruka *et al*. (38) reported that the spore and parasporal body of *P. popilliae* had catalase activity, whereas the vegetative cells lack catalase activity, and that the appearance of catalase could be used as a sporulation marker since catalase is detected as cell refractility increases. Mitruka *et al.* (38) suggested that the parasporal body was the result of an aborted sporulation process. However, this seems unlikely as some other bacilli produce paraspores in the course of normal sporulation. The metabolism and nutritional requirements of *P. popilliae* have been studied in an effort to explain lack of sporulation *in vitro*. Costilow and Coulter (14) proposed that *P. popilliae* requires carbohydrate concentrations that limit growth or inhibit sporulation. St. Julian and Bulla (52) speculated that sporulation does not occur because once protein synthesis stopped in stationary phase, lipid synthesis and membrane formation halted which prevented further growth and development. Black (8) suggested that lack of *in vitro* sporulation could be due to a mutation or repression of an essential metabolic step.

Vancomycin resistance in *Paenibacillus popilliae*

Vancomycin resistance has been used as a phenotypic trait for distinguishing between *P. popilliae* and *P. lentimorbus*, the former being resistant to vancomycin. Stahly *et al*. (59) found that vancomycin could be used in a selective medium for the quantitation of *P. popilliae* spores in soils and in commercial spore powders. The emergence of vancomycin resistant enterococci has

prompted important medical concerns because of the possibility that the genes for vancomycin resistance may be transferable to other gram-positive bacteria, especially *Staphylococcus aureus* (40). Walsh (65) noted that vancomycin is one of a few antibiotics that provide an effective treatment against ß-lactam antibiotic resistant streptococcal and staphylococcal infections. Vancomycin acts by binding to D-alanyl-D-alanine in the cell wall precursor, thus inhibiting peptidoglycan cross-linking and further cell growth (4). Vancomycin is only effective against gram-positive organisms because it cannot cross the outer membrane of gram-negative cells (4). In vancomycin resistant enterococci, a D-alanyl-D-lactate replaces the D-alanyl-D-alanine as the terminus in cell wall peptidoglycan precursor so that vancomycin can no longer bind or binds poorly (27).

Inducibility and resistance to vancomycin and the related antibiotic teicoplanin is characterized by four phenotypes. VanA is described as having acquired inducible resistance to vancomycin and a high minimum inhibitory concentration (MIC) for vancomycin and teicoplanin (1). The genes responsible for the VanA phenotype are carried by a transposon called Tn*1546*, and are usually found on a plasmid (2). VanB is also described as having acquired inducible resistance to vancomycin, but VanB has a variable MIC for vancomycin and is sensitive to teicoplanin (1). VanB genes are found on either a large conjugative chromosomal element or on a plasmid (46). VanC phenotype exhibits low-level resistance to vancomycin and can be constitutive, as seen in *Leuconostoc* and *Lactobacillus*, or inducible, as seen in enterococci (17, 50). VanD was described in enterococci and is characterized as constitutive with low-level resistance to vancomycin and teicoplanin (43). The genes for *vanA*, *vanB*, *vanC* (*vanC-1*, *vanC-2*, and *vanC-3*), and *vanD* encode for ligases. VanA, VanB, VanC-2/3, and VanD ligate a D-alanine to D-lactate, whereas VanC-1

ligates a D-alanine to D-serine (6, 9, 35, 43).

Rippere *et al.* (48) have hypothesized that past use of *P. popilliae* as a biopesticide may have provided the opportunity for transfer of vancomycin resistance genes to enterococci. Rippere *et al*. (48) have identified a putative ligase gene, designated *vanE*, in the *P. popilliae* type strain ATCC 14706 that shares sequence similarity to enterococci *vanA* and *vanB* genes. Other strains of *P. popilliae* have been screened for the *vanE* gene using PCR primers designed from the sequence of the *P. popilliae* type strain; all showed similarity to the *vanA* and *vanB* sequences in enterococci (48). Transfer of vancomycin resistance genes has not been shown to occur between *P. popilliae* and enterococci.

Molecular biology of *Paenibacillus popilliae* and *P. lentimorbus*

Genetic studies have been restricted to the isolation of plasmids (31) as there are no known transformation or conjugation systems for *Paenibacillus popilliae* or *P. lentimorbus* (3). Valyasevi *et al*. (63) isolated and described three plasmids from *P. popilliae* vegetative cells obtained from milky diseased larvae. Plasmid pBP149, pBP082, and pBP043 were estimated to be 12, 7.4, and 4.9 kb, respectively. Restriction and hybridization studies revealed that plasmids pBP149 and pBP082 shared no significant homology; the relationship of plasmid pBP043 was not determined.

