

**CHARACTERIZATION OF A BROAD HOST RANGE TAILOCIN FROM
*BURKHOLDERIA***

A Dissertation

by

IRIS DUARTE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2012

Major Subject: Plant Pathology

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Approved by:

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ABSTRACT

Characterization of a Broad Host Range Tailocin from *Burkholderia*. (August 2012)

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Chair of Advisory Committee: Dr. Carlos F. Gonzalez

Members of the *Burkholderia cepacia* complex (Bcc) are plant and human opportunistic pathogens. Essentially all Bcc isolates demonstrate *in vitro* broad-spectrum antibiotic resistance. In fact, many clinical isolates are resistant to all currently available antibiotics, rendering therapy ineffective. There is a substantial need to develop new antimicrobial therapies. The potential use of phage-tail-like high molecular weight bacteriocins, or "tailocins", as alternative anti-bacterial agents against Bcc was investigated. A tailocin, designated Bcep0425, produced by *B.cenocepacia* strain BC0425 was determined to have broad host range activity against members of the Bcc. Targeted mutagenesis of genes involved in the biosynthesis of the bacterial lipopolysaccharide (LPS) was conducted to determine the receptor site and it was determined that L-rhamnose and α -glucose associated with the LPS core were the receptors. Genetic analysis and targeted mutagenesis of the tailocin encoding genes was conducted in the host strain, *B. cenocepacia* BC0425, to determine the genetic organization of the tailocin Bcep0425 gene cluster and to confirm gene functions. We report for the first time genes involved in replication and integration that are associated with a pyocin/tailocin gene cluster. Additionally, a new class (IV) of holin was

identified as part of the lysis cassette. Genetic analysis of the tailocin encoding genes revealed a high degree of similarity to defective phages identified in sequenced *Burkholderia* genomes. Two novel transcriptional regulators, *bctN* and *bctR*, along with *recA* were found to be involved in the induction of Bcep0425. Numerous studies have focused on the characterization of pyocins from *Pseudomonas*, but there have been no molecular investigations of tailocins from *Burkholderia*. This constitutes the first molecular characterization of a phage tail-like bacteriocin from *Burkholderia*.

DEDICATION

To my parents, Jesus and Leticia, for all their sacrifices, support, and love

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CHAPTER I

GENERAL INTRODUCTION

The *Burkholderia cepacia* complex (Bcc) consists of at least 17 phenotypically similar, but genotypically distinct species of non-fermenting, Gram-negative bacteria that are found in a diverse set of niches (21, 66, 109). These species have a high degree of 16S rDNA gene (98–100%) and *recA* (94–95 %) sequence similarity, and moderate levels of DNA–DNA hybridization (30–50%) (20). Members of the Bcc are opportunistic pathogens, capable of causing disease in plants, animals and particularly causing life-threatening respiratory infections in persons with cystic fibrosis (9, 34, 63). Strains isolated from infected cystic fibrosis (29) patients indicate that the distribution of species is quite disproportionate, with *B. cenocepacia* and *B. multivorans* accounting for 85–90% of infection in this patient population (64, 65).

Infection by members of the Bcc in CF patients, results in acute pulmonary disease and sepsis (referred to as “cepacia syndrome”) or chronic respiratory tract infection associated with an accelerated decline in lung function. The resultant altered epithelial surface liquid, in some way, predisposes the respiratory tract of affected persons to bacterial infections (25). Since the mid-1980s, the rates of Bcc infections have dramatically increased. In some centers, up to 45% of patients have become infected. Some of the reasons for this increase are the increased use of antibiotics, social and

This dissertation follows the style of Journal of Bacteriology.

behavioral changes that have allowed for close contact between patients (79), and the more accurate identification of Bcc organisms (21). Most clinically relevant strains of the Bcc have demonstrated *in vitro* broad-spectrum antibiotic resistance. Even with combined antibiotic therapy, clearance of the microorganisms is not observed. There is a substantial need to develop new strategies for antimicrobial therapy against these pathogens. The use of bacteriophage or high molecular weight (HMW) bacteriocins, as potential anti-bacterial agents against the Bcc is an alternative currently being investigated in our laboratory.

Bacteriocins are bacterial products with specific bactericidal activity, generally towards species closely related to the producer strain (27). The first bacteriocin was described in *Pseudomonas aeruginosa* by Francois Jacob (49). Jacob showed that a mutagenic agent (i.e. UV irradiation) produced an inducible compound, released by bacterial lysis, specifically adsorbing on the cell surface of susceptible bacteria and provoking their death. The term pyocin has been used to describe the bacteriocins produced by strains of *Pseudomonas*. Pyocins produced by *P. aeruginosa* are chromosomally encoded and their synthesis is induced by the SOS response (75). Cell lysis, a process similar to phage-mediated lysis, then releases the particles into the surroundings. The two types of bacteriocins released into the surroundings are either high-molecular weight (HMW) or low-molecular weight (LMW) bacteriocins.

HMW bacteriocins are thermolabile, trypsin resistant, and sedimentable by ultracentrifugation and they have been identified in many families, such as *Enterobacteriaceae*, *Vibrionaceae*, and *Pseudomonadaceae* (14, 19, 52). LMW

bacteriocins are soluble and protease sensitive (78). Electron microscopy has revealed that HMW bacteriocins have a structure resembling that of many bacteriophage tails. The most thoroughly studied phage tail-like bacteriocins are the F-type and R-type pyocins produced by *P. aeruginosa*. R-type pyocins have a contractile sheath resembling the T-even coliphage tail whereas the F-type pyocins have sheathless, flexible rod-like structures resembling the lambda phage tail (81).

R-type pyocins are rod-like structures that correspond to a double hollow cylinder, which consists of a sheath and core (47, 105, 125). The sheath is constituted by protein subunits arranged in helix symmetry, capable of contraction. The core is the same length and located inside the sheath. Pyocins also contain a baseplate at the distal end and have six tail fibers attached. These tail fibers are responsible for specificity. Pyocins bind to a cell surface receptor via their tail fibers, which is then followed by sheath contraction and insertion of their core through the cell envelope. Since pyocin genes are chromosomally located and their gene organization clearly demonstrates they possess an ancestral origin common with bacteriophages, it has been suggested that pyocins have evolutionarily specialized as phage tails, rather than being just simple defective phages (81).

Morphologically and genetically, R-type pyocins resemble tails of *Myoviridae* bacteriophages, but have no head structure and thus no nucleic acid (47, 53, 54, 98). Pyocins bind to targeted bacterial surface molecules and insert a core or needle that dissipates the bacterial membrane potential (108). This bactericidal event can result from the binding of a single pyocin particle. Their mechanism of action, high

bactericidal potency, and focused spectrum suggest that R-type pyocins can be developed as antibacterial agents (96). The term tailocin will be used to refer to R-type pyocins produced by *Burkholderia* species. Our laboratory has isolated a tailocin from *B. cenocepacia* strain BC0425, designated Bcep0425. Tailocin Bcep0425 can only be induced upon exposure of BC0425 to UV irradiation. Other mutagen agents, such as mitomycin C, are not able to trigger induction of Bcep0425. The lysis of BC0425 upon induction is associated with the production of tailocin Bcep0425. Tailocin Bcep0425 measures 140 nm in length and similar to other reported HMW bacteriocins, is resistant to proteases and is thermolabile (78).

Studies in the early 1970s showed R-type pyocins to be effective as antimicrobial agents of peritonitis in mice (37, 77, 95, 119). Unfortunately, due to the effectiveness of antibiotics at the time, there was little interest in pursuing pyocins or bacteriophages as anti-bacterial agents. With the emergence of multi-drug resistant bacteria, this avenue is now being reexamined. Currently R-type pyocins are being re-engineered to generate novel bactericidal protein complexes (95, 119). Pyocins that were once only effective against *Pseudomonas* species have now been modified to extend their adsorption range, with the alteration of the C-terminus of their tail fibers, and are effective against *Escherichia coli* and *Yersinia pestis* strains (95, 119). Although the bactericidal spectra of pyocins can now be altered, it still remains limited to specific species and even strains (119). Tailocin Bcep0425 is unique because it exhibits broad host range activity, not only against members of the Bcc, but other genera as well. The characterization of

Bcep0425 is vital to understanding its specificity and will give insight into the evolutionary relationship between pyocins/tailocins and bacteriophage.

CHAPTER II

ORGANIZATION OF THE GENE CLUSTER ENCODING FOR TAILOCIN BCEP0425

INTRODUCTION

Pyocins are produced by more than 90% of *P. aeruginosa* strains (78). Each strain may produce several pyocins, which are chromosomally encoded. The R2-type pyocin genes are located on the *P. aeruginosa* PAO1 chromosome between the *trpE* and *trpGCD* genes (101). The locus is approximately 13 kb and contains 16 open reading frames (ORFs) (101). These ORFs have been assigned to five gene clusters: 1) genes regulating gene expression; 2) genes of unknown function; 3) genes related to lysis; 4) R-type pyocin structural genes; and 5) F-type pyocin structural genes (81). Regulatory and lysis genes are generally shared by both the R-type and F-type pyocins and are transcribed as two independent transcriptional units (75). Function was assigned to pyocin genes based on significant sequence similarity to bacteriophages P2, CTX, and other P2-like phages (81). This sequence similarity, along with the identification of lysis cassettes, has led to the notion that pyocins were derived from a common ancestral origin, bacteriophage P2, and suggests that pyocins are not simple defective phages, but are phage tails that have been evolutionarily specialized as bacteriocin (81).

R-type pyocins resemble inflexible and contractile tails of bacteriophages (52). They are similar to each other in their structural and serological properties, but different

in receptor specificity. The tail fiber protein, which is responsible for binding to the receptor of a sensitive bacterial strain, has been proposed to account for the observed difference in specificity (82). R-type pyocins can be viewed as non-infectious particles, consisting of only the tail apparatus that have been adapted by the host as defensive antibacterial agents (95, 119). Since the producing bacterium is lysed in the process of releasing the pyocins, this defense system is often viewed as altruistic.

Pyocin production is induced by the SOS response, which yields about 100 to 200 particles per cell (75). The cells then lyse, by a process similar to phage-mediated lysis, to release the accumulated pyocin particles to the environment. A physical barrier consisting of the host cytoplasmic membrane, cell wall, and, in the case of Gram-negative bacteria, the outer membrane must be overcome. Pyocins are found to encode a lysis gene cassette similar to those used by lytic bacteriophage (81) .

Bacteriophages are lytic, except for filamentous phages, which extrude from their host. There are at least two general strategies for host lysis (120). Single-stranded DNA and single-stranded RNA phages of some Gram-negative hosts, accomplish lysis with a single lysis gene that does not encode muralytic enzyme activity. In this system, the lysis protein is an inhibitor of murein synthesis (8). Double-stranded DNA phages of eubacteria resort to active degradation of the peptidoglycan with a muralytic enzyme or endolysin to achieve lysis (122, 123). One of the most studied lysis systems is that of bacteriophage λ . The λ lysis cassette encodes four genes: the holin, endolysin, Rz, and Rz1 (116). The holin and endolysin are two proteins that are essential for bacteriophage host lysis. Endolysin is a generic term describing an enzyme that degrades the bacterial

peptidoglycan cell wall and that accumulate in the cytosol after induction of the lytic cycle (122). Holins are small membrane proteins that control lysis by accumulating in the membrane and causing lesions in the cytoplasmic membrane, until at a specific time that is “programmed” into the holin gene, the membrane suddenly becomes permeabilized and the endolysin escapes to the periplasm (117). A third class of proteins, the spanins (Rz and Rz1), are involved in disrupting the outer membrane (OM). Rz and Rz1 are an inner membrane protein and an OM lipoprotein, respectively, which span the entire periplasm by C-terminal interactions of their soluble domains (10).

In the study communicated in this chapter, I report on the nucleotide sequence analysis of tailocin Bcep0425 to determine the structural and genetic relationship between tailocin Bcep0425 and known pyocins or bacteriophages. This study also reports on the assignment of Bcep0425 gene functions, the location of the tailocin Bcep0425 gene cluster within the BC0425 host genome, the confirmed role of the holin, antiholin and endolysin in host lysis, and provides evidence that tailocin Bcep0425 is a defective phage.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this chapter are listed in Table 2.1. The complex medium TN broth (TNB) was used for routine maintenance of cultures (42). Solid medium, TNA, was identical except it lacked KNO₃ and was supplemented with 20 g/L agar. For soft agar

overlays, TN medium was amended with 7.5 g agar/L (TNSA). Luria-Bertani (LB) medium was used in mating experiments (11). For counter-selection, co-integrants were grown in Yeast-Tryptone (YT) broth, which consisted of 10 g tryptone and 10 g of yeast per liter of water. Sucrose was added to YT broth at a final concentration of 15% (wt/vol) and supplemented with 20 g/L agar (YTSA). *B. cenocepacia* and *Escherichia coli* strains were grown at 37°C. Antibiotics were added to the media at the following concentrations: 600 µg/ml kanamycin (Km) and 20 µg/ml tetracycline (Tc) for *B. cenocepacia*; 30 µg/ml Km for *E. coli*.

DNA manipulations. Genomic DNA was extracted using a DNeasy kit (Qiagen), small-scale plasmid preparation was accomplished using the Miniprep kit (Qiagen), and gel extraction and PCR product purification were conducted using Quiaquick gel extraction kit and Quiaquick PCR purification kit, respectively (Qiagen). All restriction endonucleases, *Taq* DNA polymerase and T4 DNA ligase were purchased from New England Biolabs and were used according to manufacturer protocols. Oligonucleotide primers were synthesized by Integrated DNA Technologies (IDT). DNA sequence analyses were conducted by the Institute of Developmental and Molecular Biology, Gene Technologies Laboratory at Texas A&M University.

SDS-PAGE gel and LC-MS/MS analysis. Tailocin preparation was analyzed for its protein components. The major proteins were separated using SDS-PAGE gel electrophoresis along with a Precision Plus protein standard (Bio-Rad) and stained with Coomassie brilliant blue R250 (60). Protein subunits were dissociated at 95°C for 5 min, in 20 µl of 50 mM Tris-HCl buffer (pH 7.5) containing 0.01 g of SDS, 1.54 mg of

Table 2.1. Bacterial strains and plasmids used in Chapter II

Strain	Relevant Characteristics	Reference or Source
<i>Burkholderia cenocepacia</i>		
BC0425	Producer of tailocin Bcep0425	J. LiPuma
J2315	ET12 epidemic lineage	Laboratory stock
PC184	Midwest epidemic lineage	J. LiPuma
BC0425Δ holin	BC0425, deleted holin gene	This study
BC0425Δ antiholin	BC0425, deleted antiholin gene	This study
BC0425Δ endolysin	BC0425, deleted endolysin gene	This study
<i>Escherichia coli</i>		
DH5αMCR	Cloning strain, F ⁻ <i>endA1 hsdR17</i> (r _k ⁻ , m _k ⁺) <i>supE44 thi-1 λ⁻ recA1</i> <i>gyrA96 relA1 deoR Δ(lacZYA-argF)</i> - U169 Φ80dlacZΔM15	Invitrogen
Plasmids		
pRK2013	Tra ⁺ Mob ⁺ ColE1 replicon, Km ^r	Laboratory stock (30)
pMo130	Suicide vector for allelic exchange in <i>Burkholderia</i> ; ColE1 <i>ori</i> , RK2 <i>oriT</i> , <i>xylE</i> , <i>sacB</i> , Km ^R	M. Voskuil (41)
pMo168	Replicative vector for <i>Burkholderia</i> ; <i>oriPBBR1</i> , <i>mob</i> ⁺ , <i>xylE</i> , Km ^R	M. Voskuil (41)
pMo130:: antiholin	pM0130 with antiholin upstream and downstream fragments	This study
pMo130:: endolysin	pM0130 with endolysin upstream and downstream fragments	This study
pMo130:: holin	pM0130 with holin upstream and downstream fragments	This study
pMo168:: anti-comp	antiholin gene cloned into pMo168	This study
pMo168:: endo-comp	endolysin gene cloned into pMo168	This study
pMo168:: holin-comp	holin gene cloned into pMo168	This study

Km^R = resistance to kanamycin. + and - = positive and negative for phenotype, respectively.

dithiothreitol, and 0.1 mg of bromophenol blue per ml. To identify the amino acid composition of the 43 kDa and 17 kDa proteins, bands were excised from a 10-20 % Tris-HCl gel, and the 100 kDa and 30 kDa bands were excised from a 7.5% gel Tris-HCl gel. The proteins were subjected to Liquid Chromatography-Mass Spectrometry (MS)/MS analysis on the Q-TOF Premier Mass Spectrometer. Protein LynX global Server and Mascot search engines were used to search the SwissProt (<http://ca.expasy.org/sprot>) and NCBI databases (<http://ncbi.nlm.nih.gov/Blast>) (1, 13).

