Evaluation of Antibiotic Production and its Regulation in *Pantoea agglomerans* Tx10

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SUPERVISORY AND EXAMINING COMMITTEE

Lucas Robinson, candidate for the degree of Master of Science in Biology**,** has presented a thesis titled, ***Evaluation of Antibiotic Production and its Regulation in Pantoea agglomerans T x 10,*** in an oral examination held on August 17, 2015. The following committee members have found the thesis acceptable in form and content, and that the candidate demonstrated satisfactory knowledge of the subject material.

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Abstract

The prevalence of antimicrobial-resistant pathogens, such as methicillin-resistant *Staphylococcus aureus* (MRSA), has prompted numerous efforts to identify new antimicrobials. One untapped source of antimicrobial products is the enterobacterial genus *Pantoea.* Although antibiotic discovery efforts in *Pantoea* have focused largely on agricultural applications, *Pantoea* remains an unexplored reservoir of antibiotics that may have utilization in a clinical setting. Using an agar overlay assay, *Pantoea agglomerans* Tx10 (Tx10)—an isolate from the sputum of a cystic fibrosis patient—was shown to inhibit the growth of several pathogens, including Gram-positive *Staphylococcus* and *Streptococcus,* and the Gram-negative *Citrobacter, E. coli, Erwinia,* and *Salmonella.* A genetic screen to identify the genes involved in the production of this antimicrobial natural product identified a predicted 6-gene biosynthetic cluster which, when disrupted, abolished antibiotic production. I called this compound, *Pantoea* Natural Product 2 (PNP-2). Subsequent assays with mutants deficient in PNP-2 production revealed that they were still able to inhibit *Erwinia amylovora*, suggesting the production of a second antibiotic, which I identified as the well-known pantocin A. A survey of resistance among the Stavrinides *Pantoea* collection revealed that the majority of isolates are susceptible to either pantocin A, or PNP-2, and only a minority were resistant to both. Analysis of the expression of PNP-2 revealed it was produced only on minimal media. Using the luciferase reporter gene, PNP-2 expression was shown to be linked to amino acid starvation, with the absence of certain amino acids enabling antibiotic production. Overall, the antibiotic production from this cystic fibrosis isolate, Tx10, shows the

potential reservoir of antibiotics from the genus of *Pantoea*, as well as insight into the production and expression of the antibiotic in the environment.

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Introduction

In 2013, there were over two million illnesses caused by antimicrobial resistant (AMR) pathogens (1). Increased antibiotic resistance is a consequence of the overuse in clinical and agricultural settings, which has led to more exposure of antibiotics to microbes over the past 50 years (2). Unfortunately, the rise of AMR is not accompanied by an increase in antibiotic development, as such, the rate at which current antibiotics are becoming obsolete is limiting effective treatment options (3). Since the year 2000 there have been only 20 new antibiotics approved, approximately half of which are synthetically derived products and the other half, natural products. A potential concern with these synthetic antibiotics is that the majority have been derived from the quinolone and oxasolidanones classes, resulting in compounds that are not very diverse and are therefore more susceptible to resistance mechanisms that target conserved chemical groups (4). There have been five new antibiotic classes (based on their mode of action or structure) discovered in this time frame: linezolid (synthetic), daptomycin (naturally derived), retapamulin (naturally derived), fidaxomicin (naturally derived) and bedaquiline (synthetic) (5). The cytoplasmic membrane depolarizing agent, daptomycin, which has activity against Gram-positive pathogens, was identified from *Streptomyces roseosporus*, and developed into an effective therapeutic (5). Similarly, retapamulin, a semi-synthetic antimicrobial whose precursor was derived from the fungus *Pleurotus mutilus,* acts to disrupt protein synthesis through binding the prokaryotic ribosome (6-8). The third class of antibiotic, fidaxomicin, a macrolytic antibiotic with activity against Gram-positive bacteria via inhibition of protein synthesis, was derived from a soil isolate of the

actinobacterium, *Dactylosporangium aurantiacum* (9-14). Lastly, a new antibiotic that also targets Gram-positive bacteria, teixobactin, was discovered from a large screen of soil microorganisms, and shows promise as a new antimicrobial agent (15). The development of new drugs is essential for continuing to treat infections caused by multi-drug resistant pathogens in the near future.

The problem of AMR is especially an issue in the nosocomial environment where many infections are transmitted between patients. The ESKAPE pathogens – *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp. – are the cause of many of these difficult-to-treat infections (16). Although the hospital setting is host to many multi-drug resistant pathogens, these same resistant bacteria are also becoming a problem for primary care physicians who treat infections at local clinics (17). One of the most problematic members of this group is Methicillin-Resistant *Staphylococcus aureus* (MRSA), which is responsible for deaths exceeding those caused by AIDS in the United States (18). MRSA has become one of the most problematic nosocomial pathogens for both healthy and immunocompromised individuals globally (19-21). It is estimated that 20% of the population carries the bacteria commensally in the nasal cavity, with up to 30% intermittently (22). There is an increase in the prevalence of vancomycin resistance—the standard treatment option for MRSA—along with emerging resistance to the alternative drugs, linezolid, tigecycline, and quinupristin/dalfopristin which now has caused substantial concern (23, 24), necessitating the continued exploration of new antimicrobials from natural sources.

Natural Products as Antibiotics

Since the discovery of penicillin, the majority of successful antibiotics have been derived from natural sources, including bacteria and fungi. Even with the shift towards synthetic techniques for antimicrobial development in the 1980's and 1990's, natural product discovery still dominated the field in terms of drug approval (3, 4, 25-29). Of the approved antibiotics between 1981 and 2006, 75% have been either natural products or a synthetic antibiotic structure based on a scaffold that had been derived from a natural compound (25). The changes following the golden age of discovery resulted in many analogues of previous scaffolds being released, creating a deficit of newly discovered antibiotic architectures. The strategy of altering known antibiotic scaffolds was initially effective, but resistance determinants that targeted the antibiotic backbone have since increased in frequency. For example, there are over 75 known beta-lactamase genes conferring bacterial resistance, rendering the usage of the beta-lactam backbone nearly obsolete as an antimicrobial agent (30). New classes and new scaffolds are therefore needed to treat infections caused by resistant pathogens, and naturally derived compounds may be the most promising source.

The natural environment hosts many bacteria and fungi in its numerous ecological niches, such that the number of bioactive secondary metabolites in the natural environment is quite high and largely underexplored (31-35). For example, within nutrient-rich mammalian dung, there is ongoing competition between *Streptomyces, Myxobacteria,* various fungi and other microbes, which yields many interesting secondary metabolites that can have potential therapeutic usage (31). Likewise, the oceans are also a large reservoir of microbes that have evolved a

metabolome with complex and unique biosynthesis pathways (35). For example, there has been a growing interest in the cyanobacteria as a new source of bioactive secondary metabolites (35, 36). In cyanobacteria, many secondary metabolites are biosynthesized in a variety of routes, which include non-ribosomal peptide synthetase and polyketide synthase, which are able to create a variety of unique molecules that have activity against many targets (35). In one study of the secondary metabolites produced by cyanobacteria where 38 compounds were tested, the majority were bioactive (only 7.18% were inactive), and these compounds had antimicrobial, antiviral, and anti-cancer properties (37). Many microbes, however, are not able to be cultured on general purpose media and, in fact, most microbes have not been cultured by any known method (38). New technologies in biology have enabled researchers to gather the genetic material from an environmental niche and pair with advances in next generation genome sequencing in order to gather new information regarding the metabolome of these unculturable microbes. Metagenomics is one strategy for antibiotic discovery that involves the study of DNA from the environment without culturing the microbe (27). These metagenomes can then be analyzed for their potential metabolome *in silico* (39), or heterologous expression of these putative antimicrobial producing genes to generate a large quantity of a potentially viable compound for further analysis (40). This technique has been used in association with antibiotic development in the past where a DNA isolated from a soil strain of *Streptomyces lividans* was cloned into *E. coli* and an antimicrobial with *Mycobacterium*-inhibiting activity, terragine, was identified (39). The use of these new techniques has opened additional avenues for antimicrobial development;

however, there are still large reservoirs of available culturable bacterial groups that are known to produce potent antimicrobials, which have not been fully explored.

The genus *Pantoea* as a reservoir of novel antibiotics

One bacterial group that has gained attention as a source for novel antimicrobials is the enterobacterial genus, *Pantoea*. Members of *Pantoea* are ubiquitous in the general environment and many of their members are epiphytes, as well as pathogens of plants and humans (41). Epiphytic isolates were identified as a source of several antimicrobials and are used as biocontrol agents in the agricultural industry to inhibit *Erwinia amylovora*, the causative agent of Fire Blight of apple and pear (42-48). Two of the most well established antibiotics derived from strains of *Pantoea* are pantocin A (previously called herbicolin O), produced by *P. agglomerans* EH318, *Pantoea vagans* C9-1, and *P. agglomerans* EH252 as well as pantocin B, which is also produced by EH318—both of which target amino acid biosynthesis in *E. amylovora* (42, 44-46, 49). Pantocin A interferes with the synthesis of L-histidine, and is therefore ineffective when this amino acid is available to the bacteria exogenously (44). Likewise, the activity of pantocin B is neutralized in the presence of L-arginine, L-lysine, and L-asparagine (45). In addition to the pantocin antibiotics, some strains of *Pantoea* have been noted to produce other antibiotics: andrimids, dapdiamides, D-alanylgriseoluteic acid (AGA), APV, herbicolins, MccEh252, and PNP-1 (42, 50-56). Andrimid and its derivatives present an interesting new antibiotic scaffold (52). Andrimid is formed step-wise from the products of a 21-gene cluster found in *P. agglomerans* Eh335, starting with a poly un-saturated fatty acid chain to which a phenylalanyl moiety, succinamide precursor,

carbonyl, and amine groups are added (52). The antibiotic targets the beta subunit of acetyl-CoA cayboxylase, disrupting fatty acid synthesis (52, 57). Likewise, *P. agglomerans* CU0119 produces dapdiamides A-E (50), in which L-2,3-diaminopropionic acid (DAP) is linked to variable units through two amide bonds, producing dapdiamides A through E (50). These resulting products have a mode of action that targets glucosamine-6-phosphate synthase, an enzyme essential in the cell wall biosynthetic pathway (50). Newly identified D- alanylgriseoluteic acid (AGA), is a phenazine antibiotic produced by *P. agglomerans* Eh1087 (formerly *P. herbicola*) (54). This antibiotic is formed from a plasmid encoded gene cluster having phenazine-forming, and phenazine-modifying genes, which likely originated from a Pseudomonad (54). Furthermore, AGA is active against a variety of Gram-positive and Gram-negative bacteria, which includes *E. amylovora.* Further evaluation of the reservoir of *Pantoea* derived antimicrobials revealed the antibiotic 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine (APV), found in *P. agglomerans* Pa48b, which is similar to the dapdiamide acting through direct colonization of the plant itself for biocontrol of *E. amylovora* (51). The herbicolin A and B antibiotics are lipopeptide antibiotics with activity against sterol-containing fungi (56). Lastly, the MccEh252 microcin, from *P. agglomerans* Eh252, has shown to be involved in the biocontrol of *E. amylovora* as well (55). Most recently, a new gene cluster found in *P. ananatis* BRT175 was shown to be responsible for the production of *Pantoea* Natural Product 1 (PNP-1), an antimicrobial with activity against *E. amylovora,* which is similar in activity and structure to 4-formylaminooxyvinylglycine (FVG) identified in the Pseudomonads (58-60).

Although many of the natural products of *Pantoea* have focused on those that target *E. amylovora*, the natural products of *Pantoea* are likely more diverse, having the capacity to inhibit a broader range of bacteria, including clinical pathogens such as *S. aureus*.

Thesis Objective

The objective of this thesis is to identify the antibiotics produced by the clinical isolate *P. agglomerans* Tx10 (Tx10), identify the genes responsible for antibiotic biosynthesis, and evaluate how antibiotic production is regulated. The first aim is to examine the spectrum of activity of antibiotics produced by Tx10, and use mutagenesis and genomics to identify genes involved in biosynthesis. The second aim is to explore the regulation of antimicrobial production, and evaluate the effects of nutrient availability and conditions on antibiotic gene expression.

Materials and Methods

Bacterial Strains, Media and Growth Conditions

All bacterial strains were cultured on lysogeny broth (LB) medium (Appendix 3), with *Staphylococcus, E. coli, Salmonella, Aeromonas, Klebsiella,* and *Streptococcus* strains being incubated at 37ºC, and all others at 30 ºC. *E. coli* HB101 (RK600) and *E. coli* VPE42 (pBSL118) (61) were supplemented with 50 µg/mL of chloramphenicol, 50 µg/mL of kanamycin respectively, and the transposon mutants were supplemented with both 50 µg/mL of kanamycin and 100 µg/mL of ampicillin. For the isolation of spontaneous antibiotic-resistant mutants, *P. agglomerans* Tx10 was grown overnight in LB, re-suspended in $MgSO_4$ and 100 µL was plated on an LB plate supplemented with the appropriate antibiotic – rifampicin (100 µg/mL) and streptomycin (300 µg/mL).

The antibiotic production assay was conducted using *E. coli* minimal medium (per liter: 0.25 g yeast extract, 1.72 g KH_2PO_4, 4.0 g K_2HPO_4, 0.5 g NaCl, 0.2 g sodium citrate, 2 g $(NH_4)_2SO_4$, 0.002 g $MgSO_4 \bullet 7H_2O$, 20 mL glycerol) (62). The 5× GA (glucose-asparagine salt solution) used for media overlays contained (per 100 mL) 1.115 g K_2HPO_4, 0.45 g KH_2PO_4, 0.012 g $MgSO_4$, 2 g glucose, 0.03 g L-asparagine, 0.005 g nicotinic acid (62). The assay was repeated with the 5× GA salt solution supplemented with exogenous amino acid, L-histidine, to a final concentration of 0.3%. For assays evaluating antibiotic production on LB, the 5× GA solution was substituted with 5× LB.