Dingman (16) isolated and characterized three plasmids from *P. popilliae* strain KLN4. Plasmids pBP68, pBP88, and pBP94 were determined to be 6.8, 8.8, and 9.4 kb, respectively. Hybridization studies showed that the three plasmids were also found in *P. popilliae* strains NRRL B-2524, BpPa, and Ch1. Dingman (16) also demonstrated interplasmid homology by comparing shared sequence homology of plasmids pBP68, pBP88, and pBP94 to other plasmids isolated from

P. popilliae and *P. lentimorbus*.

MacDonald and Kalmakoff (33) used pulsed-field gel electrophoresis fingerprinting to show the relatedness of the *P. popilliae* type strain, ATCC 14706, to strain NRRL B-2309 and to compare strains of *P. popilliae* from New Zealand. Restriction patterns were produced by rare cutting endonucleases; the more similar the restriction patterns were, the more closely related the strains were genetically. All strains displayed different fingerprint patterns, but the relatedness of the New Zealand strains could be seen when shared bands in the fingerprints were examined. MacDonald and Kalmakoff (33) suggested that, in addition to studying the genetics of *P. popilliae*, this type of study also could be used to study the spread of the organisms.

Longley *et al*. (31) isolated a 5.6 kb plasmid, plasmid pBP614, from a New Zealand strain of *P. popilliae* and found that the plasmid replicates by a rolling circle mode, a characteristic shared with a family of rolling circle plasmids found in many Gram-positive bacteria. Plasmid pBP614 is the first *P. popilliae* plasmid to be sequenced and shows closest homology to the pC194 family of rolling circle plasmids (31). The role of plasmids found in *P. popilliae* and *P. lentimorbus* is unknown (16, 63).

Zhang *et al*. (69) cloned and sequenced the gene that encodes the parasporal protein from *P. popilliae* subsp. *melolonthae* H1. The parasporal gene, *cry18Aa1*, was found in a putative operon on the bacterial chromosome. The operon consists of an open reading frame (*orf1*) that encodes a 19.6 kDa protein, and the *cry18Aa1* gene that encodes a 79 kDa protein. The function of the *orf1* is unknown, but shares sequence similarity to *orf1* of the *cry2Aa-cry2Ac* operon, *orf1* of the *cry9Ca* operon, and *p19* of the *cry11Aa* operon of *Bacillus thuringiensis*. Cry18Aa1 shares sequence similarity to *B. thuringiensis* and *Clostridium bifermentans* Cry proteins. Zhang et al. (70) found

that transcription of the *orf1* and *cry18Aa1* genes is driven by σ^E and σ^K types of RNA polymerase at a single transcription start site.

Taxonomic classification of *Paenibacillus popilliae* and *P. lentimorbus*

Classification of and differentiation between *Paenibacillus popilliae* and *P. lentimorbus* has been based on insect host, differences in phenotypic characteristics of the bacteria, and more recently, differences at the molecular level. *Paenibacillus popilliae* and *P. lentimorbus* were originally named by Dutky (18) based on the host from which they were isolated, *Popilliae japonica*. *Bacillus melolonthae* was isolated from the common cockchafer grub *Melolontha melolontha* (24). A number of varieties have also been described including *P. popilliae* var. *rhopaea*, which was isolated from *Rhopaea verreauxi* (36).

Dutky (18) recognized two distinct species based on differences in color of the hemolymph in infected larvae. *Paenibacillus popilliae* was responsible for type A disease, and diseased larvae turned milky-white due to the buildup of spores in the hemolymph. *Paenibacillus lentimorbus* caused type B disease, and diseased larvae were muddy-brown in color due to the clot formations in the larvae's appendages. Dutky (18) also differentiated between the two species based on the presence or absence of a parasporal body; *P popilliae* produced a parasporal body at the time of sporulation, whereas *P. lentimorbus* did not. Although the presence of a parasporal body in *P. popilliae* and its absence in *P. lentimorbus* appeared to be a reliable phenotypic trait in differentiating the two species, Rippere *et al*. (49) reported in a DNA similarity study that only two of the nine isolates having high similarity to the type strain of *P. lentimorbus* (ATCC 14707) failed to form a paraspore and suggested the existence of two groups within *P. lentimorbus*; one group produces a

parasporal body while the other does not.

The ability of *P. popilliae* and inability of *P. lentimorbus* to grow on medium containing 2% sodium chloride (57) has also been used as phenotypic characteristic for distinguishing between the two species. However, Rippere *et al*. (49) found exceptions for both *P. popilliae* and *P. lentimorbus*.