Annotation of Bcep0425 genes. To understand the genetic organization of Bcep0425, BC0425 genomic DNA was isolated using modifications of the CTAB method (5). BC0425 DNA was sequenced using 454 genome sequencing technology (Duke University, Institute for Genome Sciences & Policy). Sequence information was assembled into contigs using the Newbler sequence assembly program (Roche, USA). Genemark (http://exon.biology.gatech.edu/gmhmm2_prok.cgi) was used to predict all possible open reading frames. To annotate contig 77, which contains the Bcep0425 genes, Genemark (http://exon.biology.gatech.edu/gmhmm2_prok.cgi) was first used to identify all possible ORFs (12). The Artemis Comparison Tool (Sanger Centre) was then used to identify the most likely start codon for each ORF, by searching for the presence of a potential ribosomal binding site (RBS) (18). Each identified ORF was then compared to the NCBI protein database using Blastp (<http://ncbi.nlm.nih.gov/Blast>) to assign putative functions (1). InterProScan (<http://www.ebi.ac.uk/Tools/webservices/services/interproscan>), LipoP (<http://www.cbs.dtu.dk/services/LipoP/>), and TMHMM

(<http://www.cbs.dtu.dk/services/TMHMM/>) were used to identify conserved domains, lipoprotein processing signals, and transmembrane domains (TMDs), respectively.

Genome maps were drawn utilizing the programs DNA Master (<http://cobamide2.bio.pitt.edu/computer.htm>).

Determining the insertion site of Bcep0425. A draft genomic sequence of *B. cenocepacia* strain BC0425 was used for this study. Primer walking was used to determine the insertion site location of Bcep0425 in the BC0425 host chromosome. Primer sets C77L (contig77-left and C77L-F2) and C77R (contig77-right and C77R-R1) (Table 2.2) were designed based on the sequence data of *B. cenocepacia* strain J2315 produced by the Pathogen Sequencing Group at the Sanger Institute, Hinxton, Cambridge and can be obtained from <http://www.sanger.ac.uk/Projects/B.cenocepacia/>. Primers were designed to extend out from either side of contig 77. Sequencing was conducted by the Institute of Developmental and Molecular Biology, Gene Technologies Laboratory at Texas A&M University. Using the sequence obtained, a search of the remaining contigs of BC0425 was conducted and contigs flanking contig77 were identified. Once both contigs were identified, the sequences were annotated using the methods described above.

Plasmid construction for allelic exchange. Deletion of the antiholin, endolysin, and holin genes of BC0425 were constructed using the system developed by Hamad et al. (41). PCR primers for regions flanking the antiholin were designed based on the annotated sequence of BC0425. To construct plasmid pMo130:: antiholin used to delete the antiholin from BC0425, a 1.0 kb fragment upstream of the antiholin gene was

amplified with primers anti-up-F and anti-up-R (Table 2.2) and cloned into multiple cloning site (MCS) 1 of plasmid pMo130, digested with *NheI* and *BglIII*, to obtain plasmid pMo130:: antiholin-up. The 1.0 kb fragment downstream of the antiholin gene was amplified using primers anti-down-F and anti-down-R (Table 2.2). The PCR product was cloned into pMo130:: anti-up, digested with *BglIII* and *HindIII*, to obtain pMo130:: antiholin.

PCR primers for regions flanking the endolysin were designed based on the annotated sequence of BC0425. To construct plasmid pMo130::endolysin used to construct a deletion of the endolysin gene, a 1.0 kb fragment upstream of the endolysin was amplified with primers endo-up-F and endo-up-R (Table 2.2) and cloned into MCS1 of plasmid pMo130, digested with *NheI* and *BglIII*, to obtain plasmid pMo130:: endolysin-up. The 1.0 kb fragment downstream of the endolysin was amplified using primers endo-down-F and endo-down-R (Table 2.2). The PCR product was cloned into pMo130:: endolysin-up, digested with *BglIII* and *HindIII*, to obtain pMo130:: endolysin.

PCR primers for regions flanking the holin were designed based on the annotated sequence of BC0425. To construct plasmid pMo130:: holin used to delete the holin gene from BC0425, a 1.0 kb fragment upstream of the holin was amplified with primers holin-up-F and holin-up-R (Table 2.2) and cloned into MCS1 of plasmid pMo130, digested with *NheI* and *BglIII*, to obtain plasmid pMo130:: holin. The 1.0 kb fragment downstream of the holin was amplified using primers holin-down-F and holin-down-R (Table 2.2). The PCR product was cloned into pMo130:: holin-up, digested with *BglIII* and *HindIII*, to obtain pMo130:: holin.

Table 2.2. Primers used in Chapter II

Primer	Relevant Characteristics	Reference
contig77-left	5'-GCTGGGAGACGATGGAAAT-3'	This study
C77L-F2	5'-TCTTCAGCAGCGTGTCGAAG-3'	This study
contig77-right	5'-CTTGTGCAACCTTGGTTTTG-3'	This study
C77R-R1	5'-AGCGCTTGTATTCGTCGATC-3'	This study
anti-up-F	5'-CTGG <u>GCTAGC</u> GTGAGCCGCACCTGCTGTT-3'	This study
anti-up-R	5'-CTCAGATCTGAGCGAATGACGATGAAAACC-3'	This study
anti-down-F	5'-CTCAGATCTCGGGAACGCCACGTCACCCT-3'	This study
anti-down-R	5'-GACAAGCTTCGCAAAATCGCCGTGCGGCAA-3'	This study
endo-up-F	5'-GAGG <u>GCTAGC</u> CGAGAGGGTTGGCTGCGACG-3'	This study
endo-up-R	5'-CTCAGATCTTCCCGCGCATGAACGGACTCGC-3'	This study
endo-down-F	5'-CTCAGATCTGGTTTTTCATCGTCATTTCGCTCCT-3'	This study
endo-down-R	5'-CACAAGCTTCATGAAGCGTAAGGCGCGCC-3'	This study
holin-up-F	5'-CTGG <u>GCTAGC</u> CGAAAGCGCGTGCGAGCTTC-3'	This study
holin-up-R	5'-GTCAGATCTTGACGTGGCGTTCCCGTTCGC-3'	This study
holin-down-F	5'-GACAGATCTGGCCATGACGGCGATTCCAG-3'	This study
holin-down-R	5'-GTGAAGCTTCCGGCGATAGCTGTGCGAGCG-3'	This study
anti-comp-F	5'-GTCTGGATCCCGCTCGATCTCCTGGCGCTG-3'	This study
anti-comp-R	5'-CGGTTCTAGACCGAGGCGGCGGGTTTTTCAT-3'	This study
endo-comp-F	5'-CAGTGGATCCTGGCGATGTTTCGTCTGCGGC-3'	This study
endo-comp-R	5'-CGGTTCTAGATTTTCGCGGCGAGTCCGTTCA-3'	This study
holin-comp-F	5'-CTGTGGATCCCCGTCTACCTGCCGCCGTTC-3'	This study
holin-comp-R	5'-CGGTTCTAGACGAACGGGAACGCCACGTCA-3'	This study

Added restriction site is underlined

PCR reactions were conducted for 30 cycles with parameters as follows: denaturation at 95°C for 1 min; annealing at 62°C, 65°C, 65°C, 60°C, 64°C, 63°C for primer sets anti-up, anti-down, endo-up, endo-down, holin-up, and holin-down, respectively; and extension at 72°C of 1 min for all reactions. All constructed plasmids were confirmed by restriction endonuclease digestion. All plasmid constructs were performed using *E. coli* DH5 α MCR as a host. Plasmid constructs were introduced by chemical transformation (92), followed by selection on LB agar amended with 30 μ g/ml Km at 30°C.

Site-directed mutagenesis. Plasmids pMo130::antiholin, pMo130::endolysin, and pMo130::holin were individually introduced into *B. cenocepacia* BC0425 by conjugation, using triparental matings with pRK2013 as the mobilizing plasmid. Donor, mobilizer, and recipient suspensions were made in LB broth from cultures grown on solid media under selective conditions, as appropriate for 18 h. Bacterial suspensions were adjusted spectrophotometrically ($A_{600}=0.5$), mixed at an equal ratio (1:1:1), and transferred to a positively charged sterile membrane layered on a 100 x 15 mm LB agar petri dish. Following an 18 h incubation period at 37°C, the cells from the mating and respective controls were washed twice in phosphate buffer (0.125 M, pH 7.1) by centrifugation (12,096 X *g* for 10 min at 5°C). The bacterial pellets were suspended in phosphate buffer and dilution plated to LB agar plates amended with 600 μ g/ml Km and 20 μ g/ml Tc for selection of single crossover events. Transconjugants were sprayed with 0.45 M pyrocatechol to identify colonies in which single crossover events had occurred. Co-integrates exhibited a yellow color because *xylE* encodes for catechol 2,3-

dioxygenase, which catalyzes the ring cleavage of pyrocatechol and converts the colorless substrate to an intensely yellow hydroxymuconic semialdehyde (41). YTSA was used to select for double-crossover events in transconjugants, since the *sacB* gene is contained in plasmid pMo130. Single yellow colonies were grown in YT broth medium for five hours and then plated to YTSA to select for resolved co-integrants (41). The colonies were once again sprayed with pyrocatechol. Presumptive resolved co-integrants exhibited a white phenotype. To confirm gene deletion, co-integrants were analyzed by PCR using multiple primer combinations, both internal and external to the target gene deletion.

Complementation experiments. Plasmids pMo168::holin-comp, pMo168::anti-comp and pMo168::endo-comp were individually introduced into strain BC0425 Δ holin, BC0425 Δ antiholin and BC0425 Δ endolysin, respectively, by conjugation using triparental matings with pRK2013 as the mobilizing plasmid. The antiholin gene was amplified using primers anti-comp-F and anti-comp-R (Table 2.2) and cloned into MCS2 of plasmid pMo168, digested with *Bam*HI and *Xba*I, to obtain plasmid pMo168::anti-comp. Triparental matings (1:1:1) with selection on LB media supplemented with 600 μ g/ml Km and 20 μ g/ml Tc, were performed as described above to mobilize pMo168::antiholin-comp into BC0425 Δ antiholin. Transconjugants were sprayed with 0.45 M pyrocatechol to identify colonies which contained the pMo168 derivative plasmid.

The endolysin gene was amplified using primers endo-comp-F and endo-comp-R (Table 2.2) and cloned into MCS2 of plasmid pMo168, digested with *Bam*HI and *Xba*I,

to obtain plasmid pMo168::endo-comp. Triparental matings with selection on LB media supplemented with 600 µg/ml Km and 20 µg/ml Tc were performed as described above to mobilize pMo168::endo-comp into BC0425 Δ endolysin. Transconjugants were sprayed with 0.45 M pyrocatechol to identify colonies which contained the pMo168 derivative plasmid.

The holin gene was amplified using primers holin-comp-F and holin-comp-R (Table 2.2) and cloned into MCS2 of plasmid pMo168, digested with *Bam*HI and *Xba*I, to obtain plasmid pMo168::holin-comp. Triparental matings with selection on LB media supplemented with 600 µg/ml Km and 20 µg/ml Tc were performed as described above to mobilize pMo168::holin-comp into BC0425 Δ holin. Transconjugants were sprayed with 0.45 M pyrocatechol to identify colonies which contained the pMo168 derivative plasmid.

PCR reactions were conducted for 30 cycles and parameters as follows: denaturation at 95°C for 1 min; annealing at 65°C, 63°C, and 64°C for primer sets anti-comp, endo-comp, and holin-comp, respectively; and extension at 72°C of 1 min for each reaction. All constructed plasmids were confirmed by restriction endonuclease digestion. All plasmid construction was performed using *E. coli* DH5 α MCR as a host. Plasmid constructs were introduced by chemical transformation as described by Sambrook et al. (92), followed by selection on LB agar amended with 30 µg/ml Km at 30°C.

Growth curve experiments. Deletion mutants BC0425 Δ antiholin, BC0425 Δ holin, BC0425 Δ endolysin and BC0425- wild-type (WT) were UV induced and their

growth followed by recording optical densities (OD) to observe for lysis. Suspensions were made in 20 ml TN broth from cultures grown on TNA under selective conditions, as appropriate for 18 h at 37°C. Bacterial suspensions were adjusted spectrophotometrically ($A_{600}= 0.08-0.1$) and shaken at 170-200 rpm at 37 °C. Once cultures attained an $A_{600}= 0.5$, cultures were centrifuged at 7741 X g for 10 min at 4 °C and the pellet was resuspended in 10 ml of 0.85% NaCl. Centrifugation was repeated and pellets were again resuspended in 10 ml of 0.85% NaCl. The 10 ml suspensions were then aliquoted to individual sterile petri plates and UV irradiated ($400\mu\text{w}/\text{cm}^2/\text{sec}$) for 7 sec with constant shaking of the plates. UV irradiated cells were transferred to 500 ml Erlenmeyer flasks with 10 ml 2X TN broth and 30 ml TN broth. Cultures were then grown at 37 ° C, with shaking (200 rpm) and the A_{600} recorded at 30 min intervals.

Dinitrophenol triggering. Deletion mutants, BC0425 Δ antiholin, BC0425 Δ holin, BC0425 Δ endolysin, and BC0425-WT were UV induced as described above. Suspensions were made in 20 ml TN broth from cultures grown on solid media under selective conditions, as appropriate for 18 h at 37°C. Bacterial suspensions were adjusted spectrophotometrically ($A_{600}= 0.08-0.1$) and shaken at 170-200 rpm at 37 °C until the cultures reached an $A_{600}= 0.5$. The cultures were centrifuged at 7741 X g for 10 min at 4 °C and the pellet was resuspended in 10 ml of 0.85% NaCl. Centrifugation was repeated and pellets were resuspended in 10 ml of 0.85% NaCl. The 10 ml suspensions were then aliquoted to individual petri plates and UV irradiated ($400\mu\text{w}/\text{cm}^2/\text{sec}$) for 7 sec. UV irradiated cells were combined in a 500 ml flask with 10 ml 2X TN broth and 30 ml TN broth, and grown with shaking (200 rpm) at 37 ° C. At 2 h post UV

induction, the protonophore uncoupler dinitrophenol (DNP) was added to the cultures at a final concentration of 10 mM and the culture was monitored by optical density.

Tailocin activity quantification. Deletion mutants, BC0425 Δ antiholin, BC0425 Δ holin, BC0425 Δ endolysin, and BC0425-WT were UV induced as described above. Suspensions were made in 20 ml TN broth from cultures grown on solid media under selective conditions, as appropriate for 18 h. Bacterial suspensions were adjusted spectrophotometrically ($A_{600} = 0.08-0.1$) and shaken at 170-200 rpm at 37 °C. Once cultures had grown to an $A_{600} = 0.5$, they were centrifuged at 7741 X g for 10 min at 4 °C and the pellet was resuspended in 10 ml of 0.85% NaCl. Centrifugation was repeated and pellets were resuspended in 10 ml of 0.85% NaCl. The 10 ml suspensions were then aliquoted to individual petri plates and UV irradiated ($400\mu\text{w}/\text{cm}^2/\text{sec}$) for 7 sec. UV irradiated cells were combined in a 500 ml flask with 10 ml 2X TN broth and 30 ml TN broth. Cultures were incubated with shaking (200 rpm) at 37°C. Two 50 μl samples were taken from each culture at 1, 1.5, 1.75, 2, 2.25, 2.5, and 3 h post UV irradiation. Samples were either immediately filter sterilized or 50 μl chloroform was immediately added. Chloroformed samples were vortexed and then centrifuged. The supernatant or filtrate was analyzed for tailocin activity using the overlay spot assay as described below. Samples that were filter sterilized represented total amount of extracellular tailocin activity. Intracellular tailocin activity was calculated by subtracting the tailocin activity of filter sterilized samples from tailocin activity of chloroformed samples.

Overlay spot assay. The overlay spot assay was used to assay for tailocin activity. A suspension of *B. cenocepacia* strain PC184 was made in TN broth from

cultures grown on TNA for 18 h at 37°C. The bacterial suspension was adjusted spectrophotometrically to $A_{600} = 0.3$. Molten TNSA was tempered to 55°C and 5 ml dispensed into 13 x 100 mm sterile plastic tubes (Falcon). 100 μ l of the bacterial suspension was then added to 5 ml TNSA, vortexed and overlayed onto a TNA plate. Plates were allowed to harden before being used. Serial dilutions (1:10) of preparations were spotted (10 μ l) on the overlayed plates in duplicate. Zones of inhibition were indicative of tailocin activity.