Antibiotic Production and Susceptibility Assays

Antibiotic production was evaluated using a top agar overlay assay. The base layer of the plate contained 20 mL of *E. coli* minimal media that was kept at 37ºC for one hour before the assay began. The molten top layer was prepared with 3.2 mL of molten 0.9% agar, 0.8 mL of 5× GA salt solution and 0.3 mL of the bacterial indicator strain (grown in LB overnight, pelleted and re-suspended in 10 mM $MgSO_4$). The molten solution was poured over the warm *E. coli* minimal media basal layer, and allowed to solidify. Subsequently 3 µL of the test strain (grown in LB overnight, pelleted, and re-suspended in 10 mM $MgSO_4$) was spotted onto the solidified top layer. The assay was also conducted in the presence of exogenous amino acids, added to a final concentration of 0.3% in the overlay layer. The plates were incubated at 30^{o}C for at least 24 hours, and up to 30 hours, before the development of a zone of inhibition was scored. Two rating systems were used to score the plates. The first was a 5 point rating system wherein a rating of 5 represented the zone of inhibition created by *P. agglomerans* Tx10 on *E. amylovora* EA321, and a 0 rating indicated zero inhibition of the growth of the target strain. The second was a binary system, where a target isolate was considered susceptible if a zone of inhibition of any size was observed, and resistant if no defined zone of inhibition formed. A similar approach was used for assessing antibiotic susceptibility of our collection of *Pantoea* isolates against the natural products of Tx10. Tx10 and a transposon mutant (Tx10M1) were tested against our strain collection using the standard overlay medium, with and without L-histidine (final concentration 0.3%).

Mutagenesis and Sequence Analysis

Tn5 transposon mutagenesis was carried out via triparental mating using the recipient *P. agglomerans* Tx10, the helper *E. coli* HB101 (RK600) and the donor VPE42 (pBSL118), the latter having a mini-Tn5 transposon carrying kanamycin resistance. Overnight broth cultures containing appropriate antibiotics were prepared for each of the three isolates, and 1 mL of each broth culture was pelleted and resuspended in 100 µL of 10 mM $MgSO_4$. All three suspensions were then combined in a single tube (300 µL total), and three 100 µL volumes were spotted on plain LB agar, and incubated overnight. A small loopful of the resulting bacterial lawn was then resuspended in 10 mM $MgSO_4$, 10-fold serial dilutions made, and dilutions plated on LB agar plates containing kanamycin and ampicillin. After a two-day incubation, the resulting mutants were tested using the top agar overlay assay for antibiotic production. Mutants that no longer produced a zone of inhibition on the indicator strain, *S. aureus* K5-1, were considered candidates and were retested to confirm the absence of antibiotic production. Over 2000 isolates were screened for antibiotic production in this process.

An inverse PCR approach was used to identify the Tn5-disrupted genes from candidate mutants. Total DNA was extracted from each candidate using the Omega E.Z.N.A.® Bacterial DNA kit, and 1-2 µg was digested using either HincII or HindIII (New England Biolabs) (20 000 units/mL) using the manufacturer's instructions. Enzymatic digestion was conducted for 2 hours at 37ºC, followed by a deactivation at 80ºC for 20 minutes for HindIII and 65ºC for 20 minutes for HincII. Unimolecular ligations were carried out using 15 µL of the product from these restriction reactions in a 200 µL reaction with 3 µL of T4 DNA ligase (New England

Biolabs, 400 000 units/mL), 20 µL of T4 DNA ligase buffer (10× solution), and 162 µL of ddH_2O. The ligation was left to incubate overnight at 16ºC. Following ligation the DNA was purified using Omega E.Z.N.A.® Cycle Pure Kit. The inverse PCR was then conducted using primers specific to the kanamycin resistance gene within the mini Tn-5 transposon (Table 1). Reactions contained 1 µL of the DNA ligation product, 2 µL Taq (New England Biolabs) (500 units/mL) buffer, 2 µL dNTPs (1.5 mM), 0.5 µL npt+772 primer (10 µM), 0.5 µL npt-41 primer (10 µM), 1.2 µL $MgCl_2$, 0.2 µL Taq enzyme (New England Biolabs) (500 units/mL), and 12.6 µL ddH_2O. PCR reaction conditions were 95°C for 2 minutes, followed by 35 cycles of 95°C for 30 s, 57°C for 30 s, and 68°C for 3 minutes. These PCR products were purified using Omega E.Z.N.A ® Cycle Pure Kit, and sequenced by Sanger sequencing. The sequences from the Tn5 mutants were compared to our draft genome sequence to identify the point of insertion. Additional mutants were later characterized using a PCR-based approach wherein primers pointing outward from the kanamycin gene in the mini-Tn5 were paired with primers specific to different loci within the biosynthetic cluster (Table 1). Amplicon size was used to determine the precise insertion point of the transposon.

PCR-based Antibiotic Cluster Survey

Using primers anchored in multiple regions across the cluster, PCR was used to survey 128 strains of *Pantoea* for the presence of several PNP-2 loci. Tx10 served as positive control. For *Pantoea* strains SN01080, DC434, SP05061, SP05120, and 12531, which had similar inhibition profiles to Tx10, three primer pairs were used (213+ and 1499-, 1545+ and M946+, $M_2$140+ and 6060-) to determine whether an

identical PNP-2 cluster was present (Table 1). For all other strains one primer pair was used (1545+ and M946+), which spans three of the genes in the cluster, *pnp*2B-*pnp*2D (Table 1). PCR reactions contained 2 μL 10× Taq buffer, 1.6 μL dNTPs (1.5 mM), 0.4 μL forward primer (10 μM), 0.4 μL of reverse primer (10 μM), 1 μL DMSO, 1 μL (50 ng/μL) genomic DNA, 0.1 μL Taq enzyme (New England Biolabs) (500 units/mL), and 13.5 μL ddH_2O. PCR reaction conditions were 95°C for 2 minutes, followed by 35 cycles of 95°C for 30 s, 57°C for 30 s, 68 °C for 60 s, followed by 2 minutes at 68°C.

Genomic Analysis

To identify homologous PNP-2 gene clusters in other isolates, the complete PNP-2 gene cluster was searched using blastx against the nr database, and using tblastx against a custom database containing 24 draft genomes of *Pantoea* isolates (58, 63, 64). To identify whether the 4.9 kB *P. agglomerans* Tx10 draft genome (63) contained any other known antibiotic biosynthetic clusters, we used both nucleotide (blastn) and translated BLAST (blastx) to search for the presence of the *P. agglomerans* Eh318 pantocin A and B gene clusters (accession number: U81376.2), the *P. agglomerans* Eh335 andrimid gene cluster (accession number: AY192157.1), the *P. agglomerans* 48b/90 APV gene cluster (accession number: JQ901494.1), the *P. agglomerans* Eh1087 AGA antibiotic cluster (accession number: AF451953.1), and the *P. agglomerans* Cu0119 dapdiamide gene cluster (accession number: HQ130277.1).

PNP-2 Antibiotic Regulation

To evaluate the expression of the PNP-2 antibiotic under different media conditions, the PNP-2 promoter was cloned upstream of the luciferase reporter in pCS26. The plasmid was isolated from *E. coli* K-12 W3110 using the Mobio UltraClean® Midi Plasmid Prep Kit digested using XhoI and BamHI restriction enzymes. The recognition sites for XhoI and BamHI are only separated by one base. This created difficulties in a double digestion, therefore, a sequential digestion was performed in which pCS26 was digested with BamHI first, followed by XhoI. Unlike BamHI, the XhoI enzyme has a higher capacity to digest the DNA with only one base pair surrounding the restriction site (as per manufacturer recommendations). Therefore, 1 µg of pCS26 plasmid DNA was digested in a reaction containing 1.5 µL BamHI (New England Biolabs, 20 000 units/mL), 5 µL 10x NEB restriction endonuclease buffer #3.1, and 39 µL ddH_2O for 1 hour at 37ºC, followed by the addition of 1.5 µL of XhoI (New England Biolabs, 20 000 units/mL) for an additional 1 hour at 37 ºC. This sequential digestion was followed by an additional reaction with 0.2 µL of Calf Intestinal Phosphatase (New England Biolabs, 10 000 units/mL) for 30 minutes at 37ºC. The digested pCS26 product was then immediately purified using an Omega E.Z.N.A ® Cycle Pure Kit.

The promoter for the PNP-2 gene cluster was predicted to lie within the 880 bp of DNA upstream (promoter region is named PNP-2P). The primers used in the PCR of the promoter region was designed to include the XhoI restriction site (CTCGAG) on the forward primer (x10XhoI+) (Table 1) and the BamHI restriction site (GGATCC) on the reverse primer (x10Bam -) (Table 1). PCR reactions contained 0.5 µL of Tx10 gDNA (160 ng/µL), 5 µL Taq buffer (10x), 5 µL dNTPs

(1.5 mM), 0.5 µL x10XhoI+ primer (10 µM), 0.5 µL x10Bam – primer (10 µM) (Table 1), 2.0 µL DMSO, 0.5 µL Taq enzyme (New England Biolabs, 500 units/mL), and 36 µL ddH_2O. PCR reaction conditions were 95 °C for 2 minutes, followed by 35 cycles of 95°C for 30 s, 61°C for 30 s, 68°C for 1 minute 5 seconds (Table 1). These PCR products were then purified using Omega E.Z.N.A ® Cycle Pure Kit. The constitutive promoter from the kanamycin resistance gene, *nptII* was used as a control for the expression studies. To amplify the *nptII* promoter identical PCR reaction conditions were used as those for the amplification of the PNP-2 promoter, except for a shortened annealing time to 45 seconds, and the puc18tk plasmid was used as template (32.6 ng/µL). The primers for the *nptII* promoter were also designed with the XhoI restriction site (CTCGAG) on the forward primer (Npt+_XhoI+) (Table 1) and the BamHI restriction site (GGATCC) on the reverse primer (Npt5′+41Bam-) (Table 1), in order to facilitate directional cloning into the pCS26 reporter plasmid.

The purified PNP-2 promoter and the *nptII* promoter PCR products were ligated to the pCS26 plasmid that had been digested with the restriction endonucleases BamHI and XhoI. The ligation reaction was designed based on a 3:1 molar ratio of insert to vector. The reaction contained 8 µL (80 ng) of the digested pCS26, 3 µL (35 ng/µL) of the promoter region (PNP-2P) or the *nptII* promoter (24.1 ng/µL), 2.5 µL of 10x NEB ligase buffer, 1 µL NEB T4 DNA ligase (New England Biolabs, 400 000 units/mL), and 10.5 µL ddH_2O. The ligation was incubated for 18 hours at 16 ºC. The ligation reaction was then transformed into electrocompetent *E. coli* CC118, which were made by growing a fresh culture of in a 250 mL Erlenmyer

flask to a density of 0.4 OD600. The culture (70 mL) was cooled in an ice water bath for 15 minutes before these cells were spun down at 4500 rpm for 10 minutes and resuspended in an equal volume ice cold 10 % w/v glycerol. This process was repeated for three total glycerol washes and finally resuspended in a final volume of 3.5 mL and 100 µL was aliquoted into 1.5 mL epitubes. The ligations of the pCS26 plasmid (2.5 µL) as well as an empty pCS26 plasmid (40 ng/µL) were transformed into the CC118 electro-competent cells. Electrocompetent cells (100 µL) were put into an ice-cold 1 mm cuvette with the 2.5 µL of the ligation or plasmid. The cells were electroporated with the Biorad Micro Pulser ™ using the Ec1 protocol: (1 mm cuvette, 1.8 kV, 1 pulse). This was followed by immediate resuspension of the cells in 900 µL of SOC media (per 500 mL: 10 g tryptone, 2.5 g yeast extract, 5 mL 1 M NaCl, 1.25 mL 1 M KCl, 1.8 g glucose, 2.4 g $MgSO_4$) and letting shake for 1 hour at 37ºC. The transformation (100 µL) was then plated on an LB agar plate supplemented with kanamycin (50 ng/µL) and grown overnight. Colonies on the plate were confirmed for successful *nptII* and PNP-2P ligation using a colony PCR method. The PCR reactions contained: a very small amount of bacterial colony using a pipetted tip touched against the bacteria, 2 µL Taq buffer (10x), 2 µL dNTPs (1.5 mM), 0.5 µL x10XhoI880+ or Npt+XhoI+ primer (10 µM), 0.5 µL x10PBam- or Npt5′41BamHI- primer (10 µM)(Table 1), 1.0 µL DMSO, 0.2 µL Taq enzyme (New England Biolabs, 500 units/mL), and 13.4 µL ddH_2O. PCR cycling conditions were 95 °C for 2 minutes, followed by 35 cycles of 95°C for 30 s, 61°C for 30 s, 68°C for 1 minute 5 seconds.

Plasmid isolations were performed using the Mobio UltraClean® Mini Plasmid Prep Kit. These plasmids with the *nptII* or PNP-2P inserts were then transformed into wild type Tx10, along with empty pCS26 plasmid using the transformation protocol as above. This yielded a positive control (Tx10 pCS26-*nptII*), a negative control (Tx10pCS26) and the test isolate with the promoter region (Tx10pCS-PNP-2P). These three isolates were tested each time before each assay by growing an overnight culture of each in *E. coli* minimal media (EMM) and evaluating luminescence in the standalone luminometer, the Promega Glomax®, using an extended protocol to measure luminescence for 6 seconds in a 1.5 mL epitube. 96-well plates were used to test a number of media conditions for their effects on the expression of the PNP-2 promoter in Tx10. Cultures of Tx10pCS26-*nptII*, Tx10pCS26 and Tx10pCS26-PNP-2P were grown to log phase (OD_{600} 0.4) and resuspended in 10 mM $MgSO_4$ at a 1:4 dilution. The 96 well plates were organized to test 8 media types simultaneously including the control media LB and EMM. The test media conditions involved supplementing EMM with amino acids (Table 2). Each well contained 250 µL of media and 5 µL of cell culture, which was then overlaid with 50 µL of silicone oil (ibidi Anti-Evaporation Oil) to prevent evaporation over the time course, but to also allow for some gas exchange. These plates were then incubated overnight in the BioTek Synergy HT® incubating plate reader at 30ºC with shaking at medium strength in 10 minute intervals. At each interval, the OD_{600} and the endpoint luminescence (Emmisson: Hole, gain: 130) was taken. The test strain, Tx10pCS26-PNP-2P was replicated five times in each medium.

