CHAPTER TWO

Materials and Methods

Media and reagents

MYPGP broth	1.5% yeast extract, 1.0% Mueller-Hinton broth, 0.3% K_2HPO_4, 0.1% sodium pyruvate, 0.2% glucose
MYPGP agar	1.5% yeast extract, 1.0% Mueller-Hinton broth, 0.3% K_2HPO_4, 0.1% sodium pyruvate, 0.2% glucose, 2.0% agar
1X TE buffer	10 mM Tris-HCl [pH 8.0] and 1 mM EDTA
Cell suspension buffer	10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 0.35 M sucrose, and 1 mg/ml lysozyme
Lysing solution	100 mM Tris-HCl [pH 8.0], 20 mM EDTA, 0.3 M NaCl, 2.0% [v/v] ß-mercaptoethanol, 2.0% [wt/v] SDS, and 100 µg/ml proteinase K
RNase	1 mg/ml RNase A dissolved in 0.15 M NaCl [pH 5.0] and 4000 U/ml T_1 RNase
Sodium acetate buffer	3.0 M sodium acetate [pH 6.0]
Sodium perchlorate buffer	5.0 M sodium perchlorate
1 M Sodium phosphate buffer	0.50 M $Na_2HPO_4 \cdot H_2O$ and 0.50 M $Na_2HPO_4 \cdot 7H_2O$ [pH 6.8]
Hydroxyapatite (HA) buffer	0.14 M sodium phosphate buffer and 0.5% SDS
Hydroxyapatite (HA) suspension	Bio-Rad DNA grade HA (calcium phosphate) and 0.01 M sodium phosphate buffer
Tris buffer	1.0 M Tris-HCl [pH 8.0]
TE + SDS buffer	10 mM Tris buffer, 1 mM EDTA, and 0.1% SDS
Salmon sperm DNA	salmon sperm DNA (Sigma D-1626) dissolved in TE buffer, sheared to 400-600 bp, and diluted to 1.2 mg/ml non-denatured, 0.4 mg/ml non-denatured, and 0.4 mg/ml

	denatured
S_1 nuclease reaction buffer	0.3 M NaCl, 0.05 M acetic acid, and 0.5 mM $ZnCl_2$ [pH 4.6]
1 M HCl buffer	1.0 M HCl, 1.0% $Na_4P_2O_7 \cdot 10H_2O$, and 1.0% $NaH_2PO4 \cdot H_2O$
Acid wash buffer	1:5 dilution of 1 M HCl buffer
S_1 nuclease storage buffer	20 mM Tris [pH 7.5], 50 mM NaCl, 0.1 mM $ZnCl_2$, and 50% glycerol
High salt buffer	3.2 X SSC and 5 mM HEPES [pH 7.0]
1X SSC	0.15 M NaCl and 0.015 M trisodium citrate
Nuclease S_1	dilute to 10 U/µl with S_1 nuclease storage buffer
50X TAE	2.0 M Tris, 57.1 ml/L glacial acetic acid, 100 ml/L 0.5 M EDTA [pH 8.0]
Gel loading dye	30% sucrose in 1X TE buffer with bromophenol blue
10X PCR buffer	500 mM KCl, 100 mM Tris-HCl [pH 9.0], 1% Triton X-100 (Promega)
$MgCl_2$ (Promega)	25 mM $MgCl_2$
10X restriction enzyme buffer B	10 mM Tris-HCl [pH 7.4], 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mg/ml BSA, 50% glycerol (Promega)

Pure culture isolation of bacterial strains

Bacterial strains were isolated in pure culture from glass slides smeared with the hemolymph of milky disease infected scarab larvae. Twenty-nine strains from Central and South America and 2 strains from Mexico were received from Philip Shannon at the Centro Agronomico Tropical De Investigacion Y Ensenanza (CATIE) in Costa Rica. Bacterial strain H1 was received as a spore suspension from Jianbing Zhang at the University of Heidelberg in Germany. A suspension of the

hemolymph was heated at 60°C for 20 min to kill vegetative cells and then one hundred microliters of 10^{-4} to 10^{-7} dilutions were plated on MYPGP (1) agar and incubated at 30°C for seven to fourteen days. Colonies that resembled *P. popillae* growth were transferred to MYPGP broth and then back to MYPGP plates to insure the isolation of a pure culture. Growth was examined under a phase-contrast microscope and hydrogen peroxide was used to verify that strains were catalase negative. Stock cultures are stored at −80°C in 10% glycerol.