Quantitative tailocin assay. Quantitative tailocin assays were performed by counting surviving bacteria, using a method slightly modified from that described by Kageyama et al. (53) and modified by Scholl and Martin (96). In a typical assay, fivefold serial dilutions of tailocin in P-buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 8 mM $MgSO_4$) was incubated in a microtiter well with a known quantity of target bacteria in P-buffer, for example, 5×10^8 CFU/ml, for 40 min at 37°C. The samples were then serially diluted and 100 μ l aliquots spotted on TN agar plates to count surviving bacteria. Killing units were calculated based on calculations proposed by Scholl and Martin (96). A bactericidal event is the death of a bacterium from contact with a tailocin particle(s). In order to accurately count tailocin particles, one must consider the fact that multiple particles may, by chance, attach to a single cell and that some cells may not be contacted by a tailocin at all at a given tailocin-to-cell ratio. The number of bactericidal events is related to the fraction of bacterial survivors in a Poisson distribution, $m = -\ln(S)$, where m is the average number of bactericidal events per bacterial cell, and S is the fraction of survivors. The total number of bactericidal events per ml equals m times the number of

bacterial cells per ml. The microtiter well that has an average of 1 tailocin particle per bacterium will yield at or near equilibrium 37% survivors, and the well with an average of 2.3 tailocin particles per bacterium will yield 10% survivors. It was within this survival range that the m values were calculated (96).

Calculation of efficiency of killing. *Burkholderia cenocepacia* strains BC0425 and BC0425 Δ bctN were UV induced as described above. Tailocin lysates, along with an ovalbumin standard, were run on a 10-20% Tris-Tricine SDS gel (BioRad) at 80 volts for 4 h. The gel was stained with Coomassie blue and destained as described by the manufacturer (BioRad). The gel was then scanned and the intensity of the ovalbumin standard bands was analyzed using the BioRad Quantity One program. A standard curve was plotted as nanograms vs. intensity. The intensity of the sheath protein was compared to the standard curve to calculate the total amount of sheath protein loaded on the gel in nanograms. Since the molecular weight of the sheath protein was known (43kDa), the total number of molecules that were loaded on the gel could be calculated. The amount of sheath molecules in one tailocin particle was then estimated based on the length and number of sheath proteins contained in bacteriophage P2. Bacteriophage P2 has a tail that measures 135 nm and contains 200 sheath proteins/phage tail (62). Bcep0425 was determined to be 140 nm in length, which calculated to ~ 207 sheath molecules/tail. The total number of tailocins/ml was then calculated to be equal to the total # of sheath molecules/ 207 sheath proteins/tail. The efficiency of killing (EOK) was then calculated by taking the total number of killing events (described above)/ total number of tailocins/ml.

Transmission Electron Microscopy (TEM). TEM was performed using high titer UV induced lysates spotted onto 400 mesh carbon-coated copper grids and negatively stained with 2% (wt/vol) uranyl acetate. The samples were visualized with a JEOL 1200EX at an accelerated voltage of 100 kV.

RESULTS

Mass spectrometry of tailocin proteins. To identify tailocin Bcep0425, a 20 X 20 test matrix of *B. cenocepacia* clinical isolates, in which all isolates served as producers and indicators, was used to determine production of bacteriocins. Evaluation of strains grown on a solid medium matrix, followed by chloroform treatment, and overlayed with each indicator strain revealed that *B. cenocepacia* strain BC0425 produced a small zone of inhibition when evaluated as a producer. The observation of a small zone indicated the potential production of a tailocin by strain BC0425. The presence of a tailocin in UV induced lysates was confirmed by Guichun Yao by UV induction studies and examination of concentrated lysates by electron microscopy. The electron microscopy studies showed the presence of a tailocin (Fig. 2.1)

A purified tailocin preparation was analyzed for its protein components. The molecular weights of the Bcep0425 major protein subunits were approximated to be 17 kDa, 30 kDa, 43 kDa and 100 kDa (Fig. 2.2). The major protein subunits were excised from gels and subjected to Liquid Chromatography- MS/MS. Analysis of peptide sequences of tailocin Bcep0425 determined that the 17 kDa protein had highest homology to a major tail tube phage protein. The peptide sequence for the 43 kDa

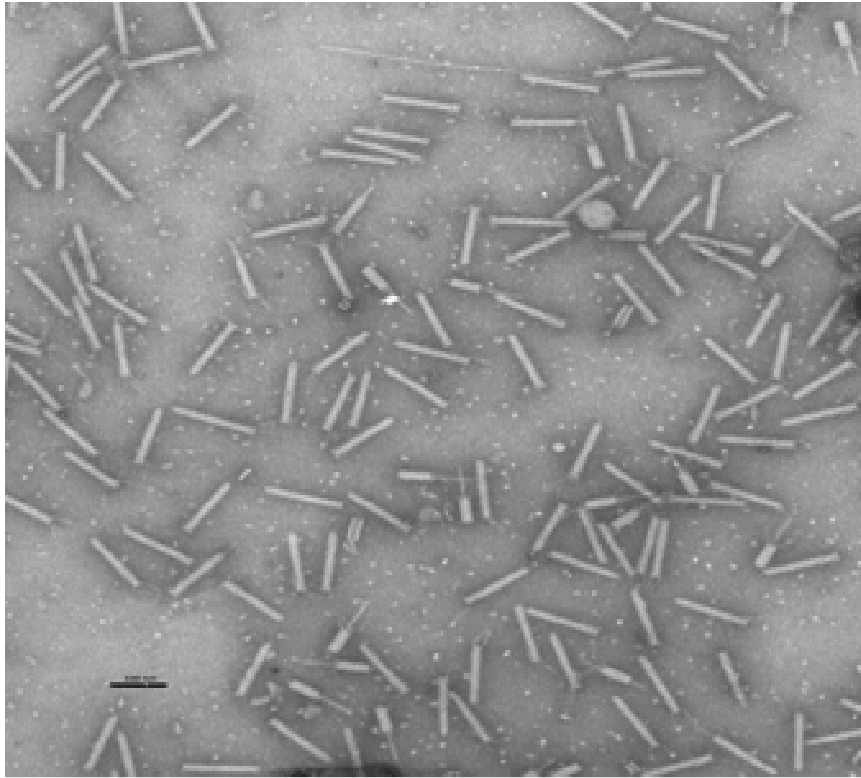


Fig. 2.1. Electron micrograph of Bcep0425 tailocin following UV induction.

Samples were stained with 2% (wt/vol) aqueous uranyl acetate. Bar =100 nm

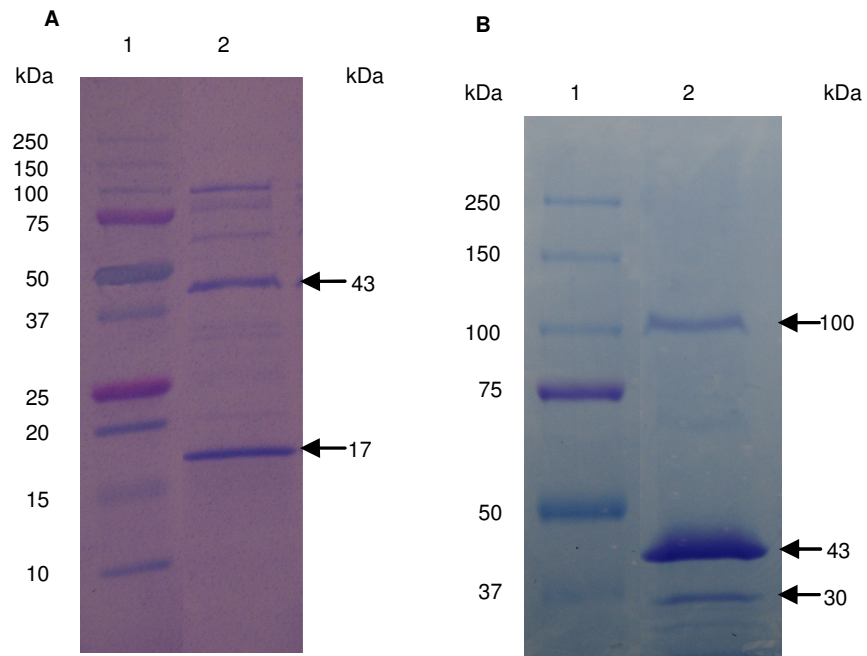


FIG. 2.2. Molecular weights of Bcep0425 major proteins. Protein subunits of tailocin Bcep0425 were dissociated as described in Materials and Methods and loaded on (A) 10-20% Tris-HCl SDS gel and (B) 7.5% Tris-HCl SDS gel. Proteins were visualized using Coomassie blue staining.

protein had significant homology to a putative phage major tail sheath protein, the 30 kDa protein to a tail fiber assembly protein and the 100 kDa protein to a tail fiber protein (Fig. 2.3). All four proteins revealed striking homology to putative phage proteins encoded by *B. cenocepacia* J2315 chromosome I. A 23 kb region on chromosome 1 of *B. cenocepacia* J2315, spanning position 100,400 to 124,000 (BCAL0081 to BCAL0107), encodes for 27 phage related proteins. The proteins in this region consist mainly of tail proteins and do not consist of proteins related to capsid formation or DNA metabolism, indicating that the region may code for a tailocin. This is consistent with the genetic organization of pyocins from *Pseudomonas*.

Overview of the tailocin Bcep0425 gene cluster. A total of 537 contigs were predicted from the assembled BC0425 sequence data. Known protein sequences, obtained from the MS data of Bcep0425 (Fig. 2.3), were used to search and identify the potential contigs containing the tailocin genes. A 23 kb contig (contig 77) was determined to contain the Bcep0425 sequence. Blast analysis of contig 77 revealed that the tailocin sequence had 99% nucleotide sequence identity to an identified putative tailocin found on chromosome I encoded by *B. cenocepacia* strain J2315 (BCAL0081 to BCAL0107). Interestingly, the J2315 putative tailocin contained a 1.9 kb insertion, identified as a Group II intron, between genes showing homology to Bcep0425 *gene8* and *gene9*.

The identified Bcep0425 gene cluster had an average GC content of 69%, which was slightly higher than the 66% GC content of *B. cenocepacia* strain J2315 and the 67% GC content of BC0425. A total of 29 protein coding genes were predicted to be

A.		
Bcep0425 (17kD)	35	MEDYQGGMSGPIKVDFGQEGIQLE 59
<i>B. cenocepacia</i> (J2315) phage major tail tube	35	MEDYQGGMSGPIKVDFGQEGIQLE 59
<i>B. ubonensis</i> (Bu) phage major tail tube	33	MEDYQGGMSGPIKVDFGQEGIQLE 57
B.		
Bcep0425 (43kD)	36	ADADASAFPLNTPVLLTNVVAALGK 60
<i>B. pseudomallei</i> (13177) phage major tail sheath	36	ADADASAFPLNTPVLLTNVVAALGK 60
<i>B. cenocepacia</i> (J2315) phage major tail sheath	36	SDADATAFPLDTPVLLTNVVAALGK 60
Enterobacteria phage P2 major tail sheath	35	SDADAETFP LNKPVLITNVQSAISK 59
C.		
Bcep0425 (30kD)	36	TLDAPPARTPTTWPFYRNDVWTLKP 60
<i>B. cenocepacia</i> (J2315) hypothetical protein	36	TLDAPPARTPTTWPFYRNDVWTLKP 60
<i>B. pseudomallei</i> (13177) tail fiber assembly	36	TLDAPPARTPTTWPFYR DGAWFLLP 60
D.		
Bcep0425 (100kD)	56	SFGGANVAPDTIHTTLK 72
<i>B. cenocepacia</i> (J2315) phage tail protein	56	SFGGANVAPDTIHTTLK 72
<i>B. thailandensis</i> (E264) phage-related tail fiber	56	TFGGENVAPDTVHVVIQ 72

FIG 2.3. Amino acid sequence alignment of Bcep0425 proteins. Sequence alignment of the (A) 17 kD Bcep0425 subunit (B) 43 kD Bcep0425 subunit (C) 30 kD Bcep0425 subunit and (D) 100 kD Bcep0425 subunit aligned with putative phage proteins. Amino acid residues that are identical are shaded.

encoded by the Bcep0425 gene cluster (Fig. 2.4; Table 2.3). Bcep0425 genes were organized into two transcriptional units with all genes, except *gene8* and *gene9*, transcribed from the reverse strand (Fig. 2.4). The Bcep0425 gene cluster was divided into four functional clusters, beginning from the left end (negative strand): replication regulation, tail/tail fiber morphogenesis, and host lysis (Fig. 2.4). The order of these functional clusters and the arrangement of genes within the clusters was the same as the putative tailocin found in strain J2315 (BCAL0081 to BCAL0107).

Functional assignments for predicted Bcep0425-encoded proteins. (i)

Integration and replication. Genes responsible for phage integration are among the predicted genes expected to be found within temperate phage genomes. Integration occurs by site-specific recombination mediated by the phage-encoded integrase (38). Pyocins are often regarded as phage tails that became evolutionarily specialized as bacteriocins, rather than as simple defective phages (81). Genes for head formation, replication, or integration have not been associated with pyocin gene clusters in *P. aeruginosa* (81). *Gene1* of tailocin Bcep0425 is predicted to encode an integrase (Table 2.3). gp1 has a conserved C-terminal integrase domain and significant sequence similarity to the integrase from phage P22. Thirteen Bcep0425 gp1 homologues exhibiting over 70% identity in sequenced *Burkholderia* genomes were identified. To our knowledge, this is the first report of a phage integrase to be associated with a high-molecular weight bacteriocin gene cluster, providing direct evidence that tailocin Bcep0425 is a defective phage.

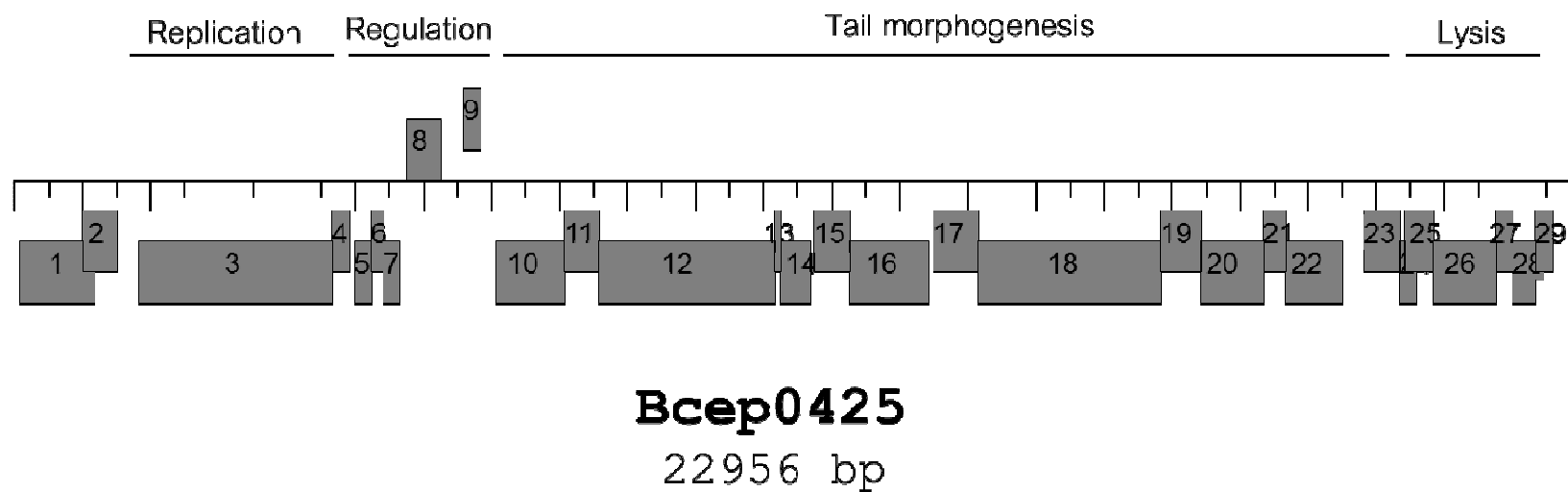


FIG. 2.4. Bcep0425 tailocin gene cluster. Map of tailocin Bcep0425 gene cluster with genes drawn to scale on forward and reverse strands. The Bcep0425 gene cluster is divided into four functional clusters: replication, regulation, tail morphogenesis and lysis. The assigned function of each indicated gene is listed in Table 2.