Table 1: List of primers used for PCR

Primer Name	Locus	*Priming Site	Primer Sequence (5′ to 3′)
X10 213+	*pnp2A*	1-24 (F)	ATGGACATGCTGGCCTACCTTAAG
X10 1499-	*pnp2B*	237-259 (F)	CCTCAGGCCATTCACCATATCC
X10 1545+	*pnp2B*	379-357 (R)	CTGCTCATCCACAGCGATAATG
X10 M946+	*pnp2D*	591-562 (R)	ATCTTCAGCCATCACTTTGAGATAGTCTG
X10 M2140-	*pnp2F*	800-775 (R)	GACGTACCATTACGAATGCCGAGTG
X10 6060-	*pnp2F*	**64-40 (R)	TTGCATAAAGATAATCGCCATTGG
NPT+772	*nptII*	(F)	TTCGCAGCGCATCGCCTTCTATC
NPT-41	*nptII*	(R)	AGCCGAATAGCCTCTCCACCCAAG
X10XhoIPNP-2P+	Upstream *880bp*	(F)	GTACCTCGAGCGCTACCTATAAGCCACTAT
X10Bam-	*pnp-2*	(R)	ATCGGATCCAATCTTCTCCTTAGATATTGATT
Npt+XhoI+	*nptII*	(F)	TATCTCGAGGGTCTGACGCTCAGTGGAACGAAAAC
Npt5′41BamHI-	*nptII*	(R)	TATGGATCCAGCCGAATAGCCTCTCCACCCAAG

*Indicates the nucleotide position of the priming site within each locus. F=forward. R=Reverse. **Primer X10 6060- anneals 64 bases downstream of the predicted stop codon of *pnp2F*.

Table 2. Nitrogen and/or carbon source supplemented into EMM

Nitrogen or Carbon Source	Concentration (% w/v)
Glucose	1%
Sucrose	1%
L-alanine	1%
L-arginine	1%
L-asparagine	0.5%
L-aspartic acid	0.46%
L-cysteine	0.04%
L-glutamine	1%
L-glycine	0.86%
L-histidine	1%
L-isoleucine	0.5%
L-leucine	0.5%
L-lysine	1%
L-methionine	1.5%
L-phenylalanine	0.72%
L-proline	1.1%
L-serine	1%
L-threonine	1%
L-tyrosine	0.13%
L-tryptophan	0.46% (DMSO used for solubility)
L-valine	1%

Results

Antibiotic Activity

Screening of multiple isolates for the production of antibiotics effective against *S. aureus* using a soft agar overlay identified *P. agglomerans* Tx10 as an antibiotic producer (Figure 1). Subsequent testing of Tx10 against other pathogens showed inhibition of Gram-positive clinical pathogens *Staphylococcus hemolyticus* and *Streptococcus mutans,* Gram-negative clinical pathogens *Escherichia coli, Aeromonas sp., Citrobacter sp., Enterobacter sp.* and plant-associated isolates of *E. amylovora*, and *Erwinia billingiae* (Table 3). Tx10 was not able to inhibit *P. aeruginosa* or *Klebsiella pneumoniae*. The size of the zone of inhibition for *Staphylococcus sp., P. vulgaris, Citrobacter sp., Enterobacter sp.* and *E. coli* ranged from 1.5 mm to 3.5 mm, with those for *Erwinia* isolates reaching up to 5 mm (Table 3). Antibiotic production was observed on *E. coli* minimal media, but not on LB media. I called this antibiotic, *Pantoea* Natural Product 2 (PNP-2). Furthermore, the spontaneously generated mutants with resistance to rifampicin as well as streptomycin resulted in the loss of antibiotic production in Tx10 (Table 4).

Identification of the antibiotic biosynthetic cluster and distribution across *Pantoea*

A library of 2000 transposon mutants were screened for loss of PNP-2 production using the PNP-2 susceptible strain, *S. haemolyticus* K5-1, which resulted in the identification of 27 candidate mutants. Of these mutants, 12 were

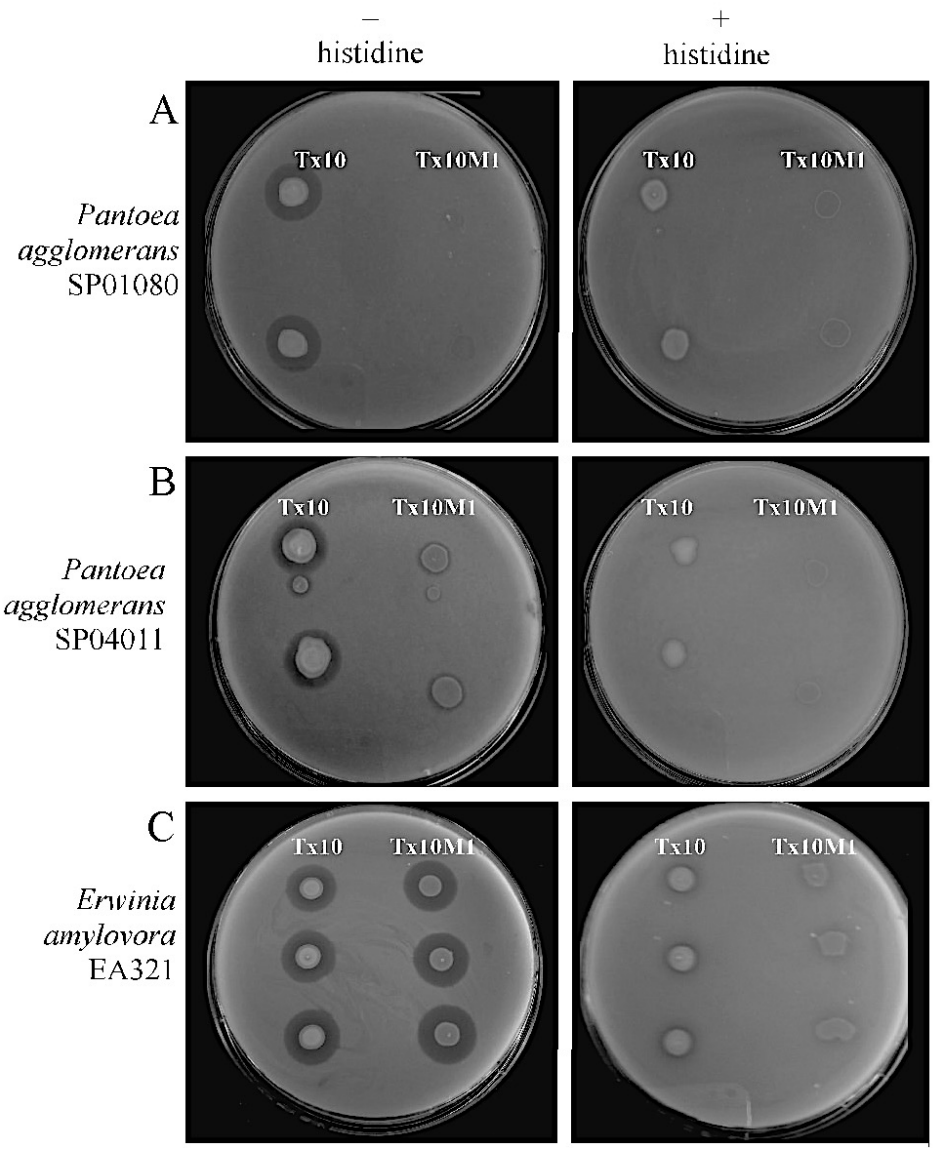

Figure 1. Inhibition of representative target strains by wildtype *P. agglomerans* Tx10, which produces PNP-2 and pantocin A, and mutant Tx10M1, which produces only pantocin A using Agar overlay. A. and **B** *P. agglomerans* SP01080 and *P. agglomerans* SP04011 represent the phenotype in which there is susceptibility to PNP-2 and pantocin A, but with the addition of L-histidine, the Tx10 wildtype is reduced to an intermediate phenotype.**C.** *E. amylovora* EA321 represents a phenotype in which there susceptibility to Tx10 and Tx10M1 in the absence of L-histidine, but there continues to be some level of susceptibility with exogenous L-histidine. (Each spot was replicated at least twice).

Table 3. Inhibitory activity of wildtype *P. agglomerans* Tx10 and PNP-2 mutant, Tx10M1, against Gram-positive and Gram-negative pathogens.

Indicator strain	Tx10*	Tx10M1*
Aeromonas sp.	2	2
Cronobacter sakazakii	2	1
Citrobacter sp.	2	0
Enterobacter sp.	1	0
Escherichia coli	3.5	0
Klebsiella pneumoniae	0	0
Pseudomonas aeruginosa	0	–
Salmonella enterica	2	0
Staphylococcus aureus (MRSA)	3	0
Staphylococcus epidermidis	3	0
Staphylococcus haemolyticus	3	0
Streptococcus mutans	3	0

*Rating system (1-5) described in materials and methods, – not tested.

Table 4. Inhibitory activity of wildtype *P. agglomerans* Tx10 and antibiotic resistant Tx10 strains against *Erwinia amylovora* EA321

Indicator strain	*Erwinia amylovora* EA321
P. agglomerans Tx10	S
P. agglomerans Tx10 RifR	R
P. agglomerans Tx10 StrepR	R

*S, susceptible; R resistant;

characterized, and the disrupted genes identified by comparison to the Tx10 draft genome (63). All 12 mutants had transposon insertions in one of six genes that formed a predicted 5.7 kb operon having a %GC of 41% (Table 5, Figure 2). The first gene identified in the cluster, *pnp2A* codes for an arylamine *N*-acetyltransferase (NAT) (Table 5). NATs have many described functions, including transferring an acetyl group from acetyl CoA to either an amino group, part of a heterocyclic amine, aromatic amine or a hydrazine compound (65). The second gene, *pnp2B*, codes for a predicted serine hydrolase (SerH), which can hydrolyze acyl, amide, and ester functional groups (66). The third gene in the cluster, *pnp2C*, codes for a member of the major facilitator superfamily transporter (MFS), which can transport sugars, peptides, and amino acids across membranes (67). The MFS transporters are also known to be a transporters of drugs and can function as a resistance determinant for some antibiotics (67). The fourth gene in the cluster, *pnp2D*, is a predicted *p*-aminobenzoate N-oxygenase (AurF), which is homologous to a gene in *Streptomyces thioluteus* that is involved in the oxidation of *p*-aminobenzoate to *p*-nitrobenzoate during the biosynthesis of the antibiotic, aureothin (68). The fifth gene in the cluster, *pnp2E*, belongs to the ferredoxin reductase-like superfamily (FNR), members of which facilitate the reduction of NADP+ and oxidation of ferredoxin (69). The sixth gene in the PNP-2 cluster, *pnp2F*, is predicted to code for an ATP-grasp domain-containing protein that has an atypical ATP-binding site called the ATP-grasp fold, and can catalyze ATP-dependent ligation of various enzymes that have an atypical ATP-binding site called the ATP-grasp fold (70) (Table 5).

The distribution of the PNP-2 cluster was evaluated using BLAST against completed and draft genomes available in the databases, which led to the identification of similar clusters in *P. agglomerans* IG1 (IG1) and *P. agglomerans* SL1 M5 (SL1). The IG1 cluster is almost identical to that of Tx10, having nucleotide identity ranging between 98% and 100% across all six genes, although one gene in the cluster (*pnp2E*) has not been annotated (Table 5). The SL1 cluster was more divergent, with nucleotide identities around 57% for the *pnp2E* (FNR), and 59% for the *pnp2A* (NAT). A portion of the PNP-2 cluster also resembled a cluster in *Proteus penneri* ATCC35198, with only *pnp2B*, *pnp2C*, and *pnp2D* matching in the translated protein database. An analysis of the regions flanking these three genes revealed that the first gene *pnp2A* and *pnp2F* were present but degenerate, while only a small remnant of *pnp2E* remained. A comparison of %GC content showed that although Tx10, IG1 and SL1 have an average genomic %GC of approximately 55%, their PNP-2 clusters are 41%, 42%, and 45%, respectively. In contrast, the *P. penneri* genome has a %GC content of 38%, while that of its degenerate cluster is much closer to that of the genome at 34%.

We then extended our search for the PNP-2 cluster to our collection of *Pantoea* isolates with a PCR-based screen. Initially *P. agglomerans* strains SN01080, DC434, SP05051, SP05120, and 12531, which inhibited similar bacterial species as Tx10 (58), were screened for the presence of *pnp2A* (arylamine N-acetyltransferase), *pnp2B* (serine hydrolase), and *pnp2F* (ATP-grasp domain-containing protein) (Table 5). These regions could not be PCR amplified from any of these select isolates, indicating that the PNP-2 biosynthetic cluster was not widely

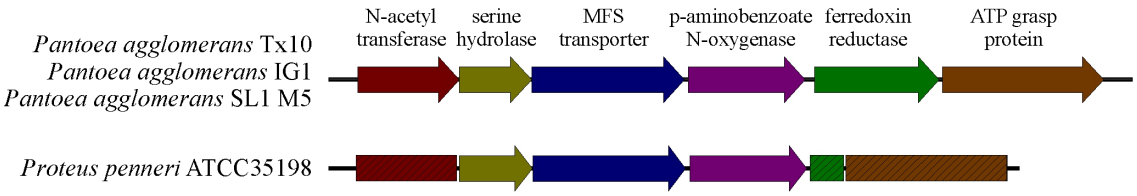

Figure 2. The *Pantoea* Natural Product 2 (PNP-2) predicted biosynthetic cluster. The PNP-2 gene clusters of *P. agglomerans* IG1 (Accession: BAEF01000001), and *Pantoea agglomerans* SL1 M5 (Accession: ADWZ00000000) are similar in genetic organization and content to those of *P. agglomerans* Tx10. A similar, degenerate cluster was identified in *Proteus penneri* (Accession: SAMN00000019). Homologous genes share similar colouring, and hatching indicates degenerate loci.

Table 5. Homologous genes of the putative PNP-2 biosynthetic cluster.

Mutants Recovered	Gene	Name	Closest BLAST homolog	Size (aa)	Similarity (%)	E value	Accession
M8, M20, M23, M24, M25, M26, M27	*pnp2A* (NAT)	arylamine *N*-acetyltransferase	*Pantoea agglomerans* IG1	262	100	0	WP_010670507
		arylamine *N*-acetyltransferase	*Pantoea agglomerans* SL1 M5	264	59	2e-105	ZP_09513813
M19, M21	*pnp2B* (SerH)	hypothetical protein	*Pantoea agglomerans* IG1	186	99	5e-137	WP_010670506
		hypothetical protein	*Pantoea agglomerans* SL1 M5	186	69	1e-93	ZP_09513814
M9	*pnp2C* (MFS)	major facilitator superfamily	*Pantoea agglomerans* IG1	394	98	0	WP_010670505
		major facilitator superfamily	*Pantoea agglomerans* SL1 M5	394	82	0	ZP_09513815
	pnp2D (AurF)	*p*-aminobenzoate *N*-oxygenase	*Pantoea agglomerans* IG1	301	99	0	WP_010670504
		p-aminobenzoate *N*-oxygenase	*Pantoea agglomerans* SL1 M5	301	99	0	ZP_09513816
M1	*pnp2E* (FNR)	Ferredoxin reductase-like	*Pantoea agglomerans* IG1	340	99	0	not annotated
		Ferredoxin reductase-like	*Pantoea agglomerans* SL1 M5	327	57	3e-130	ZP_09513817
M2	*pnp2F* (ATP-grasp)	hypothetical protein	*Pantoea agglomerans* IG1	363	100	0	WP_010670503
		hypothetical protein	*Pantoea agglomerans* SL1 M5	418	71	0	ZP_09513818

distributed across the collection. The remainder was screened only for the presence of the MFS transporter-encoding gene, *pnp*2C, spanning from *pnp*2B-*pnp*2D (Table 1, Table 5), but this region could also not be amplified from any isolate. To complement the PCR, standalone nucleotide and translated BLAST was used to compare the PNP-2 cluster against the draft genomes of 24 isolates of *Pantoea* (58, 63, 64), but no other homologous clusters could be identified. I found evidence for the production of a second natural product with antibiotic activity when conducting overlay assays using the mutant Tx10 M1.