Based on physiological characteristics, Wyss *et al*. (68) suggested that organisms causing milky disease were a homogenous group and proposed two *Paenibacillus* species, *popilliae* and *euloomarahae*, with three subspecies in the former: *popilliae*, *lentimorbus*, and *melolonthae*. Krywienczyk and Luthy (29) supported Wyss' proposal of three *popilliae* subspecies based on shared common antigens.

Milner (37) suggested a single *Paenibacillus* species, *popilliae*, with four subgroups based on the size, shape, and position of the paraspore in the sporangium. *Paenibacillus popilliae* was divided into four subgroups, A_1, A_2, B_1, and B_2. Subgroup A1 is described as having a large spore with a parasporal body that is often small and overlaps the spore. Subgroup A_2 has a large spore with a parasporal body, but the parasporal body is often large and separated from the spore. Subgroup B_1 has a large central spore and no parasporal body and subgroup B_2 has a small spore in a large sporangium and no parasporal body.

De Ley (15) stated that the GC contents within a bacterial species should not vary by more than 2%. Stahly *et al*. (57) reported that *P. popilliae* had a GC content of 41.3 mol% and *P. lentimorbus* had a GC content of 37.7 mol%, supporting the idea that these are two separate species. However, Bergey's Manual (13) cited a number of cases in which the GC contents of *Bacillus* species varied by up to 14%. Rippere *et al*. (49) examined isolates from North America, Europe, and New Guinea using DNA similarity and random amplified polymorphic DNA (RAPD) analyses and

found two related but distinct species, *P. popilliae* and *P. lentimorbus*.

DNA similarities

Johnson (25) and Logan (30) describe DNA: DNA reassociation as the formation of duplexes between single-stranded DNA fragments that allows for the comparison of entire genomes of organisms, and stated that the amount of similarity can be used to express the relatedness between species. Stackebrandt and Goebel (56) recognized DNA hybridization as the best method for clarifying relationships between strains and species. Stackebrandt and Goebel (56) also reported that 16S rRNA should not replace DNA reassociation for determining relationships between strains and species because of the highly conserved primary structure of 16S rRNA and because species with 70% or more DNA similarity usually have more than 97% rRNA sequence similarity. McCarthy and Bolton (34) mentioned that the presence of mutual genes in organisms could be insufficient in determining relationships because many genes may be dormant and may not contribute to the function of an organism.

Logan (30) defined a bacterial species as one that shows relatedness of 70% or more and with 5°C or less ΔT_m. The $T_{m,}$ or melting temperature that causes double-stranded DNA molecules to denature, is linearly related to the mol% GC of an organism and can be used to determine mismatches in hybrid molecules by comparing the T_m values of heterologous and homologous duplexes (30). Ursing *et al*. (62) considered strains with a 50 to 70% DNA reassociation value and a 5 to 7°C difference in thermal stability of homologous and heterologous duplexes to be within a species. Vandamme *et al*. (64) stated that the 70% rule should not be absolute based on differences in methods and conditions used to determine DNA reassociation values. Salt and formamide

concentrations, reassociation temperature, and mol% GC can affect duplex formation. Johnson and Ordal (26) stated that homologous duplexes showed thermal stability similar to native DNA when a reassociation temperature of 25°C below the T_m was used. By decreasing the salt concentration and/or increasing the reassociation temperature, the stringency of the reactions is improved and interspecies base pairing will only occur between homologous sequences (67). Rippere *et al*. (49) used a DNA reassociation value of 70% or greater to either the *P. popilliae* type strain or to the *P. lentimorbus* type strain for determining speciation of bacteria isolated from milky diseased larvae. It should be noted that 32 of the 34 strains used in the studies by Rippere *et al*. (49) were isolates from the United States. DNA similarity studies are needed on strains from more diverse geographic areas with comparisons to a wider number of reference strains in order to gain a better understanding of the relationships and diversity among the strains within these two species.

DNA similarity experiments rely on the fact that DNA can denature and renature back into the native DNA molecule. Johnson (25) described two methods for measuring DNA similarity, a free solution method and a membrane method. In the free solution method, unlabeled competitor DNA and labeled reference DNA are denatured, mixed, and incubated in solution to allow the formation of duplexes. Any remaining single stranded DNA is degraded using S1 nuclease and the percent similarity is measured by radioactive counts. In the membrane method, unlabeled competitor DNA is denatured, fixed to a membrane, and incubated with labeled reference DNA that has also been denatured. Duplexes that form are stable and bound to the membrane, whereas labeled DNA that did not hybridize can be washed away. The percent similarity is measured by radioactive counts. In both methods, competitor DNA is used in excess to reduce reassociation of the labeled reference

DNA. Salmon sperm DNA is used as a control to measure background reassociation because salmon sperm DNA shares no sequence similarity to bacterial DNA. The amount that the competitor and reference DNA molecules hybridize correlates to the amount of similarity between the two molecules.

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