Table 2.3. Bcep0425 putative genes and homologues

GP	Annotation	Start	End	Size (AA)	Code. Strd.	Representative Homologue (Query genome, accession gene)	E-value
1	Integrase	69	1145	358	-	<i>Burkholderia cenocepacia</i> J2315, gil206558485	0
2	Conserved Hypothetical	1012	1462	149	-	<i>Burkholderia cenocepacia</i> J2315, gil206558486	7.57E-54
3	Zinc finger CHC2-family protein	1840	4362	930	-	<i>Burkholderia ubonensis</i> Bu, gil167589039	0
4	Conserved Hypothetical	4638	4895	85	-	<i>Burkholderia cenocepacia</i> J2315, gil206558488	7.74E-37
5	Phage transcriptional activator protein	5023	5271	82	-	<i>Burkholderia cenocepacia</i> J2315, gil206558489	3.44E-39
6	Conserved Hypothetical	5258	5438	59	-	<i>Burkholderia cenocepacia</i> J2315, gil206558490	1.10E-24
7	Conserved Hypothetical	5453	5668	71	-	<i>Burkholderia cenocepacia</i> J2315, gil206558491	5.02E-31
8	Putative BctR	5759	6250	163	+	<i>Burkholderia glumae</i> BGR1, gil238025796	1.61E-35
9	Putative BctN	6568	6864	98	+	<i>Burkholderia cenocepacia</i> J2315, gil206558494	7.21E-48
10	Phage late control gene D protein	7052	8122	356	-	<i>Burkholderia cenocepacia</i> J2315, gil206558495	0
11	Tail Assembly	8119	8550	143	-	<i>Burkholderia cenocepacia</i> J2315, gil206558496	7.65E-77
12	Tape Measure	8573	11143	856	-	<i>Burkholderia cenocepacia</i> J2315, gil206558497	0
13	Tail associated protein	11159	11272	37	-	<i>Burkholderia cenocepacia</i> J2315, gil206558498	4.10E-12
14	Tail associated protein	11281	11652	123	-	<i>Burkholderia cenocepacia</i> J2315, gil206558499	9.08E-61
15	Tail Tube/Core	11728	12237	169	-	<i>Burkholderia cenocepacia</i> J2315, gil206558500	7.30E-94
16	Tail Sheath	12266	13438	390	-	<i>Burkholderia cenocepacia</i> J2315, gil206558501	0
17	Tail Fiber Assembly	13493	14191	232	-	<i>Burkholderia ubonensis</i> Bu, gil167589026	2.23E-96
18	Tail Fiber	14207	16858	883	-	<i>Burkholderia cenocepacia</i> J2315, gil206558503	0
19	Tail associated protein	16865	17407	180	-	<i>Burkholderia cenocepacia</i> J2315, gil167589024	2.76E-87
20	Baseplate	17400	18314	304	-	<i>Burkholderia cenocepacia</i> J2315, gil206558505	7.83E-169
21	Baseplate Assembly	18670	19479	269	-	<i>Burkholderia cenocepacia</i> J2315, gil206558506	1.41E-61
22	Baseplate Assembly	18311	18673	120	-	<i>Burkholderia cenocepacia</i> J2315, gil206558507	4.89E-151

Table 2.3- Continued

23	Tail Completion	19865	20314	149	-	<i>Burkholderia cenocepacia</i> J2315, gil206558508	1.03E-77
24	Rz1	20310	20594	95	-	<i>Burkholderia</i> phage phi52237, gil53722100	6.92E-17
25	Rz	20431	20871	146	-	<i>Burkholderia cenocepacia</i> J2315, gil206558509	1.09E-73
26	Endolysin	20868	21725	285	-	<i>Burkholderia cenocepacia</i> J2315, gil206558510	8.97E-158
27	Antiholin	21722	21988	88	-	<i>Burkholderia cenocepacia</i> J2315, gil206558511	2.21E-40
28	Holin	21990	22334	114	-	<i>Burkholderia cenocepacia</i> J2315, gil206558512	2.78E-54
29	Tail Protein X	22351	22557	68	-	<i>Burkholderia ubonensis</i> Bu, gil167589010	5.07E-27

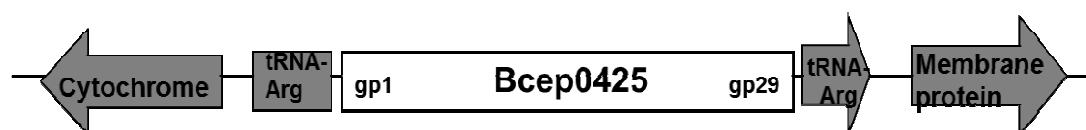
Abbreviations: GP, gene product; Code. Strd., coding strand; AA, amino acid

The Bcep0425 tailocin cassette was found to have integrated into a tRNA-Arg gene within the host chromosome (Fig. 2.5A). Nucleotide sequence analysis for *attP*, *attB*, *attL* or *attR* revealed a 31 bp core sequence (Fig. 2.5B). In *attB* this 31 bp sequence consists of the 3' end of a putative tRNA-Arg(AGG) gene. Phage DNA integration restores the putative tRNA-Arg(AGG) gene in *attL*. Phages often integrate into essential genes without inactivating them by repeating the 3' end of the gene within the phage *attP* site with very few changes, so that when the phage integrates, the normal 3' end of the gene is replaced by the very similar phage-encoded sequence (110).

gene3 was predicted to encode a zinc finger CHC2-family protein (Table 2.3). This group of proteins is recognized to bind DNA, RNA, protein and/or lipid substrates (32, 39). Gp3 had a conserved topoisomerase-primase (TOPRIM) domain that has been reported in the active site regions of bacterial DnaG-type primases and their homologs (4). Primases synthesize RNA primers for the initiation of DNA replication.

(ii) Regulation. *gene5* encoded a phage transcriptional activator protein (Table 2.3). Gp5 contained a conserved Ogr/Delta domain similar to that of phage P2. P2 phage Ogr proteins possess one domain but function as dimers, whereas the P4 phage delta protein contains two such domains attached covalently (40). The Ogr protein belongs to a class of zinc-binding transcriptional activator proteins (61) and in phage P2 the Ogr protein functions as a positive regulator of transcription from the P2 late promoters (36).

A.



B.

```

attB  ...cgaagtgacag-gtgGAGGCTTCCGTCCCCAACGACCcctacgcgagcgcta...
attP  ...gtgcgcgtctat-tttGAGGCTTCCGTCCCCAACGACCtcggcccgcgcggt-...
attL  ...cgaagtgacag-gtgGAGGCTTCCGTCCCCAACGACCtcggcccgcgcggt-...
attR  ...gtgcgcgtctat-tttGAGGCTTCCGTCCCCAACGACCcctacgcgagcgcta...

```

Fig. 2.5. Site specific integration of tailocin Bcep0425 in *B.cenocepacia* BC0425 genome. (A) The tailocin Bcep0425 gene cluster was found to be located in the middle of a tRNA-Arg gene. The diagram is not drawn to scale. (B) DNA sequences within *attB*, *attP*, *attL* and *attR*. The 31 bp core sequences, where recombination occurs, are shown in bold. Short segments of sequence surrounding core sequence are shown in lower case letters. Bacterial sequences are in blue and phage sequences are in red.

Gp8 was identified as a lambda cI-like repressor based on the presence of a helix-turn-helix domain. DNA-binding domains are characteristic of repressors such as Cro and CI (2, 84).

(iii) Tail morphogenesis. The tail genes (gp11 to gp23) of Bcep0425 possess protein and gene order similarity to tail genes of bacteriophage P2 (Fig. 2.6). The tail homologues from phage P2 ranged from 49% to 77% in similarity at the amino acid level. Eleven Bcep0425 tail encoded proteins could confidently be correlated to P2 equivalents. The tape measure (gene12), tail fiber assembly (gene17) and tail fiber (gene18) genes were the only genes that did not show significant homology to P2, but were identified based on homology to putative phage tail structural genes identified in *Burkholderia* sequences.

A large body of experimental results has shown that the host range of tailed phages/pyocins is determined by the tail fibers (38, 119). During adsorption of the virion to the host, the tail fiber recognizes and binds reversibly to a receptor on the surface of the bacterial cell. Since the tail fibers determine specificity, it is common to find significant differences among the tail fibers of phage/pyocins. The amino acid sequences, of the R-type pyocin tail fiber assembly (TFA) proteins, differ considerably in the C-terminal region (119). A comparison of the Bcep0425 TFA amino acid sequence with that of a putative tailocin, found on chromosome 1 of *B. cenocepacia* strain J2315, (BCAL0096) revealed large differences within the C-terminal region of the TFA, as might be expected (Fig.2.7). Electron microscopy studies of UV induced J2315

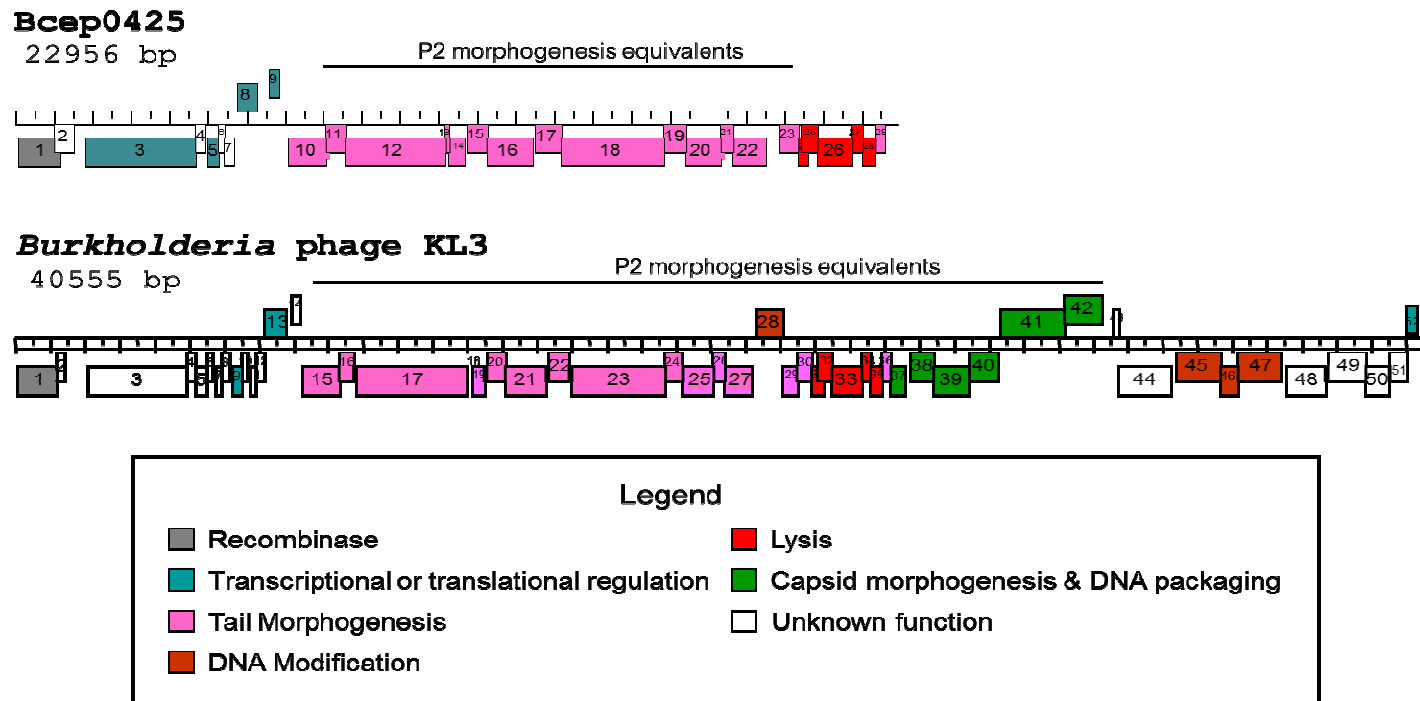


Fig. 2.6. Genome maps of tailocin Bcep0425 and *Burkholderia* phage KL3 prophage elements. Genome maps of Bcep0425 and KL3 with genes drawn to scale on forward and reverse strands are shown. Gene organization of Bcep0425 and phage KL3 are nearly identical. Bcep0425 does not contain genes involved in capsid morphogenesis and DNA modification. Homologues of genes indicated in pink are present in phage P2.

<i>Bcep0425/1-232</i>	1	MLIHHSYSTGEYLS	SSQPDADPRNPERWLIPASATLDAPPARTPTTWPF	50
<i>J2315/1-244</i>	1	MLIHHSYSTGEYLS	SSQPDADPRNPERWLIPASATLDAPPARTPTTWPF	50
<i>KL3/1-207</i>	1	MLIHHSYSTGEYLT	SSQPDADPRNDGRWLIPSSATIDAPPARTPTTWPF	50
<i>Bcep0425/1-232</i>	51	YRNDVWTL	LLPDFRGRICYRTDTGEPVEIATAGKTPDELGLTTEPRPSRH	100
<i>J2315/1-244</i>	51	YRNDVWTL	LLPDFRGRICYRTDTGEPVEIATAGKTPDELGLTTEPRPSRH	100
<i>KL3/1-207</i>	51	YRDGAWF	LLPDFRGRVCYRTDTGEPVEIAIAGKTPDDLELTTEPRPSRH	100
<i>Bcep0425/1-232</i>	101	AWLDGAWTVPEL	LEREKRDAAAEFEQRLETARKANAGKADAYAAGLLD	150
<i>J2315/1-244</i>	101	AWLDGAWTVPEL	LEREKRDAAAEFEQRLETARKANAGKADAYAAGLLD	150
<i>KL3/1-207</i>	101	AWIDDAWTVPEL	IAREKRDAAAAEFERLMEIARRANAGKADAYAAGLLD	150
<i>Bcep0425/1-232</i>	151	DEGVYYFKAWSAYQMALVRV	IQADTFDAIAWPATPAPYVPPPTPVPPA	200
<i>J2315/1-244</i>	151	DEGVYYFKAWSAYQMALVRV	IQADTFDAIAWPATPAPYVPPPTPVPPA	200
<i>KL3/1-207</i>	151	DEGTYYFKAWSAYQMALVSA	INADTFPAAVAWPATPAPYV.....A	191
<i>Bcep0425/1-232</i>	201	PSEP.....	IEPPAPTEPSSPPADPATPAVPVADSAA	232
<i>J2315/1-244</i>	201	PSEPSEPSEPSEPIEP	IEPPAPTEPSSPPADPATPAVPVADSAA	244
<i>KL3/1-207</i>	192	PAEP.....	IEPQNP.....APVAST..	207

FIG. 2.7. Alignment of the amino acid sequence of Bcep0425 tail fiber assembly (TFA) with the TFAs of tailocin J2315 and phage KL3. The TFA gene product amino acid sequence deduced from the nucleotide sequence is aligned with those of tailocin J2315 and phage KL3. Amino acid residues that are identical are shaded. Gaps are shown with dashes.

supernatants revealed the presence of a tailocin; however, the activity was not confirmed due to lack of a sensitive host (Fig. 2.8). Additionally, the BCAL0081 to BCAL107 sequence was not confirmed to encode for the detected tailocin. The TFA of Bcep0425 showed 73% homology to the annotated sequence of the TFA of the recently identified temperate phage KL3, isolated from *B. cenocepacia* strain CEP511 (71). Phage KL3 was reported to only have one sensitive host. In a three-way alignment of the amino acid sequence of the TFA proteins of tailocin Bcep0425, putative tailocin J2315, and phage KL3, the largest divergence was observed at the C-terminal sequence.

A further comparison of tailocin Bcep0425 to phage KL3 showed that they had identical gene organization in genes involved in transcription/translation, regulation, tail morphogenesis and lysis (encoded from left to right on the negative strand) (Fig. 2.6). However, tailocin Bcep0425 as compared to phage KL3, lacked genes involved in capsid formation and DNA modification (Fig. 2.6). Greater than 60% identity was observed in the 29 identified Bcep0425 genes with that of KL3, except for *gene1* (putative integrase), *gene2* (hypothetical protein) and *gene6* (hypothetical protein), which had 6, 3, and 12 %, identity, respectively.

(iv) Host cell lysis. The Bcep0425 lysis cassette consisted of *gene24*, *gene25*, *gene26*, *gene27*, and *gene28* and is organized similarly to the lambda lysis cassette with *SRRzRzI* equivalents. Only the endolysin is usually identifiable by a homology search. The endolysin, *gene26*, was identified based on the presence of a peptidoglycan binding domain (PFAM01471). This domain is found at the N or C terminus of a variety of enzymes involved in bacterial cell wall degradation (31, 59).