We carried out several validation tests of our mutants to confirm that they no longer produced PNP-2. An overlay assay of the Tx10M1 mutant (disrupted in the FNR gene) showed that it no longer had activity against *Staphylococcus* or *E. coli*. We carried out the same assay using *E. amylovora* EA321 as the indicator strain, and noted that Tx10M1 retained inhibitory activity, suggesting the production of a second natural product. We examined the Tx10 draft genome (63) for the presence of known *Pantoea* antibiotic biosynthetic genes, including the gene clusters coding for pantocins A and B, andrimid, APV, AGA, and dapdiamide (42, 50-56), and identified only the three-gene, co-regulated pantocin A gene cluster (*paaA*, *paaB*, *paaC*) (45). To determine if the inhibition of *E. amylovora* by the transposon mutant Tx10M1 was due to pantocin A, the overlay was repeated with L-histidine, which is known to neutralize the effects of pantocin A (44). L-histidine dramatically reduced the inhibition of *E. amylovora* in the overlay assay (Figure 1C). On plates containing L-histidine, the zone of inhibition surrounding wild type Tx10, which we attributed

to PNP-2 production was also noticeably reduced, though significantly more than that around Tx10M1.

Antibiotic Survey

To evaluate the array of *Pantoea* and *Erwinia* isolates that were susceptible to PNP-2 and/or pantocin A, we assayed Tx10 and Tx10M1 in the presence and absence of L-histidine against our collection of environmental and clinical isolates representing nine different species groups of *Pantoea* and two species groups of *Erwinia* (Appendix 1, Table 6). Isolates were scored as susceptible to pantocin A if they were inhibited by Tx10M1 in the absence of L-histidine. Isolates were scored as susceptible to PNP-2 if they were inhibited by Tx10 in the presence or absence of L-histidine. Isolates that were inhibited by Tx10, but not by Tx10M1 were also scored as susceptible to PNP-2.

The first group of isolates was scored as resistant to both PNP-2 and pantocin A. These isolates were not inhibited by either Tx10 or Tx10M1, whether or not L-histidine was present. This group included *P. agglomerans* SP01230, SP03310, 7373, SP03231 and EH318, *Pantoea eucalyptii* SP03372, *Pantoea stewartii* 626, *Pantoea ananatis* M232A, and Cit30-11 *Pantoea dispersa* 625 and *Pantoea calida* B021323 (Table 6). The second group consisted of strains that were scored as susceptible to PNP-2, but resistant to pantocin A. Tx10 inhibited these isolates in the presence or absence of exogenous L-histidine, but not by Tx10M1 (Figure 1A). This group included nine isolates of *P. agglomerans*, *P. eucalyptii* B011489, *P. ananatis* 15320, *P. eucrina* Tx6 and *P. septica* BB350028A, G2291404, and G3271436 (Table 6). In this category were also those isolates that were resistant to Tx10M1, inhibited

by Tx10 without L-histidine, and the zone of inhibition around Tx10 was reduced in the presence of L-histidine. Those with this phenotype included five *P. agglomerans* isolates, along with *P. eucalyptii* SM03214 and B014130, *P. ananatis* 26SR6 and BRT98, and *P. septica* Tx4, B016375, 101150 and G4071105 (Table 6). The third group of strains was represented by those isolates that were resistant to PNP-2, but susceptible to pantocin A. These isolates were inhibited by both Tx10 and Tx10M1 in the absence of L-histidine, but were fully resistant in the presence of L-histidine. Isolates with this phenotype included five isolates of *P. agglomerans,* along with *P. eucalyptii* SP04031, *P. brenneri* B011483 and four isolates of *P. septica* (Table 6). The fourth group consisted of those isolates that were predicted to be susceptible to both PNP-2 and pantocin A. These isolates were inhibited by Tx10 with and without L-histidine, and inhibited by Tx10M1 in the absence of L-histidine (Figure 1). This group of isolates included 11 *P. agglomerans* isolates, along with *P. eucalyptii* SP03391, *P. brenneri* B024858 and B016381, *P. eucrina* 06868, *P. dispersa* M1657B, *P. calida* BB956721B1, *P. septica* BB350028B, 081828, and both *Erwinia billingae* isolates (Table 6). Also included in this category are those strains that were inhibited by both Tx10 and Tx10M1 in the absence and presence of L-histidine, but exhibited reduced zones of inhibition on the L-histidine plates, with the Tx10 inhibition being more pronounced than that of Tx10M1 (Figure 1C). This group includes eight *P. agglomerans* isolates, *P. eucalyptii* F9026, *P. stewartii* DC283, *P. eucrina* Tx5, *P. calida* BB957621A2 and BB957621C1, *P. septica* 7M1517 and X44686, and *E. amylovora* EA321 (Table 6). An evaluation of resistance across the 87 isolates tested revealed that 11 were resistant to both antibiotics, 28 susceptible to

PNP-2 only, 11 susceptible to pantocin A only, and 37 susceptible to both. With the combination of both antimicrobials, Tx10 was able to inhibit the growth of 76 of the 87 *Pantoea* isolates tested.

Table 6. Inhibition of representative target strains by wild type *P. agglomerans* Tx10, which produces PNP-2 and pantocin A, and mutant the Tx10M1, which produces pantocin A.

Bacterial Species	- Histidine*		+ Histidine*		Predicted Resistance	
	Tx10	**M1**	**Tx10**	**M1**	**PNP-2**	**PAA**
Pantoea agglomerans SP01230	R	R	R	R	R	R
Pantoea agglomerans SP03310	R	R	R	R	R	R
Pantoea agglomerans SP03231	R	R	R	R	R	R
Pantoea agglomerans EH318	R	R	R	R	R	R
Pantoea agglomerans 7373	R	R	R	R	R	R
Pantoea eucrina SP03372	R	R	R	R	R	R
Pantoea stewartii 626	R	R	R	R	R	R
Pantoea ananatis M232A	R	R	R	R	R	R
Pantoea ananatis Cit30-11	R	R	R	R	R	R
Pantoea dispersa 625	R	R	R	R	R	R
Pantoea calida B021323	R	R	R	R	R	R
Pantoea agglomerans B025670	s	R	R	R	S	R
Pantoea agglomerans B026440	S	R	R	R	S	R
Pantoea agglomerans 3581	S	R	R	R	S	R
Pantoea agglomerans 1574	S	R	R	R	S	R
Pantoea agglomerans 240R	S	R	R	R	S	R
Pantoea eucrina SM03214	S	R	R	R	S	R
Pantoea brenneri B014130	S	R	R	R	S	R
Pantoea ananatis S4SR6	s	R	R	R	S	R
Pantoea ananatis BRT98	S	R	R	R	S	R
Pantoea septica Tx4	s	R	R	R	S	R
Pantoea septica B016375	S	R	R	R	S	R
Pantoea septica 10-1150	S	R	R	R	S	R
Pantoea septica G4071105	s	R	R	R	S	R
Pantoea agglomerans 3-770398	S	R	S	R	S	R
Pantoea agglomerans B016395	S	R	s	R	S	R
Pantoea agglomerans SN01080	S	R	S	R	S	R
Pantoea agglomerans SP04022	S	R	s	R	S	R
Pantoea agglomerans 17124	S	R	s	R	S	R
Pantoea agglomerans DC434	S	R	S	R	S	R
Pantoea agglomerans DC556	s	R	s	R	S	R
Pantoea agglomerans 1512	S	R	s	R	S	R
Pantoea agglomerans 308R	S	R	s	R	S	R
Pantoea eucrina B011489	S	R	S	R	S	R
Pantoea ananatis 15320	s	R	s	R	S	R

Pantoea eucrina Tx6	S	R	s	R	S	R
Pantoea septica BB350028A	S	R	S	R	S	R
Pantoea septica G2291404	S	R	S	R	S	R
Pantoea septica G3271436	s	R	s	R	S	R
Pantoea agglomerans SP00202	s	s	R	R	R	S
Pantoea agglomerans SP03383	s	s	R	R	R	S
Pantoea agglomerans SP02243	S	S	R	R	R	S
Pantoea agglomerans SS02010	S	s	R	R	R	S
Pantoea agglomerans 12531	S	S	R	R	R	S
Pantoea eucrina SP0413	s	s	R	R	R	S
Pantoea brenneri B011483	S	s	R	R	R	S
Pantoea septica Tx3	s	s	R	R	R	S
Pantoea septica VB38951 A	S	S	R	R	R	S
Pantoea septica VB38951 B	S	S	R	R	R	S
Pantoea septica 09-1457A	S	S	R	R	R	S
Pantoea agglomerans B015092	S	s	s	R	S	S
Pantoea agglomerans BB834250	S	S	S	R	S	S
Pantoea agglomerans SP05061	S	S	s	R	S	S
Pantoea agglomerans SP05120	S	s	s	R	S	S
Pantoea agglomerans SP05130	S	s	s	R	S	S
Pantoea agglomerans SP04021	S	S	s	R	S	S
Pantoea agglomerans SP05092	S	S	S	R	S	S
Pantoea agglomerans SP05051	s	s	s	R	S	S
Pantoea agglomerans DC432	S	S	s	R	S	S
Pantoea agglomerans 7612	S	S	S	R	S	S
Pantoea eucrina SP03391	S	s	S	R	S	S
Pantoea brenneri B024858	S	S	S	R	S	S
Pantoea brenneri B016381	S	S	S	R	S	S
Pantoea eucrina 6868	S	s	S	R	S	S
Pantoea dispersa 2M1657B	S	S	S	R	S	S
Pantoea calida BB956721B1	S	s	S	R	S	S
Pantoea septica BB350028B	S	s	s	R	S	S
Pantoea septica 08-1828	S	S	S	R	S	S
Erwinia billingae EhWh18	S	S	S	R	S	S
Erwinia billingae EhWh9	S	S	S	R	S	S
Pantoea agglomerans SP00303	S	S	S	s	S	S
Pantoea agglomerans SN01170	S	S	S	s	S	S
Pantoea agglomerans SN01122	S	s	s	s	S	S
Pantoea agglomerans SP04011	S	S	S	s	S	S
Pantoea agglomerans SP04010	S	S	S	s	S	S
Pantoea agglomerans 13301	S	S	S	s	S	S
Pantoea agglomerans 12534	S	s	S	s	S	S
Pantoea agglomerans 788	s	s	S	s	S	S

Pantoea agglomerans 5565	S	S	s	s	S	S
Pantoea eucrina 5-F9026	S	S	s	s	S	S
Pantoea stewartii DC283	S	S	S	s	S	S
Pantoea eucrina Tx5	S	S	S	S	S	S
Pantoea calida BB957621A2	S	s	S	S	S	S
Pantoea calida BB957621C1	S	S	s	s	S	S
Pantoea septica 7-M1517	S	s	S	s	S	S
Pantoea septica X44686	S	S	s	s	S	S
Erwinia amylovora EA321	S	S	S	s	S	S

*Target isolates were scored as either “resistant” (R) if no defined zone of inhibition formed, “susceptible” (S) if a larger defined zone of inhibition was observed, and “somewhat susceptible” (s) if there was a less-defined or more diffuse zone of reduced growth observed in the lawn surrounding the test isolate. Strains in the table are sorted based on similar phenotype.

Regulation of PNP-2 expression

In order to elucidate the conditions for antibiotic expression, the PNP-2 promoter was cloned into a plasmid containing a luciferase reporter gene cassette. Along with controls, the luminescence produced by the luciferase gene cassette under the control of the PNP-2 promoter was used to identify the media conditions for PNP-2 expression. The luciferase genes were therefore under the control of the PNP-2 promoter, and would be expressed when PNP-2 was expressed. The level of the luminescence as a result of PNP-2 promoter activation was evaluated in both LB and EMM media, as shown by luminescence (Table 7). In EMM, where antibiotic production is usually assayed, Tx10pCS26-PNP-2P luminescence ranged between 50000 and 200000 units (Table 7). In LB, Tx10pCS26-PNP-2P had low levels of luminescence (<1500 units) (Table 7). In both LB and EMM the control construct, Tx10pCS26-*nptII*, had high levels of luminescence (>5000000 units), and the negative control Tx10pCS26 had low levels (<500 units). These experiments were then repeated using the plate reader to compare growth versus luminescence (Figure 3). The control conditions using the 96 well assay in the plate reader were consistent with previous results that were completed using the standalone luminometer (Figure 4). These assays were carried out using the plate reader luminometer, which reported significantly lower levels of luminescence than the standalone luminometer. However, the trends in both experiments were similar.

The experiment was extended to evaluate the effects of amino acid supplementation on the regulation of the PNP-2 promoter. Luminescence varied between media types, but certain amino acids caused a change in expression.

Supplementation of EMM with L-isoleucine, L-leucine, L-glycine, L-valine, L-phenylalanine, L-tryptophan, L-methionine, L-histidine and L-cysteine repressed PNP-2 expression, with luminescence not exceeding 900 units (Figure 4). When the EMM was supplemented with the amino acids: L-lysine, L-proline, L- threonine, L-alanine, L-arginine and L-tyrosine there was peak expression levels between 900 and 1500 units (Figure 4). Supplementation with L-asparagine, L-lysine, L-glutamine, L-serine and L-aspartic acid did not reduce the expression of the PNP-2 compared to the EMM alone, reaching peak levels above 2000 units. In the case of supplementation with L-glutamine, L-asparagine and L-aspartic acid, there was an increase of expression of the PNP-2 promoter compared to the EMM (Figure 4).

Table 7. Luminescence of PNP-2 constructs in LB and EMM relative to controls

Construct	Medium	Range of Luminescence
Tx10pCS26-*nptII*	EMM	>5000000
Tx10pCS26 (empty plasmid)	EMM	<500
Tx10pCS26-PNP-2P	EMM	50000-200000
Tx10pCS26-*nptII*	LB	>5000000
Tx10pCS26 (empty plasmid)	LB	<500
Tx10pCS26-PNP-2P	LB	<1500

- These readings were taken using 1 mL of bacterial culture over the course of 6 seconds.