Growth conditions and phenotypic characterization of bacterial strains

The bacterial strains used in this study are listed in Table 1. Bacteria used to inoculate flasks for DNA isolation were grown overnight in 5 ml of MYPGP broth with shaking at 30°C. Four, two-liter erlenmeyer flasks containing 500 ml of MYPGP broth each were inoculated with 10 ml of culture and incubated for 12-15 h in a New Brunswick G25 shaker at 30°C with shaking at 175 rpm. Cells were harvested by centrifugation at 9820 x g and 4°C for 5 min and the cell pellet was stored at −20°C.

Phenotypic characteristics were determined by plating bacterial strains on MYPGP agar containing either 2% NaCl or 150µg/ml vancomycin. The plates were streaked from a MYPGP broth grown overnight and then incubated at 30°C for 72 h before determining NaCl tolerance and vancomycin resistance. All plates were done in triplicate. Growth was determined by visual examination of the plates. The presence or absence of a parasporal body was determined visually by observing the hemolymph smears under a phase-contrast microscope.

Table 1. *Paenibacillus popilliae* and *P. lentimorbus* strains and their geographic and environmental sources

Strain	Host Insect	Geographic Source	Environmental Source
ATCC 14706[T]	*Popillia japonica*	USA[1]	unknown
ATCC 14707[T]	*Popillia japonica*	USA[1]	unknown
H1	*Melolontha melolonthae*	Germany[4]	unknown
NRRL B4081	*Melolontha melolonthae*	Europe[2]	unknown
266	*Phyllophaga elenans*	Costa Rica[3]	sugarcane
283	*Phyllophaga elenans*	Costa Rica[3]	sugarcane
285	*Phyllophaga menetriesi*	Costa Rica[3]	sugarcane
289	*Phyllophaga menetriesi*	Costa Rica[3]	sugarcane
290	*Phyllophaga menetriesi*	Costa Rica[3]	sugarcane
292	*Phyllophaga vicina*	Costa Rica[3]	insectary
299	*Phyllophaga obsoleta*	Costa Rica[3]	unknown
381	*Phyllophaga menetriesi*	Costa Rica[3]	insectary
382	*Phyllophaga menetriesi*	Costa Rica[3]	insectary
393	*Phyllophaga elenans*	Costa Rica[3]	soursop
394	*Phyllophaga menetriesi*	Costa Rica[3]	insectary
395	*Phyllophaga vicina*	Argentina[3]	maize
470	*Anomala sp*	Colombia[3]	coffee
479	*Phyllophaga obsoleta*	Colombia[3]	arracacha
491	*Phyllophaga valeriana*	Honduras[3]	unknown
492	*Anomala sp*	Honduras[3]	unknown
493	*Phyllophaga obsoleta*	Honduras[3]	maize
494	*Phyllophaga obsoleta*	Honduras[3]	maize
495	*Phyllophaga obsoleta*	Honduras[3]	maize
497	*Phyllophaga hondura*	Honduras[3]	unknown

499	*Phyllophaga obsoleta*	Honduras[3]	maize
502	*Phyllophaga valeriana*	Honduras[3]	unknown
503	*Phyllophaga obsoleta*	Nicaragua[3]	weeds
504	*Phyllophaga menetriesi*	Nicaragua[3]	weeds
508	*Phyllophaga crinita*	Mexico[3]	sorghum
510	*Anomala flavipennis*	Mexico[3]	sorghum
518	*Phyllophaga menetriesi*	Costa Rica[3]	pasture
522	*Phyllophaga menetriesi*	Costa Rica[3]	insectary
524	*Phyllophaga menetriesi*	Costa Rica[3]	insectary
525	*Phyllophaga menetriesi*	Costa Rica[3]	insectary
527	*Phyllophaga menetriesi*	Costa Rica[3]	insectary