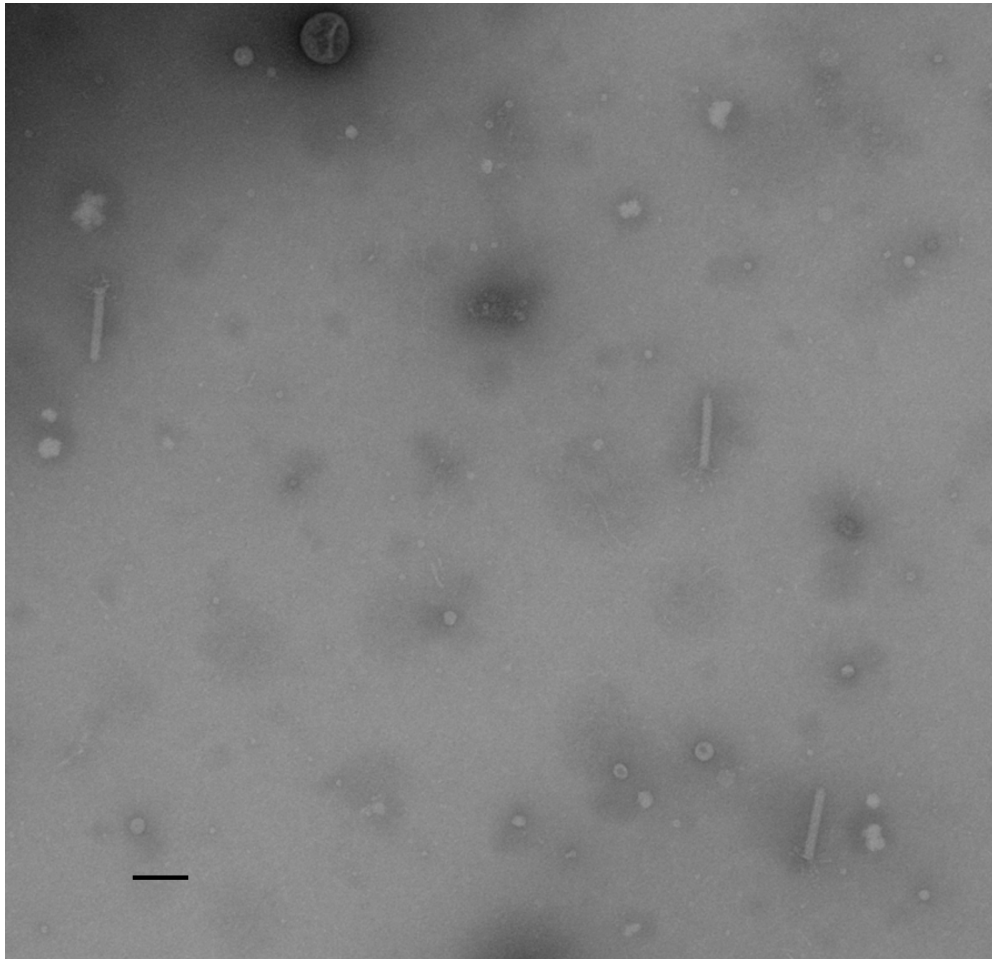


Fig. 2.8. Electron micrograph of concentrated filtered lysate of UV induced *B. cenocepacia* strain J2315. The putative tailocin particles produced by J2315 are shown in micrograph. Sample was stained with 2% (wt/vol) aqueous uranyl acetate. Bar = 100 nm

Bcep0425 *gene27* and *gene28* encoded the antiholin and holin, respectively. The holin and antiholin are two entirely separate genes, which is characteristic of the P2 lysis cassette (127). Holins are a very diverse group of proteins that contain at least one transmembrane domain (TMD). To date, three different types of canonical holins have been identified: class I (3 TMDs), class II (2 TMDs), and class III (1 TMD) (121). The Bcep0425 holin contained 4 TMDs, with a N-in and C-in topology (Fig. 2.9), which was designated as a Class IV holin. The Bcep0425 holin spanned 114 residues and contained a hydrophilic, cytoplasmic C-terminal domain.

Antiholins are specific inhibitors of holins and like holins, are very diverse (121). The only characteristic of antiholins is they always contain at least one TMD. The antiholin of Bcep0425 contained two TMDs with an N-out and C-out topology (Fig. 2.9). The presence of an aspartic acid residue at position 63 interrupted a third TMD.

The spanin complex, which consists of the Rz and Rz1, span the periplasm during lysis. The proteins are thought to undergo a conformational change and cause disruption of the outer membrane (OM) (56, 124, 126). The spanin components, *gene24* (*Rz1*) and *gene25* (*Rz*), were identified by their characteristic gene architecture. The *Rz1* encodes an OM lipoprotein, with a signal-peptidase II cleavage site, embedded out of frame within *Rz* (Fig. 2.10). Like other Rz proteins, the Bcep0425 *Rz* had an N-terminal TMD and a C-terminal periplasmic domain that was exceptionally rich in acidic and basic residues (Fig. 2.10). However, the Bcep0425 *Rz1* was not completely embedded within the *Rz*, like the lambda spannin genes (126). The *Rz1* of Bcep0425 overlapped *Rz*, which is characteristic of the phage P2 spanin genes (104). The *Rz* and *Rz1* of

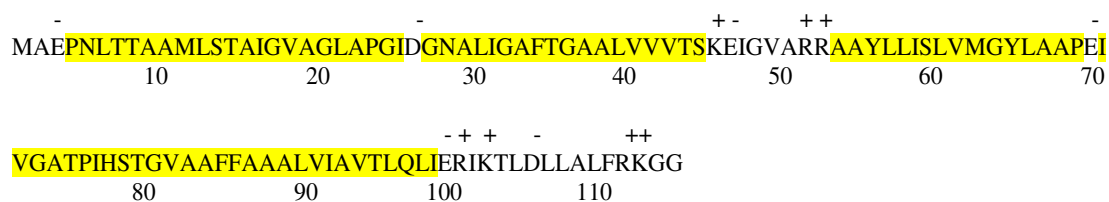
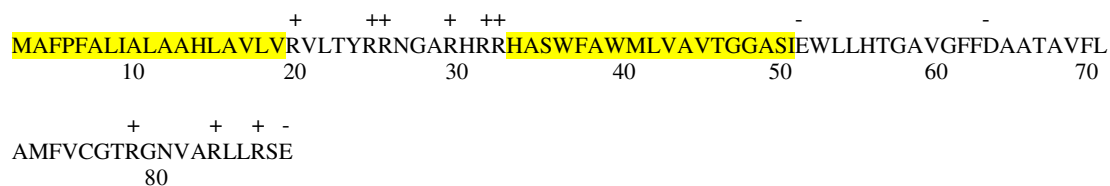
(A) Holin**(B) Antiholin**

FIG. 2.9. Predicted transmembrane domains (TMD) of the Bcep0425 holin and antiholin. The predicted TMDs of the Bcep0425 holin and antiholin are indicated in yellow, and the charged residues are indicated by + or -.

(A) Rz

```

ATGAACGGACTCGCCGCGAAAAATCATCGCGTGGATCGTCGCGCTCGCCACGTGCGCCGTC
M N G L A A K I I A W I V A L A T C A V
+
GTCGCGCTGTACGTGCATGGGCTGCGCGCCGAGCGCGGACCGCGCAACGGCAACGCGTC
V A L Y V H G L R A E R A T A Q R Q R V
+ - + +
GAGGCGCAACAGGCGCTCGCCGCGCGGACGGCGTCATTGCGCGCCTGCGGCAGGACGCC
E A Q Q A L A A R D G V I A R L R Q D A
- + - + +
GCCGAACGCGCGCAACAGCAGGTGCGGCTCGACCACGCGCAAACGCCATCGCGTCGAAG
A E R A Q Q Q V R L D H A Q T A I A S K
- + + - +
CTCGACGCCATTTCGATTTGAAAACCGGAGATTGACCGATGAAAACGCCGCGCTTCGCGCC
L D A I R F E N R R L T D E N A A L R A
- + - + - - +
TGGGCTGATACTCGCCTGCCTGACGACGTTGTCCGCTGCAAGCCAGTCCCGCTCTCACC
W A D T R L P D D V V R L Q A S P A L T
- + - - +
GGGCGCGCGGATTATGTCGAACACGTGCCAGACGGTGAAAACCGTGCACGCTGCCGAGGCT
G A G D Y V E H V P D G E T V H A A E A
- - - -
CGCGCCGCGGACCAACGGTGA
R A A D Q R *
+ - +

```

(B) Rz1

```

+ + + + + - - +
MKTPRFAPGLILACLTLSA[KPVPLSPAPAIMSNTCQTVKPCTLPRLAPRTNGELDGA]TLVKAAWATC
10 20 30 40 50 60 70

- - -
AATVDMIAACQAGAAAPDHGAHSHD
80 90

```

Fig. 2.10. The primary structure of Bcep0425 Rz and Rz1. (A) Bcep0425 *Rz* gene is shown with predicted translation below. The predicted N-terminal transmembrane domain and *Rz1* start codon are highlighted in yellow and underlined, respectively. (B) The predicted lipoprotein signal sequence is highlighted in grey with the processed Cys residue highlighted in red. Charged residues are indicated by + or -.

Bcep0425 also showed homology to the *lysB* and *lysC* spanin genes of phage P2. This homology, combined with the structural features, allow the conclusion that genes 24 and 25 were the *RzI* and *Rz*, respectively.

Confirming the role of the holin, antiholin, and endolysin in host lysis. To confirm the role of the putative holin, antiholin, and endolysin in host lysis, deletions of each gene in BC0425 were made using the system developed by Hamad et al. (41) that generated unmarked and in-frame deletion mutants. This system relies on the use of a suicide plasmid pMo130, which harbors a reporter *xylE* that allows for reliable visual detection of *Burkholderia* transformants and a modified *sacB* that allows for the resolution of co-integrants.

BC0425Δ holin, BC0425Δ antiholin, BC0425Δ endolysin, and BC0425-wild type (WT) were UV induced and their growth followed by recording optical density at 600 nm (OD₆₀₀) to observe for lysis, if any. UV induction of BC0425-WT resulted in an increase in OD₆₀₀ for 2 h post induction with an observed sudden drop in OD₆₀₀, which indicated host lysis. Inactivation of the lambda holin results in a distinctive phenotype, in which respiration and macromolecular synthesis continue past the normal lysis time (122). The cells continue to elongate and not lyse due to the inability of the endolysin to reach the periplasm (122). BC0425Δ holin exhibited a similar phenotype as that of a λ holin mutant. At 2 h post-induction, the OD₆₀₀ did not suddenly decrease as observed with BC0425-WT (Fig. 2.11). No lysis of the cells was observed until 2.5 h post induction, which has been attributed to the presence of a temperate phage harbored by BCO425 (unconfirmed) (Chapter IV).

The deletion of the endolysin should also result in the inability of cells to lyse. In λ , deletion of the endolysin does not result in lysis, but cell respiration and macromolecular synthesis stop at the normal lysis time (122). Deletion of the endolysin in BC0425 did not result in lysis (Fig. 2.11). The OD₆₀₀ continued to increase past the normal lysis time (2 h). These results were consistent with that previously reported for λ lysis mutants (33, 46, 87). It was expected that deletion of the antiholin should result in premature lysis of cells since the antiholin would not inhibit holin activity. This phenotype was observed with the BC0425 Δ antiholin mutant (Fig. 2.11). Lysis began to occur approximately 30 min earlier than observed for BC0425-WT UV-induced at the same time.

Lysis triggering using dinitrophenol. Holins accumulate within the cytoplasm and cause lysis of the host cell at a precise genetically programmed time. Release of the endolysin to the periplasm occurs when holin concentrations accumulate to a critical level that leads to formation of an oligomeric complex that disrupts the membrane (117). Dinitrophenol (DNP) is an uncoupling protonophore that dissipates the proton-motive-force (PMF) across the bacterial plasma membrane (51, 87, 122). A common feature of all holin systems is that all holins can be prematurely triggered by membrane depolarization with energy poisons such as cyanide and DNP (26). To further confirm the function of the holin in BC0425, DNP was added to cultures at 2 h post-UV induction. Immediately after addition of DNP to BC0425-WT and BC0425 Δ antiholin cultures, the OD₆₀₀ dropped, indicating lysis (Fig. 2.12). This was expected as both strains contained a functional holin protein.

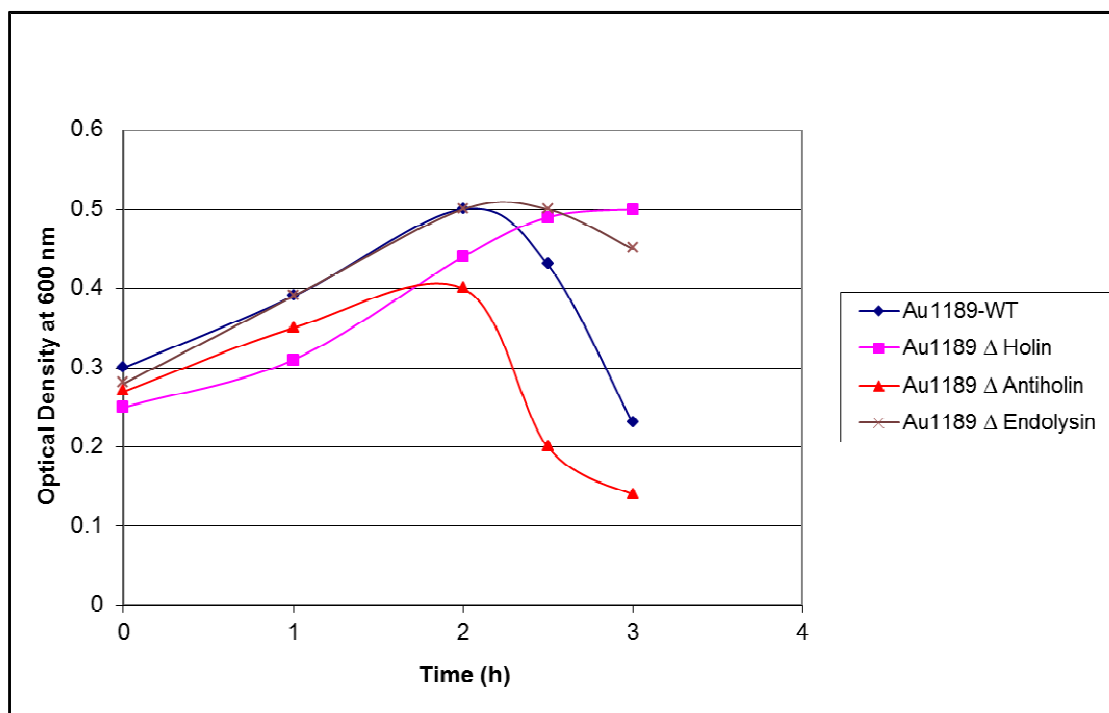


FIG. 2.11. Growth curve of BC0425 and lysis mutants post-UV induction. Lysis of BC0425-WT coincides with sudden drop in OD₆₀₀. The OD₆₀₀ was sampled and recorded at 60 min intervals. Results are representative of duplicate experiments.