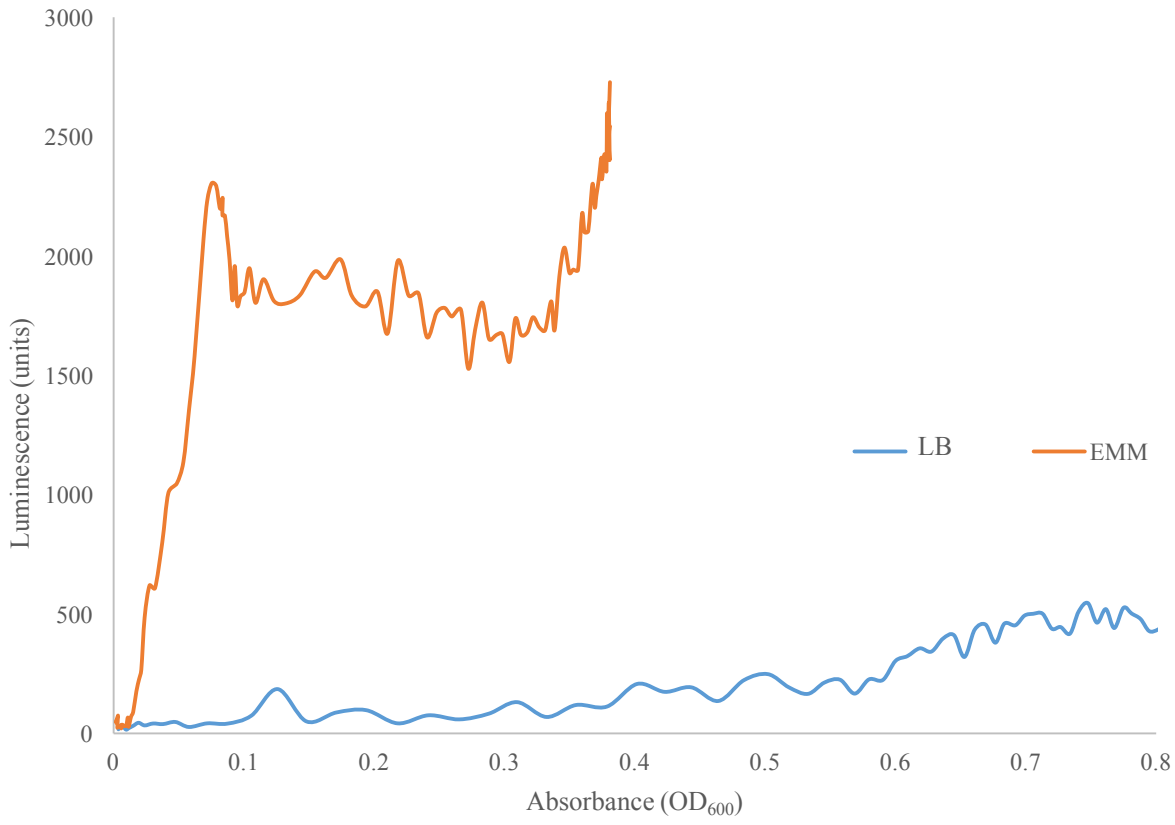

Figure 3. Expression of the PNP-2 promoter measured using a luciferase reporter gene cassette in LB and EMM media. The graph depicts luminescence of Tx10pCS26-PNP-2P under LB and EMM media conditions. The graph compares the growth the bacterial culture (Absorbance, OD_{600}) of Tx10pCS26-PNP-2P between LB and EMM. The EMM has a significantly higher level of expression, reaching over 2500 units, while the LB represses expression.

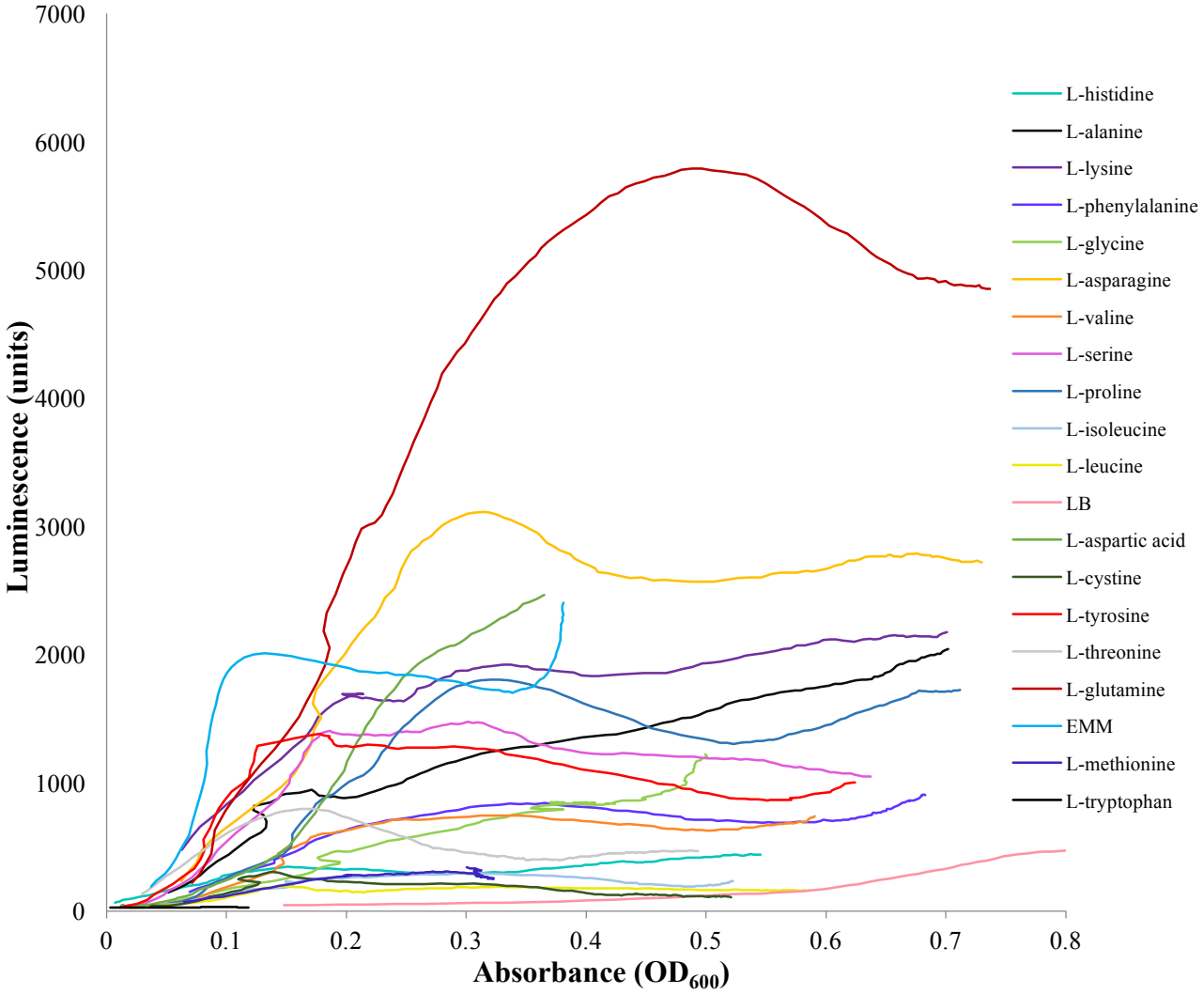

Figure 4. Expression of the PNP-2 promoter measured using a luciferase reporter gene cassette with amino acid supplementation. The graph depicts luminescence of Tx10pCS26-PNP-2P plotted against bacterial optical density (OD_{600}). The EMM is supplemented with each of amino acids individually, as well as LB and EMM as controls. Each line represents average trendlines of the luminescence and absorbance data. The trendline is truncated for EMM and L-tryptophan as the OD began to decrease over time.

Discussion

Isolates of *Pantoea* produce of a variety of antibiotics, including pantocins, andrimids, herbicolins, APV, AGA, dapdiamides and PNP-1 (45, 50-52, 58, 71, 72). *P. agglomerans* Tx10 carries a predicted biosynthetic cluster comprising six genes that have a role in the production of PNP-2, an antimicrobial natural product that has activity against both Gram-positive and Gram-negative bacteria. The cluster has a limited distribution, with homologous clusters being present only in *P. agglomerans* IG1 and *P. agglomerans* SL1 M5, along with a partial degenerate cluster in *P. penneri.* None of these clusters have been annotated as being involved in the biosynthesis of a natural product. With the exception of these three isolates, this cluster was not identified in any strains in our collection, as shown by both PCR and comparative genomics approaches of fully sequenced strains. There were limitations to our PCR-based approach, given that genetic variation in the primer binding sites could prevent amplification. Although our primers are largely in conserved regions, we screened for the presence of multiple loci in those isolates that had similar inhibitory profiles, but no homologous clusters were identified. Together with our genomic survey, these results suggest that the PNP-2 cluster has an overall limited distribution across *Pantoea*.

Antibiotic Survey

Tx10 as well as the transposon mutant Tx10M1 were able to antagonize a broad array of microbes. *Citrobacter sp.*, *Enterobacter sp.*, *E. coli, S. enterica, S. aureus, S. epidermidis, S. haemolyticus,* and *S. mutans* were susceptible to PNP-2, as indicated by their inhibition by Tx10, but were not susceptible to the PNP-2 mutant, Tx10M1. For *Aeromonas sp., C. sakazakii,* and *P. vulgaris* both Tx10 and the Tx10M1 were

antagonistic, although it is unclear what the relative contributions are of PNP-2 and pantocin A to this inhibition. The inhibition of growth of *E. amylovora* by Tx10M1 (no longer producing the PNP-2 antibiotic), against *E. amylovora* can be attributed to the production of pantocin A. Pantocin A acts to disrupt the endogenous production of L-histidine in *E. amylovora* such that adding exogenous L-histidine to our assays diminished the effects of pantocin A. This indicates that the pantocin A genes are functional in Tx10. No other known *Pantoea* antibiotic clusters were identified within the Tx10 genome.

The overlay assays were extended to the entire collection of *Pantoea* to determine which isolates could be inhibited by the natural products of Tx10. With and without added L-histidine, we compared wildtype Tx10 (PNP-2 and pantocin A proficient) to the Tx10M1 mutant (PNP-2 deficient), and used this as the basis for scoring isolates as resistant or susceptible to each antibiotic. The phenotype was clear in most cases, although the zone of inhibition surrounding Tx10 on isolates susceptible to PNP-2 was also reduced in the presence of L-histidine. Although this was initially attributed to PNP-2 targeting L-histidine biosynthesis, it was also possible that exogenous L-histidine does not completely neutralize the effects of PNP-2 across the isolates tested, unlike with pantocin A. In fact, there was a defined zone of inhibition even in the presence of the amino acid, but it was consistently smaller than that seen without the amino acid. Therefore, this phenotype may also be explained by PNP-2 expression being impacted by the addition of L-histidine, as shown by the luminescence assay (Figure 5). This is also supported by the absence of PNP-2 production by Tx10 on the complex medium, LB, suggesting PNP-2 production only the amino acid-limiting conditions.

Several isolates that were susceptible to pantocin A were still slightly inhibited by Tx10M1 in the presence of L-histidine. Isolates in this category had a slightly reduced growth surrounding the Tx10M1 isolate on L-histidine, which did not develop into a defined zone of inhibition. In these cases it is possible that the L-histidine is only partially neutralizing the effects of pantocin A, potentially caused by inefficient L-histidine uptake by the target isolate, or due to metabolic processing of the amino acid by the target isolate. Alternatively, these isolates may have a higher L-histidine requirement such that the concentration provided is more limiting. The results of our assays revealed that of the 87 *Pantoea* isolates evaluated, only 11 were resistant to both antibiotics, such that with the combination of both antimicrobials, Tx10 is able to inhibit the growth of 76 of the 87 *Pantoea* isolates tested. Additionally, there does not seem to be a correlation between the phylogenetic location and the potential resistance to PNP-2 or pantocin A (Appendix 1). This is contrast to PNP-1, a genetically unrelated antibiotic that was identified in *P. ananatis* BRT175, and which has a considerably more defined spectrum of activity (58). PNP-1 does not affect many isolates of *P. agglomerans* or *P. eucalypti,* but very strongly inhibiting select isolates of *Pantoea conspicua, brenneri, anthophila, stewartii, ananatis, calida, dispersa, eucrina and septica*. Lastly, the presence of either the PNP-2 cluster or the pantocin A gene cluster did not correlate with their susceptibilities to the two antibiotics. For instance, *P. eucalyptii* B011489 was resistant to pantocin A, however, the gene cluster is not present in the genome. Therefore, the mechanisms of resistance among isolates may include target modification, antibiotic exclusion, or generic efflux pumps that prevent the accumulation of the antibiotic in the cell. Furthermore, the irregularities seen during the antibiotic susceptibility screen could

also be due to potential synergies between PNP-2 and pantocin A, or varied minimum inhibitory concentrations across the strains tested. Previous work with antagonists of *E. amylovora* showed that combinations of antimicrobials were more effective than either alone (73). Additional analyses were needed to determine which of these may be contributing to resistance, and our current efforts are centered on isolation and structural determination of the natural product being produced.

Antibiotic Regulation

Spontaneous antibiotic resistant mutants of Tx10 were initially isolated for genetic manipulation; however, rifampicin and streptomycin-resistant mutants no longer produced antibiotics (Table 4). This suggested pleiotropic effects of gaining resistance to rifampicin and streptomycin. Mutations K43N and K43T in the *rpsL* gene of *Pectobacterium carotovorum* (formerly *Erwinia carotovora*), which confers streptomycin resistance, can result in the loss of carbapenem production (74). The *car* genes, which direct the biosynthesis of carbapenem were expressed at a reduced rate in the mutants as compared to wildtype (74). *Streptomyces coelicolor* produces the blue-pigmented antibiotic, actinorhodin, which acts on phospholipid bilayer integrity (75, 76). Actinorhodin production in some cases is increased or decreased by streptomycin resistance mutations, which impact the stringent response. The ppGpp biosynthetic genes, *relA* and *relC*, are affected by rifampicin resistance mutations in the beta subunit gene of the RNA polymerase (*rpoB*), as well as mutations to the 30S ribosomal gene, *rpsL* conferring streptomycin resistance (75). These regulatory mechanisms are responsible for the regulation of genes during periods of cellular stress, such as amino acid starvation (77, 78),(79). Tx10 antibiotic production showed some of the similar

pleiotropic responses after acquiring point mutations conferring resistance to rifampicin and streptomycin.