[1]ATCC; [2] L. Nakamura; [3] P. Shannon; [4] J. Zhang

DNA isolation

DNA was isolated by a modified Marmur procedure as follows (2). The cell pellet was thawed and resuspended in 25 ml of cell suspension buffer. One mg/ml of lysozyme was added and the suspension was incubated at 37°C for 2 h. The suspension was transferred to a 125 ml glass stoppered erlenmeyer flask and 20 ml of lysing solution and 10 ml of 5 M $NaClO_4$ were added to the suspension. After incubating at 55°C for 2 h, 15 ml of phenol-chloroform-isoamyl alcohol (25:24:1) was added to the suspension. The suspension was mixed vigorously by hand to homogenize and then shaken for 20 min. The mixture was centrifuged at 17,000 x g for 10 min to separate the phenol and aqueous layers. The phenol-chloroform-isoamyl alcohol extractions were repeated until the aqueous layer was clear. After the final extraction, the aqueous layer was transferred to a clean erlenmeyer flask and the DNA was precipitated with 2 ml of $NaClO_4$ and 0.6 volume of isopropanol. The isopropanol was poured off and the DNA was washed with 80% cold ethanol for 15 min. The

ethanol was poured off and the DNA was allowed to air dry before resuspending in 20 ml 1X TE buffer. Two hundred fifty µl of RNase was added to the resuspended DNA and then incubated at 37°C for 1 h to degrade any RNA present. Five ml of chloroform:isoamyl alcohol (24:1) were added and the mixture was shaken vigorously by hand to homogenize followed by shaking for 20 min. The DNA was centrifuged at 17,000 x g for 10 min, the aqueous layer transferred to a sterile 100 ml beaker, and 0.1 volume of 3 M sodium acetate and 2 volumes of 95% ethanol was added to precipitate the DNA. The DNA was spooled on a glass rod, washed with 80% cold ethanol, and allowed to air dry before resuspending in 3 ml 1X TE buffer. The DNA was quantified by the absorbance at 260nm and stored at −20°C.

Preparation of DNA samples for DNA similarity study

DNA samples were diluted in 1X TE buffer to a concentration of 0.4 mg/ml and a final volume of 4 ml. The samples were fragmented by three passages though a French pressure cell (American Instrument Co.) at 16,000 lb/in^2 which gave fragment sizes between 400-600 bp. Fragment size uniformity was verified by electrophoresis on a 1.0% agarose gel. DNA samples were denatured by boiling for five min and cooling in an ice-water slurry (0°C) for five min. After cooling, the samples were centrifuged at 17,000 x g and 4°C for 10 min and the supernatant was transferred to a new screw-capped tube. Samples were stored at −20°C.

DNA labeling

The DNA samples were labeled using the RadPrime DNA Labeling System (Life Technologies, Gibco BRL). Hydroxyapatite (HA) columns were set up the night before the labeling

reaction. HA was added to Pasteur pipettes plugged with glass wool until a 2-2.5 cm high column formed after settling. The layer of HA buffer in the columns was adjusted to 1-2 cm and the columns were placed in screw-capped tubes filled with enough HA buffer to maintain the buffer layer inside the columns. The columns were placed in a 70°C waterbath until the labeling reaction was performed. Five micrograms (12.5 µl) of the DNA to be labeled was pipetted into a 1.5 ml microcentrifuge tube. The tube was placed in a boiling water bath for 5 min and then placed in an ice-water slurry (0°C) for five min to denature the DNA. Each labeling reaction mixture consisted of: 1.0 µl each dATP, dGTP, and dTTP, 20 µl 2.5X reaction buffer, 8.5 µl water, 1.0 µl Klenow fragment, and 5.0 µl [α-^{33}P]dCTP (Dupont) for a final volume of 37.5 µl. The mixture was centrifuged for 15-30 sec and transferred to the microcentrifuge tube containing the DNA to be labeled. The tube was centrifuged for 15-30 sec and incubated at 37°C for 15 min in water-filled wells of a heat block. Five µl of 0.2 mM EDTA was added to the tube to stop the labeling reaction.