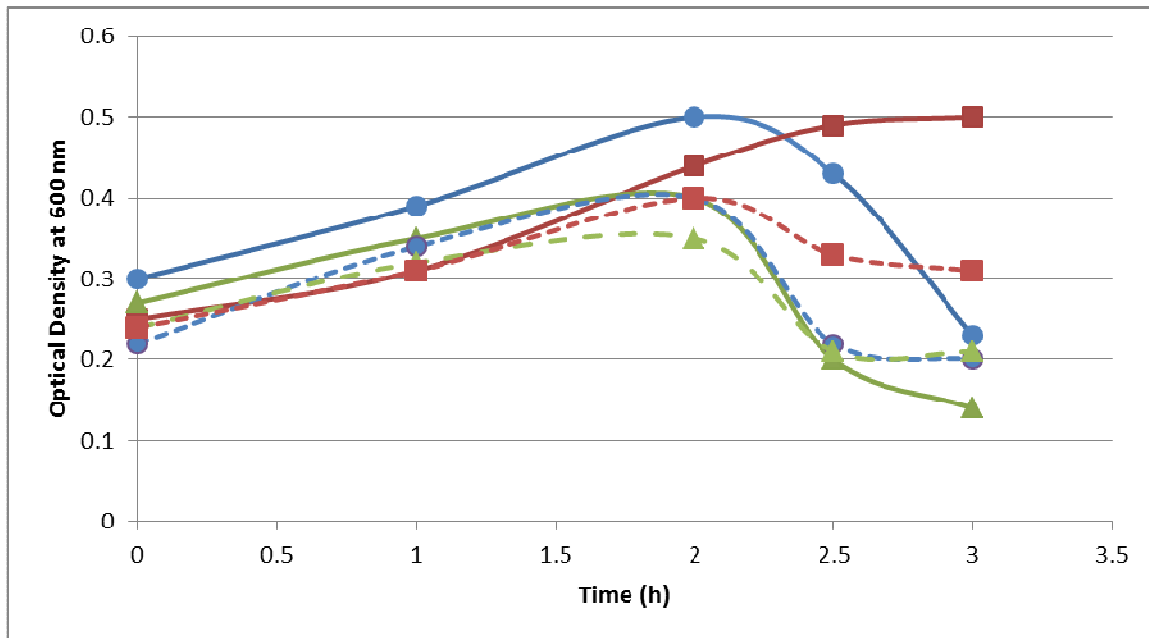


FIG. 2.12. Addition of dinitrophenol (DNP) to BC0425 Δ antiholin, BC0425 Δ holin, and BC0425-WT post-UV induction. The blue circles represent BC0425-WT, the red squares represent BC0425 Δ holin, and the green triangles represent BC0425 Δ antiholin. Dotted lines represent the addition of DNP to cultures two hours post-UV induction and solid lines represent cultures that did not receive DNP. Results are representative of duplicate experiments.

Using the same treatment, lysis was not observed for the Δ holin mutant (Fig. 2.12). Lysis was not triggered since there was no accumulation of the holin protein within the cytoplasm. However, a slight drop in OD₆₀₀ was observed in BC0425 Δ holin and may be due to a temperate phage, previously mentioned, carried by BC0425 that expresses its own lysis cassette.

Tailocin activity of UV induced lysis mutants. The holin and endolysin are the only two proteins essential for host lysis by a phage (117). Deletion of the phage holin does not result in lysis of the lysogen cells and as a result cells continue to elongate and phage continue to accumulate within the cytoplasm. In λ , adding chloroform (CHCl₃) to an induced holin mutant results in almost instantaneous lysis (7). This procedure allows for the quantification of tailocin both intracellularly and in the supernatant of UV induced cultures. BC0425-WT, BC0425 Δ holin, and BC0425 Δ antiholin were UV induced and two 50 μ l samples were taken from each culture at 1, 1.5, 1.75, 2, 2.25, 2.5, and 3 h post- UV induction. Samples were either immediately filter sterilized or 50 μ l chloroform was immediately added to assay for extracellular or intracellular and extracellular tailocin activity, respectively. Chloroform samples were centrifuged and the supernatant of both samples was analyzed for tailocin activity using the overlay spot assay. Intracellular tailocin activity was calculated by subtracting the tailocin activity of filter sterilized samples from tailocin activity of chloroformed samples. In Fig. 2.13, the intracellular tailocin activity of BC0425 Δ holin continued to increase past the normal lysis time of 2 h. This indicated that BC0425 Δ holin did not lyse and that tailocin Bcep0425 continued to accumulate within the cells. The

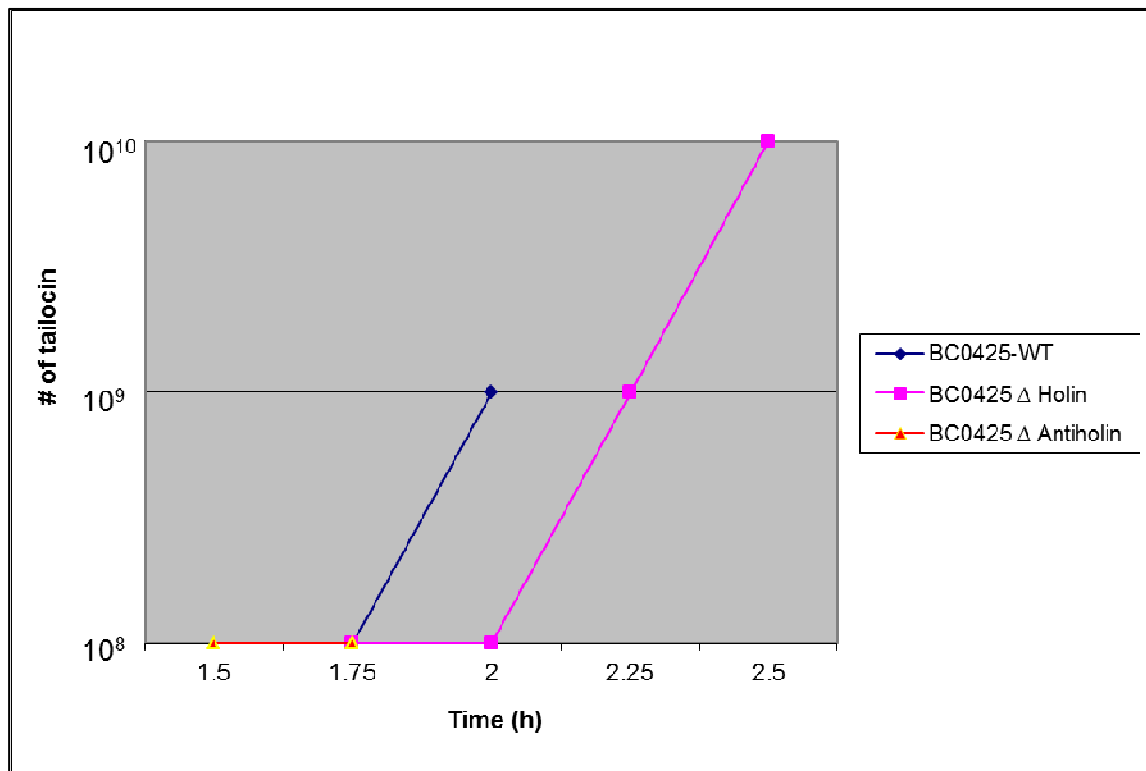


Fig. 2.13. Intracellular tailocin activity of BC0425-WT, BC0425 Δ antiholin, and BC0425 Δ holin post-UV induction. BC0425-WT, BC0425 Δ holin, and BC0425 Δ antiholin were UV induced and 50 μ l samples were taken post-UV induction. 50 μ l chloroform was immediately added to samples, mixed and centrifuged. The supernatant from samples was analyzed for tailocin activity using the overlay spot assay and assayed for zones of inhibition. Intracellular tailocin activity was calculated by subtracting the tailocin activity of samples that were filter sterilized from tailocin activity of chloroformed samples. Results are representative of duplicate experiments.

intracellular tailocin activity of BC0425-WT only continued to increase until 2 h post UV induction (Fig. 2.13). Minimal intracellular tailocin activity was observed for BC0425 Δ antiholin (Fig. 2.13), since BC0425 Δ antiholin lysed earlier than WT and tailocin Bcep0425 was not able to accumulate intracellularly, as observed for BC0425-WT.

The extracellular tailocin activity of UV induced cultures was also quantified. In BC0425-WT, a gradual increase in extracellular tailocin activity was observed as cells lysed over a 2 h time period post-UV induction (Fig. 2.14). Similar results were observed for BC0425 Δ antiholin, except reduced tailocin activity was observed since BC0425 Δ antiholin lysed earlier than BC0425-WT, which resulted in lower accumulation of tailocin within the cells (Fig. 2.14). Only minimal activity was observed in the supernatant of BC0425 Δ holin cells (Fig. 2.14). The observed slight increase at 2.25 h post-UV irradiation was attributed to cell lysis caused by the temperate phage integrated in the strain BC0425 chromosome.

Complementation of lysis mutants. In *trans* complementation of BC0425 Δ antiholin, BC0425 Δ holin and BC0425 Δ endolysin deletion mutants was achieved using plasmids pMo168::anti-comp, pMo168::holin-comp and pMo168::endo-comp, respectively. BC0425::holin-comp, BC0425::anti-comp, BC0425::endo-comp, and BC0425-WT were UV induced and their growth followed by recording OD₆₀₀ to observe for lysis, if any. UV induction of BC0425-WT resulted in an increase in OD₆₀₀ for 2 h post induction, with a sudden drop in OD₆₀₀ at 2 h indicating host lysis (Fig. 2.15). The

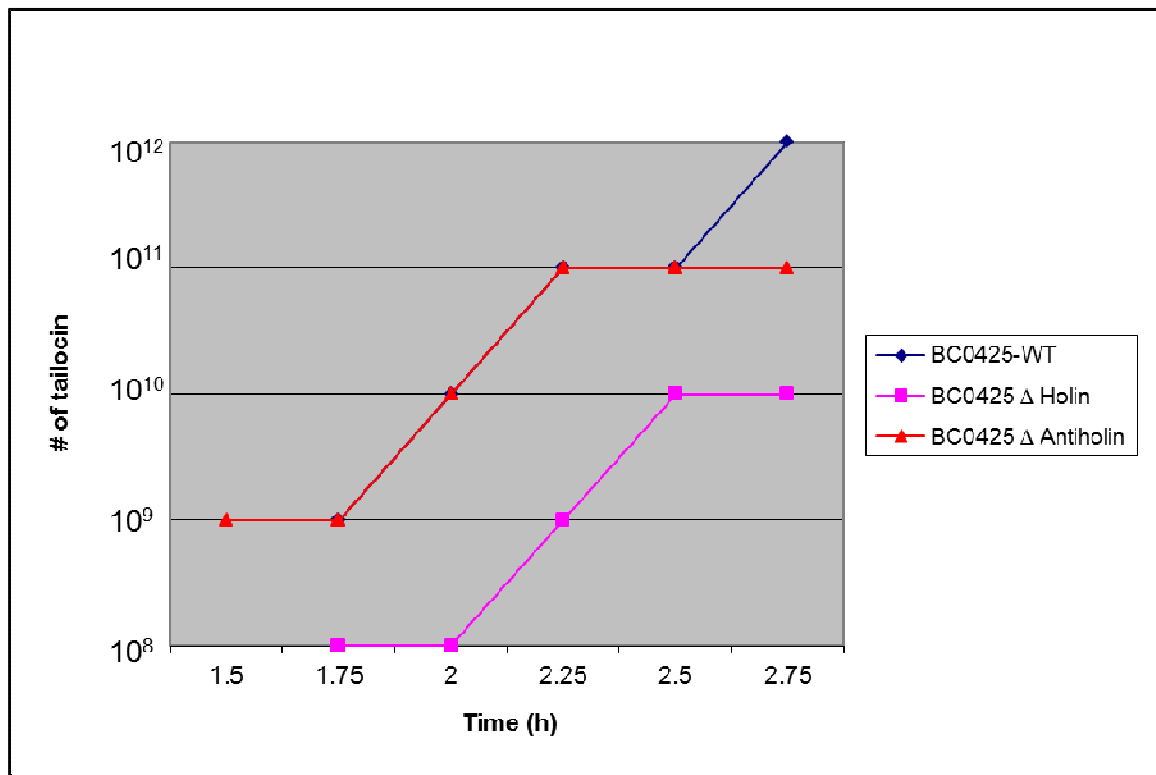


Fig. 2.14. Extracellular tailocin activity of BC0425-WT, BC0425 Δ antiholin, and BC0425 Δ holin post-UV induction. BC0425-WT, BC0425 Δ holin, and BC0425 Δ antiholin were UV induced and 50 μ l samples were taken post-UV induction. Samples were immediately filter sterilized. The samples were analyzed for tailocin activity using the overlay spot assay. Results are representative of duplicate experiments.

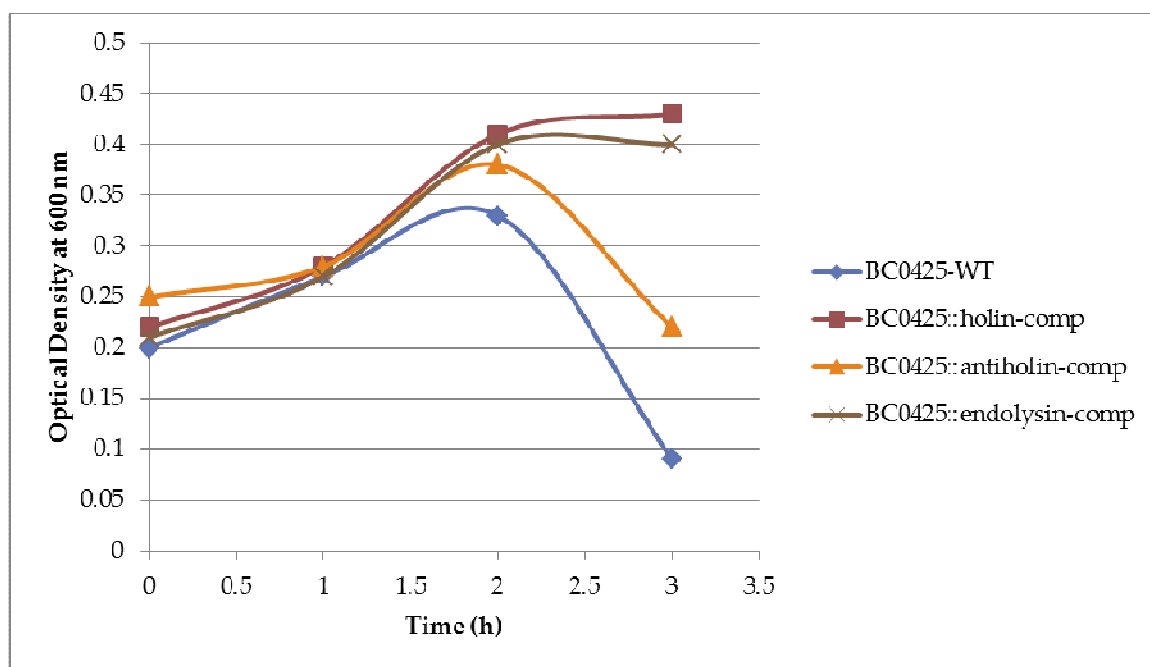


Fig. 2.15. Growth curve of Bcep0425 lysis mutant complements post-UV induction.

Lysis of BC0425-WT coincides with a sudden drop in OD₆₀₀. The OD₆₀₀ was sampled and recorded at 60 min intervals. Results are representative of duplicate experiments.

BC0425::anti-comp no longer lysed prematurely and lysis capabilities were restored (Fig. 2.15). Lysis was not restored for the BC0425::holin-comp or BC0425::endo-comp.

Bcep0425 efficiency of killing. The efficiency of killing (EOK) was calculated based on the molecular mass of the 43 kDa tailocin sheath protein and the determined amount of tailocin sheath molecules in a partially purified tailocin preparation as described in Material and Methods. Based on this data, one can calculate the number of tailocins/ml and the number of killing events/tailocin or the EOK. From multiple preparations and assays, the EOK ranged from 0.4 to 0.6, assuming 100% purity of tailocins. A typical preparation of tailocins ranged from 4×10^{11} to 1×10^{12} killing units/ml and the total number of tailocin sheath molecules ranged from 1×10^{12} to 6×10^{12} . Based on this information, the number of killing units to the number of tailocin particles was calculated. For strain PC184, cells are killed by tailocin Bcep0425 at a predicted 1:1 interaction.

DISCUSSION

Based on sequence homology and the identification of a bacteriophage lysis cassette associated with pyocin gene clusters, studies have shown that pyocin genes share a common ancestry with bacteriophages (81). However, neither a gene for capsid formation, replication, nor integration has been found to be associated with pyocin gene clusters (78). This evidence suggests that pyocins are phage tails that became evolutionarily specialized as bacteriocins, rather than as simple defective phages (81).

We have determined the genetic organization of the Bcep0425 tailocin cluster and have identified the major structural proteins and lysis cassette. *Gene1*, *gene3* and *gene5* were predicted to encode for an integrase, zinc-finger CHC2-family protein involved in replication and a phage transcriptional activator, respectively. The presence of *gene1*, *gene3* and *gene5* in the Bcep0425 cluster provides the first evidence for phage associated genes to be linked with a pyocin/tailocin gene cluster and indicates that Bcep0425 is a defective phage.

Headless mutants of bacteriophage PS17 have been demonstrated to kill sensitive cells with bacteriocin-like activity (100). Furthermore, it has been shown that component exchanges, known as phenotypic mixing, occurred between the R2 pyocin of *Pseudomonas* and phage PS17 (100). The identified gene products of the Bcep0425 tailocin cluster showed homology to *Burkholderia* phage KL3, an identified P2-like phage (71). Tailocin Bcep0425 and phage KL3 were also organized similarly, except Bcep0425 did not contain genes involved in capsid formation or DNA modification and showed greater than 60 % identity in 26 of 29 homologous genes (Fig. 2.5).