Initial antibiotic screens suggested that the PNP-2 antibiotic was being produced in the nutrient-limited *E. coli* minimal media (EMM), but not in nutrient-rich LB media. Luminescence assays confirmed that the expression was repressed in LB relative to EMM (Figure 4). Furthermore, in the early soft-agar antibiotic overlay assays shown above, it was noted that EMM supplemented with L-histidine was affecting the inhibition of the target strain compared to standard EMM; the zone of inhibition was reduced considerably (Figure 1). Initially, it was unclear whether L-histidine was responsible for neutralizing the effects of PNP-2 on the target, or whether it was decreasing its expression. To evaluate the importance of different amino acids, including L-histidine in regulating antibiotic expression, EMM was supplemented with each amino acid and luminescence measured. The results from the luminescence assay were divided into three groups of amino acids based on their effects on the expression of PNP-2 (Figure 4). Any one of the following amino acids completely repressed antibiotic expression: L-histidine, L-isoleucine, L-leucine, L-glycine, L-valine, L-methionine, L-tryptophan, and L-phenylalanine, (Table 7). The poor growth of the bacteria in the media supplemented with L-tryptophan should be noted as the media required DMSO for dissolving the amino acid, and this may be affecting the growth.

This group of amino acids that represses antibiotic production corresponds to the essential amino acids for humans, but unclear as to their role in the regulation of antibiotic expression (80). Repressing expression could indicate that these amino acids negatively regulate antibiotic expression as an indication of the surrounding nutrient

environment, such that when these nutrients are available, antibiosis no longer becomes a favourable competition strategy. Expression of this antibiotic may therefore be linked to amino acid starvation, and thus, the stringent response (81). There are a large number of regulatory changes that occur during the stringent response, such as the expression of virulence factors and secondary metabolites (81). Therefore, it is possible that the activation of the stringent response due to amino acid starvation is responsible for the antibiotic production, similar to what is seen with actinorhodin production in *Streptomyces coelicolor* (78). These amino acids can therefore be acting to bring Tx10 out of the stringent response, altering its gene expression and repressing PNP-2 production.

Two amino acids, L-glutamine, and L-asparagine, may be increasing the expression of the PNP-2 antibiotic compared to expression in EMM (Figure 4). There are a few possible explanations as to why this is occurring. Firstly, these amino acids could be precursors to the antibiotic production pathway, and may be increasing expression without affecting the regulation of the PNP-2 promoter. For instance, in the carbapenem production seen in *Pectobacterium carotovorum*, L-glutamine is a precursor in the pathway, and its availability affects the synthesis of the antibiotic (82). Furthermore, the production of dapdiamides (known *Pantoea* derived antibiotic) is observed with media supplemented L-asparagine (50). It is possible that competition through antibiosis is most selectively advantageous when certain amino acids are limited. Notably, the amino acids that repress expression correspond to the human essential amino acids, which could imply a a correlation to regulation during opportunistic human infections, although this would have to be tested.

Conclusion

P. agglomerans Tx10 is an antibiotic producing isolate. Tx10 produces PNP-2, a natural product of a predicte six-gene cluster, which exhibits antimicrobial activity against many clinical pathogens including *S. aureus* and *Salmonella*. The gene cluster is not widely distributed and appears to be relatively rare in *Pantoea*, and across the diverse bacterial and archaeal groups represented in the public databases. Tx10 produces a second natural product, pantocin A, which is known to be produced by *Pantoea* species and has inhibitory activity against many different isolates. Furthermore, the screen of both PNP-2 and pantocin A against a large collection of *Pantoea* showed that 76 of 87 isolates were susceptible to at least one of the secreted natural products of Tx10. Furthermore, the PNP-2 antibiotic is expressed differentially on different media types. A luminescence reporter system suggested that antibiotic production is reduced in nutrient-rich media, with repression being caused by the presence of certain amino acids.

The significance of the antibiotics of Tx10 is two-fold. Firstly, the newly discovered PNP-2 antibiotic may have clinical relevance. The antibiotic has inhibitory activity against some of the pathogens currently causing issues with AMR in our hospitals and primary care facilities, and with further development this antibiotic may have clinical significance. PNP-2 also encourages further research into the bioactive compounds produced by other *Pantoea* strains, which may yield additional natural products for clinical application. Secondly, the antimicrobial production seen in Tx10 is significant for its potential role in the environment. Throughout evolutionary time bacteria have been constantly competing with each other for nutritional sources and colonizing particular niches. It is possible the antibiotic production is a mechanism by

which Tx10 can outcompete other microbes. The two antibiotics produced by Tx10 have very different spectra of activity, such that the production of multiple antibiotics antagonizes a much wider range of isolates. In addition, the tight regulation of the PNP-2 antibiotic and its link to amino acid starvation may indicate its activity is used only in stress situations in order to acquire nutrients from other bacteria (if the antibiotic is bacteriolytic), or to simply eliminate competition for the limited resources that are present. This work paves the way for additional research into strategies for competition, as well as antibiotic production in *Pantoea*.

Future Directions:

In the development of any antibiotic it is crucial to identify the chemical structure of the product. PNP-2 is unknown, and its structure should be the focus of future studies. Important information can be gathered from its structure, including whether or not it is a known compound. Once the PNP-2 structure is identified and the compound can be purified, toxicity tests can be undertaken to determine the mode of action, and experimentation carried out to establish whether PNP-2 is bacteriolytic or bacteriostatic. Also, further steps will be needed to understand the regulation of PNP-2 and the role that the antibiotic production plays in the fitness and competitiveness of the microbe. To confirm if PNP-2 is regulated by the stringent response, it is necessary to complete more nutritional assays and confirm ppGpp (the strongest regulator of the stringent response) as the signalling molecule that is regulating antibiotic production in response to amino acid starvation. One potential method is to use pharmacological induction of the stringent response through serine hydroxamate supplementation in the media, as it is known to induce ppGpp production (83).

Lastly, it is important to note that in order to understand the role of PNP-2 in the fitness of the microbe, competition assays, and animal model polymicrobial infections should be conducted. A possible assay could include co-infecting a fruit fly with the Tx10 and Tx10M1 alongside *S. aureus* (MRSA), and using the luciferase reporter to monitor the microbial community. If Tx10 is able to outcompete the MRSA and overtake the fly, compared to the Tx10M1, then the role of PNP-2 in the fitness of the microbe can be evaluated. By moving into animal systems, we may be able to understand the connection between the amino acid repression and the expression of the antibiotic. These objectives have helped to characterize the antibiotics of Tx10 and further the expansion of the *Pantoea* genus as a significant antibiotic reservoir, as well as give insight into the regulation of antibiotic production.

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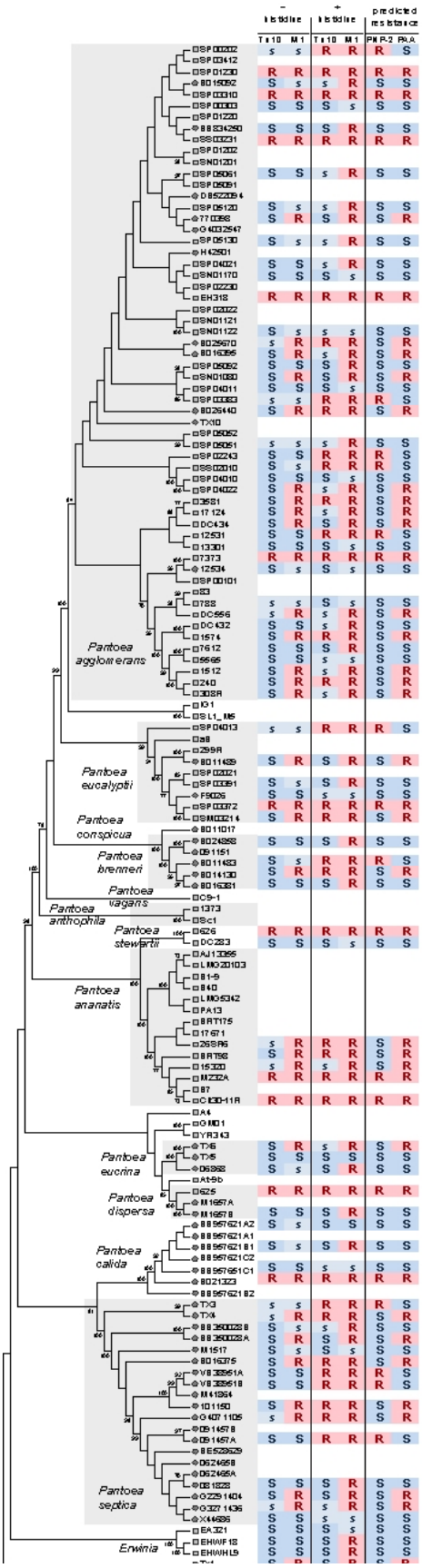
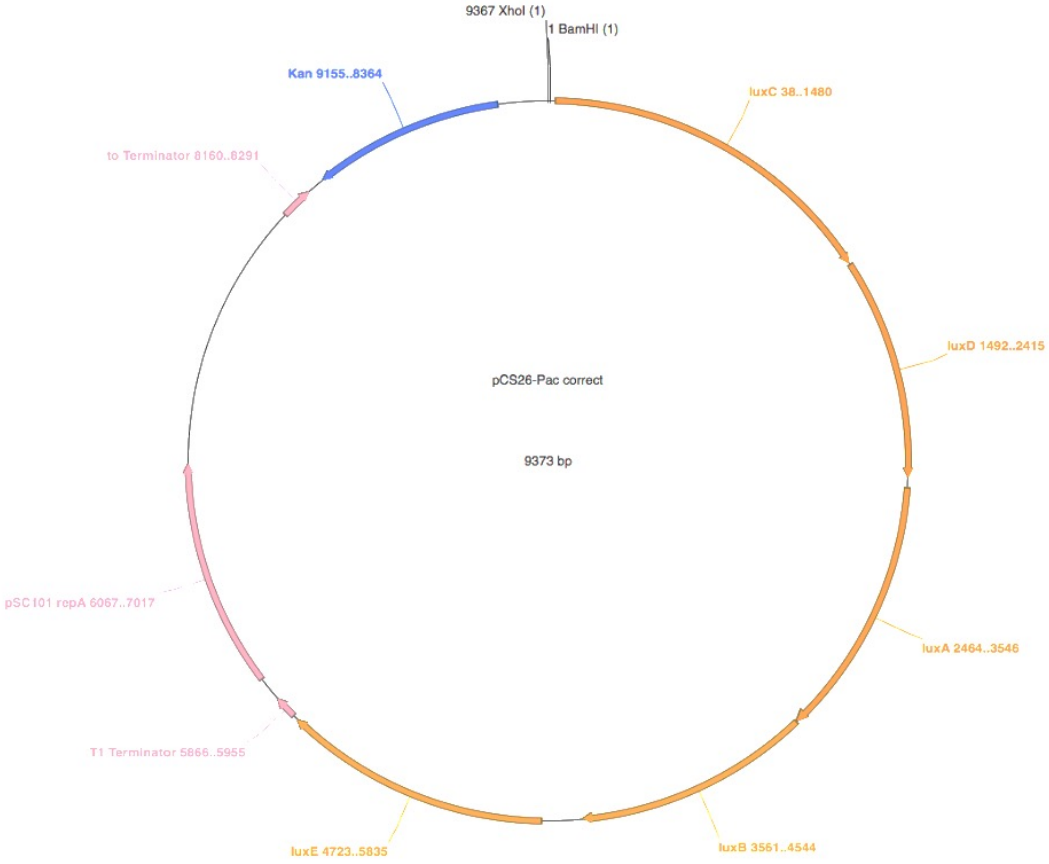
Appendix



Appendix 1. Resistance profiles of *Pantoea* and *Erwinia* isolates to natural products produced by *P. agglomerans* Tx10 (Tx10), and PNP-2 mutant, *P. agglomerans* Tx10M1 (M1), overlaid onto an MLSA species tree [33].

The interaction between the two test isolates and each target isolate in the tree was scored both in the presence and absence of L-histidine. Target isolates were either resistant (R) if no defined zone of inhibition formed, susceptible (S) if a larger defined zone of inhibition was observed, and (s) if there is a small, yet defined zone of inhibition observed in the lawn surrounding the test. Based on these phenotypes each target isolate was given a predicted resistance (R) or susceptibility (S) to both PNP-2 and Pantocin A (PAA). If the isolate showed susceptibility to Tx10M1 In the presence or absence of L-histidine, the isolate was predicted to be susceptible to Pantocin A. If the isolate showed susceptibility to Tx10 in the presence or absence of L-histidine, or, if the isolate showed any level of susceptibility to Tx10 so long as Tx10M1 showed no activity, the isolate was considered susceptible to PNP-2.



Appendix 2. pCS26 plasmid map, showing the restriction sites for XhoI and BamHI

Appendix 3. Bacterial strains used in this study.