While the mixture was incubating, a NAP-25 column (Pharmacia Biotech) was equilibrated by washing three times with HA buffer. After stopping the labeling reaction, the mixture was pipetted onto the surface of the HA column. HA buffer (2.2 ml) was added to the column and the eluent was allowed to drain into a beaker. Additional HA buffer (1.8 ml) was added to the column and the α-^{33}P-labeled DNA was collected in a screw-capped tube containing 50 µl of 0.4 mg/ml denatured salmon sperm. The α-^{33}P-labeled DNA was denatured again by boiling for 5 min and cooling in an ice-water slurry. While the DNA was denaturing and cooling, the warm HA columns were transferred from the tubes filled with HA buffer to empty tubes. The columns were drained until the level of the HA buffer in the columns was 2-5 mm above the surface of the HA. The buffer level was maintained by adding warm (70°C) HA buffer. Once the labeling reaction cooled, the HA

columns were dried, transferred to a dry collection tube, and placed back in the 70°C waterbath.

Any remaining double stranded DNA was removed by loading the α-^{33}P-labeled DNA onto a dried HA column. The movement of the labeled DNA through the column was monitored with a survey meter. Once the labeled DNA started to elute from the column, the column was transferred to a new collection tube to collect the labeled DNA. The column was washed with 0.5 ml HA buffer and the wash eluent was collected in the same tube as the labeled DNA.

A NAP-25 column was equilibrated by washing three times with TE + 0.1% SDS and drained until the surface was dry. A disposable serological pipette was used to draw up the labeled DNA and the volume was recorded. The labeled DNA was loaded onto the NAP-25 column and the eluent was allowed to drain. An additional volume of TE + 0.1% SDS was added to the column to make a 2.5 ml total volume of labeled DNA. The column was allowed to drain and 3.5 ml of TE + 0.1% SDS was added to the column. The eluent was collected in a screw-capped tube. Ten microliters of the labeled DNA was transferred to a scintillation vial containing scintillation fluid and label intensity was determined on a Beckman gamma counter. Labeled DNA was stored at –20°C.

S_1 Nuclease assay

The labeled and unlabeled DNA samples were thawed. Ten µl of labeled DNA was added to the bottom of each reaction tube. Fifty µl of unlabeled DNA was added to the tubes containing the labeled DNA as follows: four tubes contained DNA homologous to the labeled DNA, four tubes contained native salmon sperm DNA (0.4 mg/ml), and each heterologous tube was done in duplicate. Fifty µl of high salt buffer was added to each tube. The tubes were closed, vortexed 10 times, and

transferred to Nalgene reaction racks. Covers were placed over the racks and secured with rubber bands. The racks were completely submerged in a 65°C waterbath and incubated for 24 h. The reaction tubes were stored at −20°C until the next step.

After thawing the reaction tubes, 50 µl of denatured salmon sperm (0.4 mg/ml), 1.0 ml of S_1 nuclease reaction buffer, and 10 µl of S_1 nuclease (Pharmacia Biotech) were added to each reaction tube. The salmon sperm served as a substrate for the S_1 nuclease. The tubes were closed and the covers secured on the racks. The tubes were mixed by inverting the racks and incubated by completely submerging the racks in a 50°C waterbath for 60 min. After incubation, 50 µl of native salmon sperm (1.2 mg/ml) and 500 µl of cold HCl buffer were added to each tube. The salmon sperm served as a precipitation matrix for the hybridized DNA. The tubes were incubated at 4°C for 1 h.

After incubation, the reactions were filtered through Whatman glass filter strips (no. 1825 915 GF/F) and the tubes rinsed twice with acid wash buffer. The filter strips were dried in a convection oven set at 60°C. The circles where the DNA collected were removed with forceps and placed in the bottom of scintillation vials containing scintillation fluid. Each vial was counted for 1 min and 5 min with a Beckman gamma counter.

Data analysis

DNA was compared to labeled reference DNA from the *P. popilliae* type strain, ATCC 14706, the *P. lentimorbus* type strain, ATCC 14707, and initially to South American strain 522. DNA was subsequently compared to labeled DNA from South American strain 289 when data revealed a unique group of strains. DNA percent similarity data were analyzed phenetically using

the NTSYS-pc package of computer programs (6). DNA similarity matrices were obtained using the distance and Q-correlation coefficient algorithms and 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 the DNA similarity matrices and the cophenetic correlations was computed using normalized Mantel statistics z.