The presence of a lysis gene cassette in Bcep0425 similar to that contained in lytic bacteriophages further supports that tailocin Bcep0425 is a defective phage. The function of the Bcep0425 holin, along with the antiholin and endolysin, were confirmed using mutational analysis. Upon analysis of the the Bcep0425 lysis cassette, the holin was determined to contain four TMDs with a N-in and C-in topology (Fig. 2.8). Holins are a very diverse group of proteins, but only three classes of canonical holins have been

described to date (121). Class I, II and III holins contain 3, 2 and 1 TMD, respectively. We are proposing that the Bcep0425 holin represents a new class of holin, Class IV.

In complementation studies of lysis gene mutants it was possible to complement the antiholin deletion mutant. However, although the holin and endolysin complements were similarly constructed and confirmed by sequence analysis, it was not possible to complement the in-frame deletion mutants. For most bacteriophages, host cell lysis requires, at a minimum, an endolysin and a holin. Holin-dependent lysis systems are highly regulated. The precise temporal regulation is also dependent on the energy state of the cytoplasmic membrane (27, 116). Lysis requires the endolysin to accumulate within the cytoplasm until the holin reaches a critical concentration and disrupts the membrane (37). Any alteration in this concentration can retard lysis. Since pMo168 is a low copy plasmid, the BC0425::holin-comp may not have attained the critical concentration necessary for membrane disruption. For BC0425::endolysin-comp, the high concentration of endolysin necessary for cell wall degradation was also not attained due to the low copy number of the complementing plasmid.

The host spectrum determinant of pyocins resides in the tail fiber protein, which binds specific receptors on the bacterial cell surface. Currently R-type pyocins are being reengineered to generate novel bactericidal protein complexes by retargeting their spectra to other bacterial species and genera by making fusions between the pyocin tail fibers and the tail fibers of related bacteriophages (95, 119). In each example of creating novel pyocins, the formation of active particles consistently required co-expression of the cognate or nearly identical chaperone (TFA) specific for the C-

terminal portion of the tail fiber (95, 119). In a three-way alignment of the amino acid sequence of the TFA protein of tailocin Bcep0425, putative tailocin of J2315, and phage KL3, the largest divergence in the sequence was observed at the C-terminal end (Fig. 2.6). In this study, we propose that the C-terminal of the TFA gene is responsible for specificity and the broad host range of Bcep0425.

Numerous studies have focused on the characterization of pyocins, but there have been no molecular investigations of tailocins from *Burkholderia*. This chapter constitutes the first molecular characterization of a phage tail-like bacteriocin from *Burkholderia* and provides evidence that tailocin Bcep0425 is a defective phage.

CHAPTER III

IDENTIFICATION OF THE ADSORPTION SITE FOR BCEP0425

INTRODUCTION

The bacterial lipopolysaccharide (LPS) is the major component of the outer surface of Gram-negative bacteria. LPS contributes greatly to the structural integrity of the bacteria and protects the membrane from certain chemical attacks (17). LPS is composed of three different parts: lipid A, core-oligosaccharide, and in some cases the O-antigen. The LPS can also have toxic effects as the lipid A component can cause endotoxic shock that manifests as fever or other symptoms such as decreased blood pressure and inflammation.

The O-antigen consists of repetitive subunits of one to eight monosaccharides, which are responsible for much of the immunospecificity of the bacterial cell (17). The O-antigen is highly variable and only found in “smooth” bacteria. Bacteria that do not contain an O-antigen are known as “rough”. The glycosidic part, known as the core, consists of approximately ten monosaccharides and the sugar at the reducing end is always alpha-3-deoxy-D-manno-oct-2-ulosonic acid [Kdo] (17). The lipid moiety, lipid A is composed of multiple hydrophobic fatty acids, which anchor the LPS to the bacterial membrane and is highly conserved among Gram-negative genera.

The LPS plays a role in the resistance of members of the *Burkholderia cepacia* complex (Bcc) to the effects of cationic antibiotics (80). The unusual structure of Bcc

LPS lowers the anionic charge of the Bcc cell surface, which inhibits the binding and subsequent effects of cationic antibiotics. This is attributed to the fewer amounts of phosphate and Kdo found within the core oligosaccharide, as compared to other Gram-negative bacteria (22, 74, 97). Additionally, there are 4-amino-4-deoxyarabinose (Ara4N) moieties attached to the phosphate residues in the lipid A backbone (22) and D-glycero- α -D-talo-oct-2-ulopyranosylonic acid (Ko) attached to the Kdo (48). Ko is an unusual sugar, which is only found in few other bacteria such as *Acinetobacter* spp. and *Yersinia pestis* (112-114).

Both Bcc and *Pseudomonas aeruginosa* are pathogens in CF patients. Many studies of the core oligosaccharide have drawn comparisons between the two bacteria. Bcc LPS contains five times less Kdo than *P. aeruginosa* (97, 99). The Bcc LPS also contains less phosphorus and more heptose as compared to *P. aeruginosa* LPS (22, 74). There are major differences between the LPS of both genera, but both contain rhamnose and glucose as the major components of the core oligosaccharide (48, 74).

The LPS of gram-negative bacteria has been found to be a likely adsorption site for pyocins (35, 45). This study reports that the Bcep0425 receptor site was determined to be part of the core component of the bacterial LPS and that L-rhamnose and α -glucose are the surface receptor sites for tailocin Bcep0425.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this chapter are listed in Table 3.1. The complex medium TN broth (TNB) was used for routine maintenance of cultures (42). Solid medium, TNA, was identical except it lacked KNO₃ and was supplemented with 20 g/L agar. For soft agar overlays, TN medium was amended with 7.5 g agar/L (TNSA). Luria-Bertani (LB) medium was used in mating experiments (11). For counter-selection, co-integrants were grown in Yeast-Tryptone (YT) broth, which was made by dissolving 10 g of tryptone and 10 g of yeast per liter of water. Sucrose was added to YT broth at a final concentration of 15% (wt/vol) and supplemented with 20 g/L agar (YTSA). *B. cenocepacia*, *Escherichia coli*, and *P. aeruginosa* strains were grown at 37°C. Antibiotics were added to the media at the following concentrations: 600 µg/ml kanamycin (Km) and 20 µg/ml tetracycline (Tc) for *B. cenocepacia*; 30 µg/ml Km for *E. coli*.

DNA manipulations. Genomic DNA was extracted using a DNeasy kit (Qiagen), small-scale plasmid preparation was accomplished using the Miniprep kit (Qiagen), and gel extraction and PCR product purification were conducted using Quiaquick gel extraction kit and Quiaquick PCR purification kit, respectively (Qiagen). All restriction endonucleases, *Taq* DNA polymerase and T4 DNA ligase were purchased from New England Biolabs and were used according to manufacturer protocols. Oligonucleotide primers were synthesized by Integrated DNA Technologies (IDT).

Table 3.1. Bacterial strains and plasmids used in Chapter III

Strain	Relevant Characteristics	Reference or Source
<i>Burkholderia cenocepacia</i>		
BC0425	Producer of Bcep0425	J. LiPuma
K56-2	ET12 epidemic lineage, O-antigen ⁺	Sokol et al. (103)
J2315	ET12 epidemic lineage, O-antigen ⁻	Laboratory collection
PC184	Midwest epidemic lineage, O-antigen ⁻	J. LiPuma
BC0425Δ <i>waaL</i>	BC0425, deleted <i>waaL</i> gene, O-antigen ⁻ L-Rham ⁻	This study
BC0425Δ <i>waaC</i>	BC0425, deleted <i>waaC</i> gene, O-antigen ⁻ L-Rham ⁻ Glu ⁻	This study
<i>Escherichia coli</i>		
DH5αMCR	Cloning strain, F ⁻ <i>endA1 hsdR17</i> (r _k ⁻ , m _k ⁺) <i>supE44 thi-1 λ⁻ recA1 gyrA96 relA1 deoR Δ(lacZYA-argF)- U169 Φ80dlacZΔM15</i>	Invitrogen
<i>Pseudomonas aeruginosa</i>		
PAO1	LPS A ⁺ LPS B ⁺	J. Lam (16)
PAO1wbpM	LPS A ⁺ LPS B ⁻	J. Lam (16)
PAO1wbpL	LPS A ⁻ LPS B ⁻	J. Lam (91)
PAO1rmlC	LPS A ⁻ LPS B ⁻ L-Rham ⁻	J. Lam (90)
PAO1algC	LPS A ⁻ LPS B ⁻ L-Rham ⁻ Glu ⁻	J. Lam (50)
Plasmids		
pRK2013	Tra ⁺ Mob ⁺ ColE1 replicon, Km ^r	Laboratory Stock (30)
pMo130	Suicide vector for allelic exchange in <i>Burkholderia</i> ; ColE1 <i>ori</i> , RK2 <i>oriT</i> , <i>xylE</i> , <i>sacB</i> , Km ^R	M. Voskuil (41)
pMo168	Replicative vector for <i>Burkholderia</i> ; <i>ori</i> pBBR1, <i>mob</i> ⁺ , <i>xylE</i> , Km ^R	M. Voskuil (41)
pMo130:: <i>waaL</i>	pM0130 with <i>waaL</i> upstream and downstream fragments	This study
pMo130:: <i>waaC</i>	pM0130 with <i>waaC</i> upstream and downstream fragments	This study
pMo168:: <i>waaL</i> -comp	<i>waaL</i> gene cloned into pMo168	This study
pMo168:: <i>waaC</i> -comp	<i>waaC</i> gene cloned into pMo168	This study

Km^R = resistance to kanamycin. + and - = positive or negative for phenotype, respectively

DNA sequence analyses were conducted by the Institute of Developmental and Molecular Biology, Gene Technologies Laboratory at Texas A&M University.

Plasmid construction for allelic exchange. Since *B. cenocepacia* strain K56-2 is clonally related to the sequenced strain J2315 (43, 72), PCR primers for regions flanking *waaL* in strain K56-2 were designed based on J2315 sequences BCAL2404 and BCAL2406 (www.sanger.ac.uk/Projects/B_cenocepacia/). Deletion of *waaL* and *waaC* genes of strain K56-2 were constructed using the system developed by Hamad et al. (41). To construct plasmid pMo130::*waaL* to be used for deleting *waaL* from strain K56-2, a 1.0 kb fragment upstream of *waaL* was amplified with primers *waaL*-up-F and *waaL*-up-R (Table 3.2) and cloned into multiple cloning site (MCS) 1 of plasmid pMo130, digested with *NotI* and *BamHI*, to obtain plasmid pMo130::*waaL*-up. The 1.0 kb fragment downstream of *waaL* was amplified using primers *waaL*-down-F and *waaL*-down-R (Table 3.2). The PCR product was cloned into pMo130::*waaL*-up, digested with *BamHI* and *XbaI*, to obtain pMo130::*waaL*. PCR primers for regions flanking *waaC* in strain K56-2 were designed based on J2315 sequences BCAL3111 and BCAL3113 (www.sanger.ac.uk/Projects/B_cenocepacia/). To construct plasmid pMo130::*waaC*, used to delete *waaC* from BC0425, a 1.0 kb fragment upstream of *waaC* was amplified with primers *wbxy*-up-F and *wbxy*-up-R (Table 3.2) and cloned into MCS1 of plasmid pMo130, digested with *NheI* and *BglII*, to obtain plasmid pMo130::*waaC*-up. The 1.0 kb fragment downstream of *waaC* was amplified using primers *manB*-down-F and *manB*-down-R (Table 3.1). The PCR product was cloned into pMo130::*waaC*-up, digested with *BglII* and *HindIII*, to obtain pMo130::*waaC*.

Table 3.2. Primers used in Chapter III

Primer	Relevant Characteristics	Reference
waaL-up-F	5'-GT <u>GCGGCCGCT</u> CGCTTCGCCGAGCACCATG-3'	This study
waaL-up-R	5'-GTGGGATCCTCTGGGCATTTGCCGGGCTGG-3'	This study
waaL-down-F	5'-GTCGGATCCAAGACGCTCCATACGCGCCG-3'	This study
waaL-down-R	5'-GACTTCTAGAGCGCACCATCTCCTGCTCGG-3'	This study
wbxy-up-F	5'-CTGGCTAGCCCCGGGTATTGCGTCGAA-3'	This study
wbxy-up-R	5'-GTCAGATCTCCTACTGGTCGCCGAACGTCGT-3'	This study
manB-down-F	5'-GTCAGATCTCAGCCGGCGACAGACATAAAG-3'	This study
manB-down-R	5'-CGTAAGCTTCAATCGACGTCGCGGATCAGT-3'	This study
waaL-comp-F	5'-GTGGATCCCATGAGCGGGCTGTCGGTGG-3'	This study
waaL-comp-R	5'-CACTTCTAGACAGCAATCGCACGGGCTTGC-3'	This study
waaC-comp-F	5'-CACCTGCAGTCGGTTCGCGTGTGGACAGC-3'	This study
waaC-comp-R	5'-GACGGATCCTATGTCTGTCGCCGGCTGCC-3'	This study

Added restriction site is underlined.

PCR reactions were conducted for 30 cycles and parameters as follows: denaturation at 95°C for 1 min; annealing at 67°C, 63°C, 65°C, 64°C for primer sets *waaL*-up, *waaL*-down, *wbxy*-up, *manB*-down, respectively; and extension at 72°C for 1 min for all reactions. All constructed plasmids were confirmed by restriction endonuclease digestion using appropriate enzyme combinations. All plasmid constructs were performed using *E. coli* DH5 α MCR as a host. Plasmid constructs were introduced by chemical transformation (92), followed by selection on LB agar amended with 30 μ g/ml Km at 30°C.

Site-directed mutagenesis. Plasmids pMo130::*waaL* and pMo130::*waaC* were individually introduced into strain K56-2 by conjugation using triparental matings with pRK2013 as the mobilizing plasmid. Donor, mobilizer, and recipient suspensions were made in LB broth from cultures grown on solid media under selective conditions, as appropriate for 18 h. Bacterial suspensions were adjusted spectrophotometrically (A_{600} =0.5), mixed at an equal ratio (1:1:1), and transferred to a positively charged sterile membrane layered on a 100 x 15 mm LB agar petri dish. Following an 18 h incubation period at 37°C, the cells from the mating and respective controls were washed twice in phosphate buffer (0.125 M, pH 7.1) by centrifugation (12,096 X *g* for 10 min at 5°C). The bacterial pellets were suspended in phosphate buffer and dilution plated to LB agar plates amended with 600 μ g/ml Km and 20 μ g/ml Tc for selection of single crossover events. Tranconjugants were sprayed with 0.45 M pyrocatechol to identify colonies in which single crossover events had occurred. Co-integrates exhibited a yellow color because *xylE* encodes for catechol 2, 3-dioxygenase, which catalyzes the ring cleavage

of pyrocatechol and converts the colorless substrate to an intensely yellow hydroxymuconic semialdehyde. Single yellow colonies were grown in YT broth medium for 5 h and then plated to YTSA to select for resolved co-integrants. Colonies grown on YTSA were sprayed with pyrocatechol and presumptive resolved co-integrants exhibited a white phenotype. To confirm gene deletion, colonies were analyzed by PCR using multiple primer combinations, both internal and external to the target gene deletion.

Complementation experiments. Plasmids pMo168::*waaL*-comp and pMo168::*waaC*-comp were individually introduced into strain K56-2 Δ *waaL* and K56-2 Δ *waaC*, respectively, by conjugation using triparental matings, with pRK2013 as the mobilizing plasmid. The *waaL* gene was amplified using primers *waaL*-comp-F and *waaL*-comp-R (Table 3.2) and cloned into MCS2 of plasmid pMo168, digested with *Bam*HI and *Xba*I, to obtain plasmid pMo168::*waaL*-comp. Triparental matings (1:1:1) with selection on LB media supplemented with 600 μ g/ml Km and 20 μ g/ml Tc were performed as described above to mobilize pMo168::*waaL* into BC0425 Δ *waaL*. The *waaC* was amplified using primers *waaC*-comp-F and *waaC*-comp-R (Table 3.2) and cloned into MCS2 of plasmid pMo168, digested with *Bam*HI and *Pst*I, to obtain plasmid pMo168::*waaC*-comp. Triparental matings (1:1:1) with selection on LB agar supplemented with 600 μ g/ml Km and 20 μ g/ml Tc were performed as described above to mobilize pMo168::*waaC* into BC0425 Δ *waaC*. Tranconjugants were sprayed with 0.45 M pyrocatechol to identify colonies that contained the pMo168 derivative plasmid.