Species	Source of Isolation /Relevant Characteristics	Source or Reference
Aeromonas sp.		
SM02150		Regina, SK
Citrobacter sp.		
B012497	Human, female, urine midstream	Sask. Disease Control Lab
10-854	Human abdominal fluid ruptured appendix	St. Boniface General Hospital
Cronobacter sakazakii		
12202	Musk melon	ICMP
Enterobacter sp.		
Tx1	human, cf sputum	Texas Children`s Hospital
Tx2	Human cystic fibrosis sputum	Texas Children's Hospital
Erwinia amylovora		
EA321	hawthorn	Dr. George Sundin, Michigan State
Escherichia coli		
VPE42 (RK600)	conjugative plasmid	Dr. David Guttman, U of Toronto
HB101 (pBSL118)	mini-Tn5 transposon	Dr. David Guttman, U of Toronto
K-12 W3110	SGSC #447	Dr. Andrew Cameron, U of Regina
Klebsiella pneumoniae		
B011499	Human, female, cloudy urine	Sask. Disease Control Lab
G4061350	Clinical	Regina General Hospital
Lactococcus lactis		
HD1		Heather Dietz, U of Regina
Pantoea agglomerans		
83	wheat	ICMP
788	green bean	ICMP
1512	green bean	ICMP
1574	unidentified	ICMP
3581	oat seed	ICMP
5565	soybean	ICMP
7373	onion	ICMP
7612	grass grub	ICMP
12531	Gypsophila (Baby's Breath)	ICMP
12534	human, knee laceration	ICMP
13301	golden delicious apple	ICMP
17124	olive	ICMP
770398	human, female, blood	Sunnybrook Hospital
240R	pear flower	Dr. Steven Lindow, UC Berkeley
308R	pear flower	Dr. Steven Lindow, UC Berkeley
B015092	human, female, urine midstream	Sask. Disease Control Lab
B016395	human, female, superficial wound	Sask. Disease Control Lab
B025670	human, female, superficial wound	Sask. Disease Control Lab

B026440	human, male, superficial wound	Sask. Disease Control Lab
BB834250	human, female, sputum, aortic aneurysm	St. Boniface General Hospital
DB522094	human, elbow sore	St. Boniface General Hospital
DC432	maize	Dr. David Coplin, Ohio State
DC454	maize	Dr. David Coplin, Ohio State
DC556	Gypsophila (Baby's Breath)	Dr. David Coplin, Ohio State
Eh318	apple leaf	CUCPB 2140; Dr. Brion Duffy
G4032547	human, ear	Regina General Hospital
H42501	human, male, blood	Sunnybrook Hospital
SN01080	slug	(84)
SN01121	bee	(84)
SN01122	bee	(84)
SN01170	caterpillar	(84)
SP00101	raspberry	(84)
SP00202	apple	(84)
SP00303	raspberry	(84)
SP01201	strawberry leaf	(84)
SP01202	strawberry leaf and stem	(84)
SP01220	healthy rose bush	(84)
SP01230	Virginia creeper leaves and stem	(84)
SP02022	thistle	(84)
SP02230	diseased tree leaf	(84)
SP02243	unidentified tree	(84)
SP03310	diseased tree leaf	(84)
SP03383	diseased maize leaf	(84)
SP03412	diseased bean leaf	(84)
SP04010	tomato leaf	(84)
SP04011	tomato leaf	(84)
SP04021	tomato leaf	(84)
SP04022	tomato leaf	(84)
SP05051	tomato leaf	(84)
SP05052	tomato leaf	(84)
SP05061	tomato leaf	(84)
SP05091	tomato leaf	(84)
SP05092	tomato leaf	(84)
SP05120	diseased maize leaf	(84)
SP05130	diseased maize stamen	(84)
SS02010	soil - ground squirrel burrow	(84)
SS03231	soil - ground squirrel burrow	(84)
Tx10	human, cf sputum	Texas Children`s Hospital
Pantoea ananatis		
15320	rice	ICMP
17671	rice	ICMP
26SR6	maize leaf	Dr. Steven Lindow, UC Berkeley
B7	maize, rifR derivative of M232A	Dr. Steven Lindow, UC Berkeley

BRT175	strawberry	Dr. Gwyn Beattie, Iowa State
BRT98	strawberry	Dr. Steven Lindow, UC Berkeley
Cit30-11R	naval orange leaf	Dr. Steven Lindow, UC Berkeley
M232A	maize	Dr. Steven Lindow, UC Berkeley
Pantoea anthophila		
1373	balsam	ICMP
Pantoea brenneri		
91151	human	St. Boniface General Hospital
B011483	human, female, superficial wound	Sask. Disease Control Lab
B014130	human, male, superficial wound	Sask. Disease Control Lab
B016381	human, female, groin	Sask. Disease Control Lab
B024858	human, female, breast abscess	Sask. Disease Control Lab
Pantoea calida		
B021323	human, female, urine midstream	Sask. Disease Control Lab
BB957621A1	human, male, capd dialysate, peritonitis	St. Boniface General Hospital
BB957621A2	human, male, capd dialysate, peritonitis	St. Boniface General Hospital
BB957621B1	human, male, capd dialysate, peritonitis	St. Boniface General Hospital
BB957621B2	human, male, capd dialysate, peritonitis	St. Boniface General Hospital
BB957621C1	human, male, capd dialysate, peritonitis	St. Boniface General Hospital
BB957621C2	human, male, capd dialysate, peritonitis	St. Boniface General Hospital
Pantoea conspicua		
B011017	human, female, superficial wound	Sask. Disease Control Lab
Pantoea dispersa		
625	sorghum	ICMP
M1657A	human, male, blood	Sunnybrook Hospital
M1657B	human, male, blood	Sunnybrook Hospital
Pantoea eucalyptii		
299R	pear flower	Dr. Steven Lindow, UC Berkeley
B011489	human, female, superficial wound	Sask. Disease Control Lab
F9026	human, male, blood	Sunnybrook Hospital
SM03214	goose feces	(84)
SP02021	thistle leaf	(84)
SP03372	diseased maize leaf	(84)
SP03391	diseased bean leaf	(84)
SP04013	tomato leaf	(84)
Pantoea eucrina		
6686	human, headache	St. Boniface General Hospital

TX5	human, blood	Texas Children`s Hospital
TX6	human, blood	Texas Children`s Hospital
Pantoea septica		
81828	human, post hemicholectomy	St. Boniface General Hospital
101150	human	St. Boniface General Hospital
062465A	human, cerebellar cva (stroke)	St. Boniface General Hospital
062465B	human, cerebellar cva (stroke)	St. Boniface General Hospital
091457A	human, renal failure	St. Boniface General Hospital
091457B	human, renal failure	St. Boniface General Hospital
B016375	human, female, finger	Sask. Disease Control Lab
BB350028A	human, female, blood culture, fever	St. Boniface General Hospital
BB350028B	human, female, blood culture, fever	St. Boniface General Hospital
BE528629	human, peritoneal dialysis	St. Boniface General Hospital
G2291404	human	Regina General Hospital
G3271436	human, urine	Regina General Hospital
G4071105	human, urine	Regina General Hospital
M1517	human, female, blood	Sunnybrook Hospital
M41864	human, female, blood	Sunnybrook Hospital
TX3	human, blood	Texas Children`s Hospital
TX4	human, blood	Texas Children`s Hospital
VB38951A	human, female, blood culture, sore throat	St. Boniface General Hospital
VB38951B	human, female, blood culture, sore throat	St. Boniface General Hospital
X44686	human, female, blood	Sunnybrook Hospital
Pantoea stewartii		
626	maize	ICMP
DC283	maize	Dr. David Coplin, Ohio State
Pseudomonas aeruginosa		
ATCC 27853	clinical	Heather Dietz, U of Regina
Salmonella enterica		
TA100		
Staphylococcus aureus		
K1-7	clinical	Dr. Chris Yost, U of Regina
K5-4	clinical	Dr. Chris Yost, U of Regina
K7-1	clinical	Dr. Chris Yost, U of Regina
B19	clinical	Dr. Chris Yost, U of Regina
Staphylococcus capitus ureo		
K4-1	clinical	Dr. Chris Yost, U of Regina
Staphylococcus epidermids		
K10-11	clinical	Dr. Chris Yost, U of Regina

K13-11	clinical	Dr. Chris Yost, U of Regina
Staphylococcus haemolyticus		
K9-7	clinical	Dr. Chris Yost, U of Regina
K5-1	clinical	Dr. Chris Yost, U of Regina
K4-6	clinical	Dr. Chris Yost, U of Regina
K2-2	clinical	Dr. Chris Yost, U of Regina
K2-2KC	clinical	Dr. Chris Yost, U of Regina
Streptococcus mutans		
UAIS9:wt	clinical	Heather Dietz, U of Regina

Appendix 4. Spontaneous and on point: do spontaneous mutations used for laboratory experiments cause pleiotropic effects that might confound bacterial infection and evolution assays?

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Abstract

Many selectable phenotypes in microbial systems, including antibiotic resistance, can be conferred by single point mutations. This is frequently exploited in research, where the selection and use of microbial mutants that are spontaneously resistant to antibiotics like rifampicin and streptomycin, facilitates the recovery and/or quantification of a target microbe. Such mutations are often considered to be relatively neutral as compared to wild type, with often little consideration as to the ultimate systems-level impact of these single nucleotide mutations on the physiology of the microbe. This review examines some of the known pleiotropic effects of point mutations that provide selectable resistance markers, and how these mutations may impact general physiology and growth in host and non-host environments.

Introduction

There is a long history in microbiological research of isolating spontaneous mutants conferring selectable phenotypes for use in experimental assays. Assays evaluating host-microbe interactions or microbial physiology and fitness are greatly facilitated by the use of selectable markers, since they enable the researcher to isolate and recover the specific microbe of interest. The most widely used selectable markers in microbiology are those conferring antibiotic resistance. The point mutations that lead to these selectable phenotypes usually confer antibiotic resistance by altering the structure of a drug target, which prevents or reduces the ability of the antibiotic to interfere with the function this target (Lambert 2005). Spontaneous mutants carrying these mutations are isolated for experimentation by high-density plating of the wild-type isolate on the antibiotic of interest, and are generally assumed to be physiologically analogous to the wild-type isolate under the experimental conditions being evaluated. This assumption is based largely on the fact that the mutations that confer these phenotypes occur within essential genes whose products have central and often global roles in housekeeping functions (Lambert 2005). Because these mutations do not compromise the functions of these essential processes, it is expected that such mutations should not have any global impact on cellular processes and regulation. For example, streptomycin binds the S12 protein of the 30S subunit, interfering with translation mechanisms required for protein elongation and altering accuracy (Sreevatsan *et al.,* 1996). Mutations conferring streptomycin resistance result from point mutations that alter amino acid 43 within the *rpsL* gene, or from point mutations that introduce conformational changes to a pseudoknot structure in the 16S rRNA that is linked to ribosomal protein S12 (Poggi *et*

al., 2010; Olkkola *et al.,* 2010; Finken *et al.,* 1993; Springer *et al.,* 2001). Because these mutations do not compromise cellular viability and are not lethal, translation is assumed to be largely unaffected. Similarly, the target of the antibiotic rifampicin is the beta subunit encoded by the RNA polymerase gene, *rpoB,* with high-level resistance being conferred by S522L, H526N, and S574L (Jin & Gross 1988; Aubry-Damon *et al.,* 2002), while resistance to the quinolones, which target DNA gyrase, are acquired through multiple point mutations in *gyrA* and *gyrB* genes (Yoshida *et al.,* 1990). Because in all these cases the resistance-conferring mutations do not compromise base functionality of critical housekeeping proteins, the resulting mutants are expected to be physiologically equivalent to wild type and are therefore assumed to be suitable proxies for use in experimental assays. But, is this a reasonable assumption?

The use of spontaneously resistant antibiotic mutants has been expediting the recovery of microbes and facilitating the quantification of microbial growth in a variety of experimental assays across microbiological disciplines; however, there is increasing evidence that there may be systems-level effects of point mutations, which can have pleiotropic effects that can dramatically alter the physiology and response of the mutant. This review will examine the pleiotropic effects observed in spontaneous antibiotic-resistant mutants, including how point mutations can affect key regulatory mechanisms that may critically impact experimental outcomes in both host and non-host systems. We will consider some of the effects on important cellular functions linked to these regulatory systems, including production of secondary metabolites, and the overall impact on competition and virulence.

The Impact of Resistance Mutations on Bacterial Fitness and Competitive Advantage in Non-Host Environments

A single point mutation may impose pleiotropic effects on a bacterial system, fundamentally altering bacterial response, metabolism and physiology in a general, non-host environment. Spontaneous mutants have been shown to display altered fitness and growth rate due to the inherent cost of antibiotic resistance (Andersson & Levin 1999). For instance, *E. coli* strains with various spontaneous mutations conferring tigecycline resistance exhibit reduced growth rate in Mueller-Hinton broth as compared to wild type (Linkevicius et al 2013). Mutations in eight different genes were shown to lead to tigecycline resistance, including mutations that alter regulation of the tightly regulated tigecycling efflux system, AcrAB (Linkevicius *et al.,* 2013). MarA is a positive regulator of the AcrAB efflux system, with MarA being regulated by the repressor, MarR (Linkevicius *et al.,* 2013). Mutations that impair the function of either MarR or the bacterial Lon protease, which normally functions to degrade MarR, increase the concentration of MarA in the cell, thereby increasing AcrAB production and tigecycline efflux (Linkevicius *et al.,* 2013). Other spontaneously resistant mutants included those with mutations in the heptose biosynthetic pathway, which altered lipopolysaccharide maturation and reduced porin production, thus restricting the entry of tigecycline into the cell which is presumed to enter the cell via porins such as OmpF (Linkevicius *et al.,* 2013). An assessment of comparative growth of these mutants relative to wild type showed a cost of fitness as determined by growth rate in culture media (Linkevicius et al 2013). In addition, these LPS mutants are also more susceptible to other antibiotics, unlike the wild type strain (Linkevicius *et al.,* 2013).

Studies evaluating mutations in the *rpsL* gene, which confer resistance to streptomycin, also revealed pleiotropic effects on cells (Poggi et al. 2010). *E. coli* with spontaneous single nucleotide mutations conferring streptomycin resistance were shown to have reduced growth rates relative to the wild type strain (Ruusala et al. 1984). These resistance mutations resulted in hyper-accurate ribosomes with enhanced proofreading functions in the absence of streptomycin, which reduced their efficiency, and thus reduced the overall growth rate of the mutants compared to wild type (Ruusala *et al.,* 1984). Similar results were seen in *Mycobacterium smegmatis,* with L42N and L42T mutations in the *rpsL* gene conferring resistance to streptomycin (Sander *et al.,* 2002). Using a competition assay, these mutants were shown to have a 15% reduction in growth as compared to wild type in the absence of the antibiotic (Sander *et al.,* 2002). Likewise, the streptomycin resistance mutations, A523C, C526T, and C522T in the ribosomal *rrn* gene result in a reduction in relative fitness between 6% and 9% in competition assays as compared to wild type (Sander *et al.,* 2002). One mutation in the 16S rRNA, G524C, conferred a 30% growth disadvantage as compared to the wild type in the competition assay (Sander *et al.,* 2002).

Some studies have shown that the growth of spontaneously resistant mutants varies from wild type depending on environmental conditions and nutrient availability. Streptomycin resistant mutants of *Salmonella enterica* having the changes K42N and P90S in ribosomal protein S12 (*rpsL*) exhibit reduced growth on rich media, yet grow faster than wild type on lower carbon media (Paulander *et al.,* 2009). The mechanism underlying this phenotype relates to the mutations impairing capacity to induce the alternate sigma factor, RpoS. When carbon is limited, RpoS naturally inhibits the growth

of wild type cells (Paulander *et al.,* 2009). The K42N and P90S mutants are freed of this growth inhibition imposed by RpoS, enabling them to grow faster than wild type under carbon-limited conditions *et al.,* 2009). Yet in other cases, point mutations conferring antibiotic resistance may have no effect on growth, such as the L42R mutation in the *rpsL* gene of *Mycobacterium smegmatis*, which exhibited similar growth rates to the wild type in a competition assay (Sander *et al.,* 2002).