Detection of vancomycin ligase gene using PCR

Strains that were resistant to vancomycin were screened for the putative ligase gene (*vanE*) via the polymerase chain reaction (PCR). PCR reactions were set up in a laminar flow hood and the pipettors, tips, gloves, and racks were subjected to 30 min UV exposure prior to use. Each PCR reaction contained 25 ng template DNA, 5% glycerol, 1X PCR buffer, 200 μM each dNTP, 1.5 mM $MgCl_2$, 5 pmol each primer, and 0.75 U Taq polymerase (Promega). Amplification reactions were performed in a PTC-100 thermocycler (MJ Research). The temperature profile was: 94°C for 2 min, followed by 60 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 2 min and a final temperature of 72°C for 10 min. The reactions were held at 4°C until electrophoresed in a 1% (w/v) agarose gel. The location and sequences of the primers were described by Rippere *et al*. (5).

Detection of paraspore gene using PCR

All strains were screened for *cry* paraspore genes via PCR. CryBP2 primers were designed by Rippere-Lampe (4) and cover the open reading frame upstream of the paraspore gene and the gene itself. I designed the location and sequences of the CryBP4 primers based upon the published nucleotide sequence; the primers are located at the 3' end of the paraspore gene. Both sets of primers

are listed in Table 2.

Table 2. Location and sequences of primers used to detect *cry* genes in *Paenibacillus popilliae* and *P. lentimorbus* and the expected sizes of the fragments to be amplified by the primers

Primers	Sequence	Location	Expected Size
CryBP2-F	5'-AGGATGTTCCTCCGATCCCCATCAC-3'	441 - 465	806 bp
CryBP2-R	5'-GTTCCGTGGCTCGTAAAATCTCTTC-3'	1223-1247	
CryBP4-F	5'-CAACCGAACTCGATGAACTAAAAA-3'	2022-2045	616 bp
CryBP4-R	5'-GCAGCTCCAGATAACCTCAAAACT-3'	2614-2637	

PCR reactions were set up in a laminar flow hood and the pipettors, tips, gloves, and racks were subjected to 30 min UV exposure prior to use. Each reaction contained 25 ng template DNA, 5% glycerol, 1X buffer, 200 mM each dNTP, 3 mM $MgCl_2$, 25 pmol each primer (Life Technologies, Gibco BRL), and 2.5 U Taq polymerase (Promega). Amplification reactions were performed in a PTC-100 thermocycler (MJ Research). The temperature profile for the thermalcycler was: 94°C for 10 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min and a final temperature of 72°C for 7 min. The reactions were held at 4°C until electrophoresis on a 1% agarose gel.

Restriction digestion of CryBP2 PCR products

CryBP2 PCR products for *P. popilliae* strains ATCC 14706, H1, 381, and 492, and *P. lentimorbus* strains ATCC 14707 and 266 were digested with MspI in 20 µl reactions. *MspI* was

chosen based on the restriction map of *cry18Aa1* gene and location of the CryBP2 primers. Each reaction contained 10 µl of CryBP2 PCR reaction, 2 µl of 10X buffer B, 0.4 µl of bovine serum albumin (10 mg/ml), 6.6 µl sterile water, and 1 µl of MspI enzyme (10 U/µl, Promega). Reactions were incubated overnight at 37°C. Digests were electrophoresed on a 2% agarose gel.

PCR product sequencing

DNA sequencing was done at the Mayo Clinic (Rochester, MN). Six microliters of PCR reaction, 1 µl of 1 U/µl shrimp alkaline phosphatase, and 1 µl of 10 U/µl exonuclease I were incubated at 37°C for 30 min followed by incubation at 80°C for 15 min. After incubations, one microliter of sequencing primer (3.2 µM) and 1 µl of dimethyl sulfoxide were added and the DNA sequence was determined in both directions using a Taq dideoxy terminator cycle sequencing kit and a 373A DNA Sequencer (Applied Biosystems, CA). Sequence data were analyzed using version 8 of the Genetics Computer Group Sequence Analysis software (3).

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