In addition to growth rate, point mutations conferring antibiotic resistance can impact several other phenotypes, including the production of biologically active secondary metabolites. Mutations K43N and K43T in the *rpsL* gene of *Pectobacterium carotovorum* (formerly *Erwinia carotovora*) confer streptomycin resistance; however, these changes result in the loss of carbapenem production (Barnard *et al.,* 2010). The *car* genes, which direct the biosynthesis of carbapenem and whose regulation is linked directly to quorum sensing, were expressed at a reduced rate in the mutants as compared to wild type (Barnard et al 2010). Quorum sensing, sometimes called diffusion sensing, is a regulatory mechanism in which bacterial gene expression is dependent upon the environmental concentration of a bacterially-derived signalling molecule (Whitehead et al 2001; Miller & Bassler 2001). High bacterial cell densities can result in accumulation of the autoinducer, which in turn causes induction or repression of various gene pathways (Miller & Bassler 2001; Redfield 2002). In the case of *P. carotovorum*, the production of the antibiotic carbapenem as well as production of virulence factors including pectate lysases, cellullases, and proteases, all appear to be regulated by the N-(3-oxohexanoyl)-L-homoserine lactone (OHHL)-dependent quorum sensing system (McGowan *et al.,* 2005). Carbapenem production is thus activated at high culture densities (Bibb & Hesketh 2009)

(McGowan et al 1995). Carbapenem production is regulated by the *carR, carI* and *hor* genes (McGowan *et al.,* 2005; Sjöblom et al 2008). CarI produces the OHHL to activate CarR, and CarR then activates transcription of the carbapenem biosynthetic cluster (McGowan *et al.,* 2005; Sjöblom *et al.,* 2008). The product of the *hor* gene, a SlyA/MarR-like transcriptional regulator, is a global activator that is also involved in cluster regulation, although its specific roles are still unclear (McGowan *et al.,* 2005; Sjöblom *et al.,* 2008). Several streptomycin-resistant *P. carotovorum rpsL* mutants produce OHHL at wild type levels, yet do not produce carbapenem (Barnard *et al.,* 2010). It was suggested that the *rpsL* mutation reduces levels of the Hor global activator that activates the *car* biosynthetic cluster; nevertheless, exogenous addition of OHHL can trigger CarR to activate biosynthesis of carbapenem (Barnard *et al.,* 2010).

In some cases, spontaneous antibiotic resistance mutants can overproduce or underproduce a secondary metabolite relative to wild type. *Streptomyces coelicolor* produces the blue-pigmented antibiotic, actinorhodin, which was found to be effective at inhibiting a variety of Gram-positive bacteria by compromising phospholipid bilayer integrity (Hu & Ochi 2001; Lakey et al 1983). Actinorhodin production is suppressed by streptomycin resistance mutations in the ppGpp biosynthetic genes, *relA* and *relC* (Hu & Ochi 2001). The changes in actinorhodin expression appear to be linked to its regulation by the stringent response (Hu & Ochi 2001), a regulatory mechanism responsible for the regulation of an amino acid starvation response (Dalebroux et al. 2010; Hesketh et al. 2007) (Chatterji et al. 1998). During the stringent response, ppGpp, an allosteric regulator binds and disrupts the beta subunit of the RNA polymerase to disrupt transcription (Chatterji et al. 1998; Zuo et al. 2013). This, in turn, results in a reduction

in total RNA synthesis, which is generally followed by the upregulation of amino acid biosynthesis and other stress responses (Traxler et al. 2008). Thus, the streptomycin resistance mutations in the ppGpp biosynthetic genes, *relA* and *relC,* result in reduced ppGpp levels, thereby disrupting the stringent response and actinorhodin production (Hu & Ochi 2001). In contrast, rifampicin resistance mutations in the beta subunit gene of the RNA polymerase (*rpoB*), as well as streptomycin and gentamycin resistance mutations in the 30S ribosomal gene (*rpsL*) result in upregulation of actinorhodin production (Hu & Ochi 2001). It was suggested that these mutations increase production by bypassing the ppGpp binding step, or change the RNA polymerase such that it mimicks the ppGpp-bound form (Hu & Ochi 2001).

In *Streptomyces lividans*, the streptomycin resistant *rpsL* mutants, K88E and P91S, also exhibit an increase in the actinorhodin production, which was shown to be caused by enhanced translation due to increased stability of the ribosome (Okamoto-Hosoya et al 2003). Actinorhodin overproduction was found to be linked to an increase in Ribosomal Recycling Factor (RRF) (Hosaka *et al.,* 2006). RRF is a translation factor that enhances production of proteins by disassembling the 30s and 50s subunits of the ribosome, increasing protein turnaround and enabling the re-initiation of translation (Hosaka et al 2006). The increase in RRF was a result of the *rpsL* mutant upregulating the production of *ffr*, the gene that encodes the RRF; however, the mechanism of this regulatory effect is not yet understood (Hosaka *et al.,* 2006). Increased ribosome stability and translation initiation are expected to have broad impacts on cellular functions. Therefore, mutations in *rpsL* and other genes coding for ribosomal components should be evaluated for possible fitness effects that may adversely affect experimental outcomes.

The Impact of Resistance Mutations on Bacterial Fitness and Virulence in Host Environments

Virulence factors allow for bacteria to survive and multiply within a host, enabling them to cause an infection (Kirzinger & Stavrinides 2012). The pleiotropic effects of a single nucleotide mutation conferring antibiotic resistance have been shown to render mutants defective in the ability to induce certain virulence factors. *P. carotovorum* mutants that gained spontaneous resistance to streptomycin due to K43T and K43N mutations in the *rpsL* gene were shown to exhibit a significant reduction in virulence towards potato tubers (Barnard *et al.,* 2010). A proteomic analysis using 2-D difference gel electrophoresis revealed that the *rpsL* mutations resulted in the downregulation of at least 45 proteins, and upregulation of 55 proteins. The authors proposed that the reduction in virulence was related to changes in expression of the *hor* genes, which are regulators of virulence (Barnard *et al.,* 2010). One *rpsL* mutant K43R did not show any decrease in virulence compared to wild type (Barnard *et al.,* 2010), indicating that different resistance mutations in the same gene can vary in phenotype from wild type.

Similar results were obtained in studies of virulence of *Salmonella enterica* serovar Typhimurium mutants having spontaneous mutations conferring resistance to streptomycin (*rpsL*), rifampicin (*rpoB*), and nalidixic acid (*gyrA*) (Björkman *et al.,* 1998). The virulence of mutants was compared to that of wild type in an *in vivo* mouse model, wherein bacteria were injected into the host, and following incubation, the spleen harvested and bacterial titres quantified (Björkman *et al.,* 1998). Mutants showed

reduced growth in the mouse, with a doubling time as high as 58 minutes as compared to 26 minutes for wild type (Björkman et al. 1998).

A point mutation conferring antibiotic resistance can increase the fitness of a microbe in the host. In the chicken pathogen, *Campylobacter jejuni,* the *gyrA* mutation C257T conferring fluoroquinolone resistance confers increased fitness in the chicken model, with mutants not only outcompeting the wild type but also having an enhanced ability to colonize the host (Luo et al. 2005). Although the exact mechanism by which the mutation increases fitness is unknown, it does not appear to be the result of compensatory mutations (Luo et al. 2005). Likewise, a study with *Enterococcus faecium* demonstrated varying growth rate and survival of different rifampicin resistant mutants during colonization of the pig gut (Enne et al. 2004). The 12 mutants examined carried diverse mutations in the *rpoB* gene, with the majority of mutations being H489Y/Q (Enne et al. 2004). Mutants exhibited anywhere from a 2.5% increase in fitness to a 10% decrease in fitness during colonization of the pig gut as compared to the wild type, with the H489Y/Q mutation conferring increased fitness (Enne et al. 2004).

Similar fitness costs were observed by examining the impact of norfloxacin on the ability of *E. coli* to cause urinary tract infections (Komp Lindgren et al. 2005). Resistance to norfloxacin was shown to increase with mutations in *gyrA, gyrB, parC,* and *parE* (Komp Lindgren et al. 2005); however, these mutations reduced overall fitness in both *in vitro* competition assays with wild type, as well as *in vivo* competition assays in a mouse model (Komp Lindgren et al. 2005). Secondary mutations in the *gyrA, gyrB, gyrC, marOR, parC,* and *acrR* genes were also identified in mutants, which appeared to partially compensate for the reduction in fitness caused by the initial resistance mutation

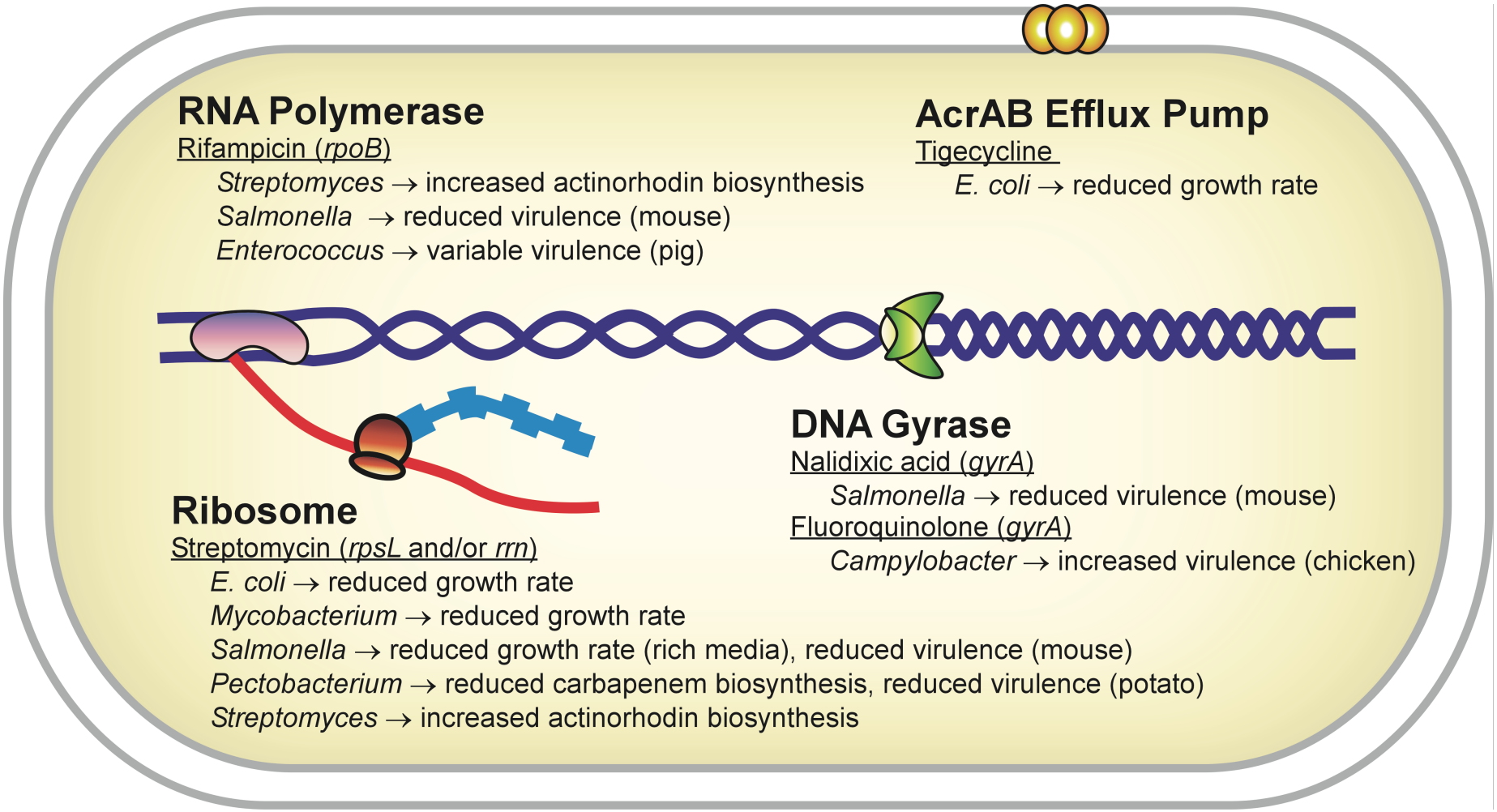

Figure 1. Point mutations conferring resistance to rifampicin, tigecycline, streptomycin, nalidixic acid, and fluoroquinolone may have pleotropic effects, affecting growth rate, virulence, and antibiotic production in bacterial mutants.

(Komp Lindgren et al. 2005). Thus in both host and non-host environments, spontaneous mutants can have increased or decreased fitness and virulence potential than the wild-type isolate, depending on the specific resistance mutation.

Implications

The pleiotropic effects of single nucleotide mutations conferring antibiotic resistance can be quite dramatic, resulting in significant phenotypic differences between mutants and wild type strains. Many laboratories use spontaneous antibiotic-resistant mutants of wild-type isolates for host assays and competition experiments, often neglecting the cellular changes that may occur with even a single point mutation. Such mutants can have a substantially divergent phenotype from wild type, including over- or under-production of secondary metabolites or virulence factors, which may directly influence microbial fitness in the specific environmental condition or niche being evaluated. Notably, because resistance to an antibiotic can arise through multiple mechanisms, phenotypes may vary depending on the gene affected and even between different mutations within that gene. Further, it is important to bear in mind that serial passage of a microbe through either *in vitro* or *in vivo* assay conditions may result in the accumulation of compensatory mutations that offset the costs of the original resistance mutation. Therefore, careful characterization of each independent mutant is recommended to establish the nature and degree of phenotypic difference, if any, between a spontaneously resistant antibiotic mutant and wild type.

There are other alternatives to using spontaneous antibiotic resistance mutants, such as the introduction of antibiotic resistance genes and fluorescent tags into wild type strains (Kreth et al. 2005), although the systems-level impact of heterologous expression

on the microbe are still largely unknown. The use of qPCR or community profiling using 16S rRNA or similar marker can also provide an alternative means for quantification, although these approaches are considerably more expensive and are often not feasible for smaller scale assays. When available, the use of selective media could be used in host or competition assays to limit the growth of other microbial species, thus eliminating the need to use selectable mutants as a proxy for wild type. Ultimately, *in vivo* and *in vitro* assays are essential to the advancement of our understanding of microbial virulence, gene regulation, and general physiology; however, it is critical that researchers carefully consider the use of any resistance mutants in their experimental designs, as these may not be appropriate proxies for the wild-type isolate.

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Conflict of Interest

All authors declare that they have no conflict of interest

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