PCR reactions were conducted for 30 cycles and parameters as follows: denaturation at 95°C for 1 min; annealing at 63°C and 66°C for primer sets *waaL*-comp-F and R and *waaC*-comp-F and R (Table 3.2), respectively; and extension at 72°C for 1 min. All constructed plasmids were confirmed by restriction endonuclease digestion. All plasmid constructs were performed using *E. coli* DH5 α MCR as a host. Plasmid constructs were introduced by chemical transformation, followed by selection on LB agar amended with 30 μ g/ml Km at 30°C.

Tailocin activity quantification. The overlay spot assay was used to assay for tailocin activity as described in Chapter II.

LPS extraction. LPS extracts from K56-2 Δ *waaL*, K56-2 Δ *waaC*, K56-2-wild-type (WT), J2315, K56-2::*waaL*-comp and K56-2-*waaC*-comp were made using the hot phenol method based on Westphal and Jann (118), with modifications from Apicella et al. (3) and Kato et al. (55). Bacterial suspensions were made in TN broth from cultures grown on solid media under selective conditions, as appropriate for 18 h. Cultures were grown in TN broth to A_{600} = 0.7-0.8 at 37°C with shaking. The cultures were then centrifuged (10,000 X *g*) at 4°C for 15 min. Pellets were resuspended in 400 ml phosphate buffered saline (PBS) and centrifugation was repeated. Pellets were resuspended in 15 ml PBS and transferred to a 50 ml polypropylene tube. After cells were resuspended, 0.5 M EDTA (pH 8) was added to a final concentration of 5 mM and lysozyme (Sigma) to a final concentration of 250 μ g/ml. Cells were then incubated at 30°C for 1 h and then overnight at 4°C. Cells were sonicated at high power, for 4 x 15 sec on ice, using a 1/8 inch probe tip (QSonica Q55). The cell sonicates were transferred

to a clean, acid-washed 50 ml flask and 1M MgCl_2 was added to a final concentration of 20 mM and DNaseI and RNaseA to a final concentration of 20 $\mu\text{g/ml}$. The mixture was incubated at 37°C, with shaking (150 rpm) for 2 h. Flasks were then placed into a 68°C water bath and incubated for 5 min. An equal volume of liquid phenol (preheated to 68°C) was added to the cell sonicates and then vigorously shaken for 3 min at 68°C. The mixture was transferred to an ice water bath and shaken for 15 min. The mixtures were transferred to 50 ml Oak Ridge tubes and centrifuged (18,000 X g) at 4°C for 15 min. The supernatant was removed and purified by dialysis against double distilled sterile water using a 3.5 kDa exclusion membrane. Dialysis was done for several hours at room temperature, then overnight at 4°C, with 3-4 water changes. The dialysate was centrifuged (124,000 X g using a Beckman 60 Ti rotor) for 3 hours at 4°C. The gel-like pellet was resuspended in 1 ml endotoxin free water (Sigma) and lyophilized. The dry weight was determined and resuspended in endotoxin free water (Sigma) to a concentration of 1-2 $\mu\text{g/ml}$.

Electrophoretic analysis of LPS. LPS samples were analyzed using the Laemmli SDS-gel electrophoresis system (60). LPS preparations were dissociated in SDS loading buffer and heated at 90-100°C for 5 min. LPS was resolved by running 1.0 μg of each extract on a 16.5% Tris-Tricine SDS minigel (Bio-rad) for 6 hours at 60 volts and visualized by silver staining (107).

LPS adsorbance assay. An LPS adsorbance assay was performed using a purified tailocin preparation. The tailocin preparation was diluted (1:10) with [P-buffer] (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 8 mM MgSO_4). Serial dilutions (1:10) of LPS

extracts were made in P- buffer from 1000 µg/ml to 10 µg/ml. 10 µl of LPS dilution and 90 µl of tailocin dilutions were mixed and incubated at 37°C for 30 min. Tailocin activity of the mixtures was assayed using the overlay spot assay as described in Chapter II using strain PC184 as the indicator strain.

Sugar inhibition assay. The effects of L-rhamnose, D-galactose, α-D-glucose, D-fructose, D-glucose, sucrose, lactose, D-cellobiose and D-raffinose on Bcep0425 adsorption to strain PC184 was determined. The sugar inhibition assay was performed as described by Dawes (24) with minor modifications. Strain PC184 was grown in 1 L TN broth (200 rpm) to an $A_{600}=1$. Cells were centrifuged ($11,337 \times g$) at 4°C for 5 min. The cells were washed in 40 ml P-buffer, centrifuged, and resuspended in 4 ml P-buffer. Two ml of the bacterial suspension (10^{11} cells/ml) was added to 500 µl of tailocin Bcep0425 (10^{10} tailocin/ml) and 2.5 ml of 1.2 M sugar (final concentration of 0.6 M). The 5 ml mixture was incubated at 37°C and 500 µl samples were taken at 0, 1, 3, 6, and 9 min post mixing. Samples were immediately centrifuged and supernatants were filter sterilized. A 1:10 dilution was made for each sample in P-buffer, followed by a 1:2 dilution series that was spotted on the overlay seeded with strain PC184, as described above. Plates were incubated and the titer was expressed as the reciprocal of the highest dilution showing inhibition.

RESULTS

Inhibitory spectrum of tailocin Bcep0425. To determine the inhibitory spectrum of tailocin Bcep0425, a panel of 102 *Burkholderia* species was tested for sensitivity to tailocin Bcep0425. Seventy-three of the isolates tested (conducted in LiPuma laboratory, U of Michigan) were representative of the species belonging to the *Burkholderia cepacia* complex (Bcc). All isolates were tested by both broth microdilution (MIC) and the overlay spot assay using 1:10 serial dilutions of tailocin supernatants (range from 1/10 to 1/100,000 dilutions). Only 17 of the 41 *B. cenocepacia* strains tested were resistant to Bcep0425 (Table 3.3). Over 70% of the Bcc strains tested showed sensitivity to Bcep0425, with 26/29 non-Bcc exhibiting sensitivity (Table 3.3). In addition, 50% of *P. aeruginosa* isolates tested showed sensitivity, as well as 1 of 2 *E. coli* tested. This is significant since phage-like bacteriocins are known to have narrow host ranges and are normally effective against bacteria within the same species (89). The results in Table 1 indicate that tailocin Bcep0425 has broad host range activity against members of the Bcc, other *Burkholderia* species and is also effective against bacteria from other genera.

LPS adsorption assay. Adsorption of pyocins to a susceptible host(s) involves specific interaction of the tail fiber with a specific sugar or protein on the surface of a susceptible host (78). In order to determine why Bcep0425 exhibited a broad host range, we needed to determine the specific adsorption site of Bcep0425. The LPS of Gram-negative bacteria is often an adsorption site for pyocins (35, 45). An adsorption assay

Table 3.3. Host range activity of Bcep0425

<u>Species</u>	<u>Sensitive/Total Tested</u>
<i>B. cepacia</i>	3/3
<i>B. multivorans</i>	9/11
<i>B. cenocepacia</i>	24/41
<i>B. stabilis</i>	2/2
<i>B. vietnamiensis</i>	3/3
<i>B. dolosa</i>	2/3
<i>B. ambifaria</i>	3/3
<i>B. anthina</i>	1/3
<i>B. pyrrocinia</i>	3/3
<i>B. seminalis</i>	1/1
<i>B. vanimaris</i>	0/1
<i>B. gladioli</i>	3/3
<i>B. glumae</i>	23/25
<i>P. aeruginosa</i>	3/6
<i>E. coli</i>	1/2
	Total 81/110

All isolates were tested by both broth microdilution (MIC) and top agar spot assay using 1:10 serial dilutions of tailocin (range from 1/10 to 1/100,000 dilutions). *B. vanimaris*, *B. gladioli* and *B. glumae* are not members of the Bcc.

was conducted to determine if Bcep0425 adsorbed to LPS. Adsorption studies using purified LPS isolated from both resistant and sensitive *B. cenocepacia* isolates indicated that the putative receptor for Bcep0425 was a component of the LPS (Fig. 3.1). Increasing amounts of LPS were incubated with tailocin Bcep0425 and allowed to adsorb. A serial dilution of unadsorbed tailocin in supernatants was spotted on a lawn of *B. cenocepacia* strain PC184. In Fig. 3.1, tailocin activity in the supernatant decreased as the amount of LPS increased, indicating that tailocin Bcep0425 was adsorbing to the purified LPS. These results indicated that the adsorption site of Bcep0425 was a component of the bacterial LPS and was not a protein moiety.

Effects of sugars on tailocin adsorption. In a classic experiment reported by Dawes (24), the interaction of bacteriophage T4 with *E.coli* B cells in the presence of a variety of mono-, di- and trisaccharides, was used to determine the putative T4 glucose-glucose receptor site. The proposed rationale was any sugar mimicking the whole or part of the phage receptor site would inhibit adsorption of the phage to the cell outer membrane. Sugars unrelated to the receptor would have little effect on adsorption. To putatively identify the sugar(s) involved in Bcep0425 adsorption, a sugar inhibition assay was conducted using methods similar to those performed by Dawes (24). The sugars were used to determine the inhibition of adsorption of Bcep0425 to susceptible *B. cenocepacia* PC184 cells by calculating the number of unabsorbed tailocin at different time points. α -D-glucose, L-rhamnose and D-galactose were chosen because they are sugars commonly found on the core component of Bcc LPS (83, 111). α -D-glucose and L-rhamnose were found to inhibit 100% of tailocin adsorption (Fig. 3.2). The control,

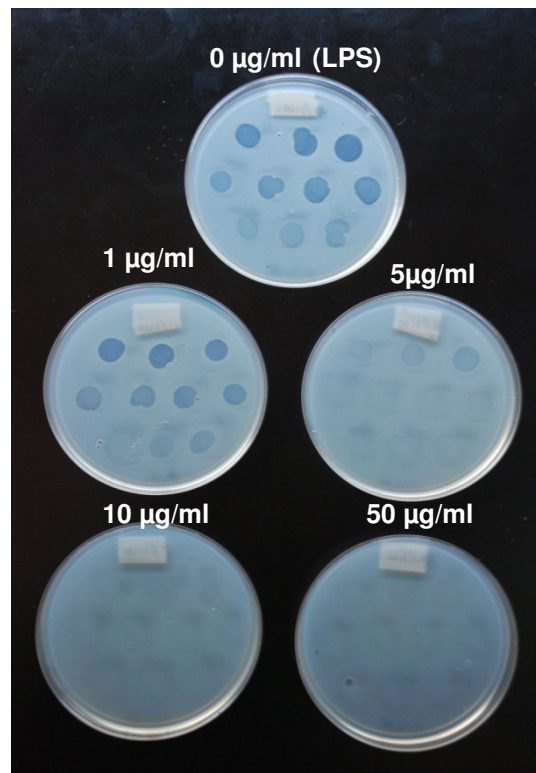


Fig. 3.1. Inactivation of Bcep0425 by LPS. Increasing amounts of LPS were incubated with tailocin and allowed to adsorb for 30 min. A serial dilution (1:2) of unadsorbed tailocin in supernatants was spotted on a lawn of PC184. A zone of inhibition is representative of tailocin activity.

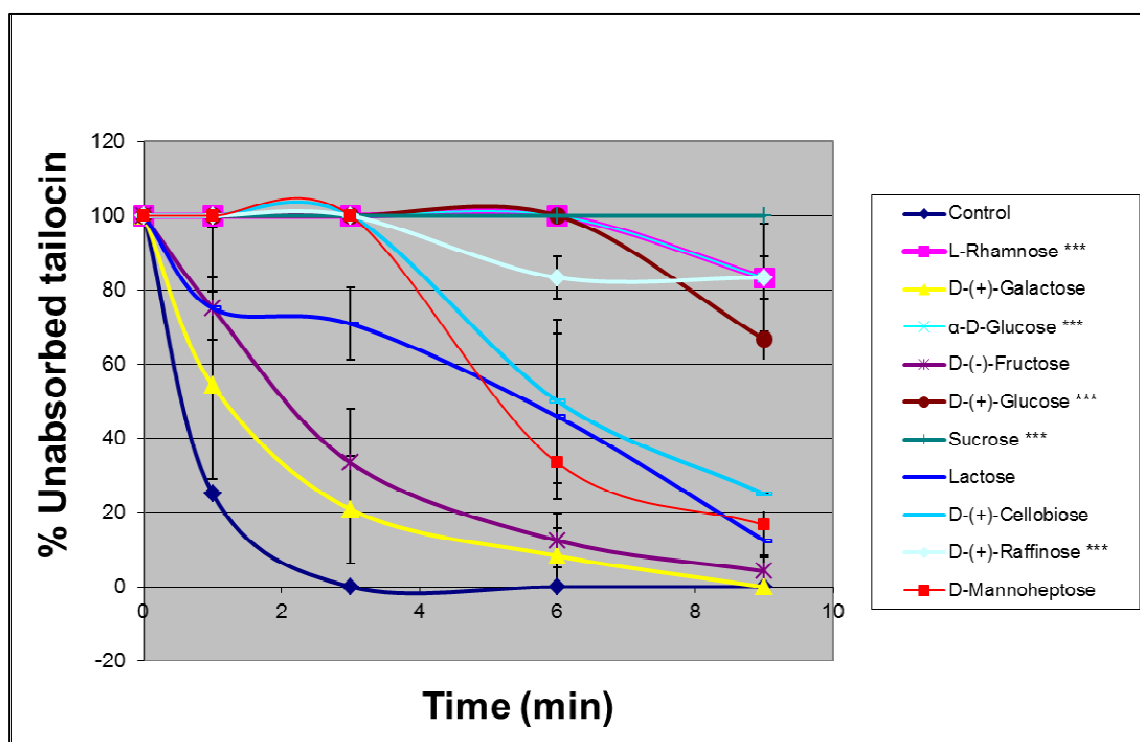


Fig. 3.2. Sugar inhibition assay. Effects of L-rhamnose, D-galactose, α -D-glucose, D-fructose, sucrose, lactose, D-cellobiose and D-raffinose on tailocin adsorption to *B. cenocepacia* strain PC184. The titer was expressed as the reciprocal of the highest dilution showing inhibition. *** Indicates sugars that inhibited Bcep0425 tailocin adsorption. Results are representative of triplicate experiments.

which did not contain a sugar, did not show adsorption inhibition. D-galactose did not inhibit tailocin adsorption and neither did D-fructose, which is not a normal component of bacterial LPS (Fig. 3.2).

The effects of a di- and tri-saccharides containing glucose were also investigated. Only those sugars in which glucose was α -linked to another residue inhibited adsorption (e.g. sucrose or raffinose); a β -linkage, as in D-cellobiose and lactose, did not inhibit adsorption (Fig. 3.2). This assay indicated that both α -glucose and L-rhamnose are the putative sugar components involved in Bcep0425 adsorption to the *B. cenocepacia* LPS.

Sensitivity of *P. aeruginosa* LPS mutants to tailocin Bcep0425. Kohler et al. (58) recently proposed that LPS plays an essential role both as a protective shield and receptor for R-pyocins in *P. aeruginosa*. The susceptibility of genetically defined isogenic *P. aeruginosa* LPS mutants to R-pyocins was assayed and the structural determinants required for R-pyocin recognition were identified for three R-type pyocins (58). We had previously determined that *P. aeruginosa* strain PA01 exhibited low sensitivity to tailocin Bcep0425. This observation suggested that LPS mutants of PA01 might show sensitivity to Bcep0425 as observed in *P. aeruginosa* for R-type pyocins (58). To determine if this was the case, defined isogenic *P. aeruginosa* PA01 LPS mutants obtained from Dr. Joseph Lam (University of Guelph) were tested for sensitivity to Bcep0425 using the overlay spot assay.

Together, the A-band and B-band comprise the O-antigen structure of PA01-wild type (WT). PA01-WT was shown to have minimal susceptibility to tailocin Bcep0425 (Fig. 3.3). In contrast, the LPS B-band deficient *wbpM* mutant was

susceptible to tailocin Bcep0425, suggesting that removing the B-band LPS moieties exposes the adsorption site to allow adsorption of tailocin Bcep0425 (Fig. 3.3). The *wbpL* mutant, deficient in both LPS B-band and A-band synthesis, was also susceptible (Fig. 3.3). A PAO1 *rmlC* mutant, devoid of L-rhamnose, was also susceptible because the α -glucose residue was still present and accessible to Bcep0425. However, an *algC* mutant exhibited no sensitivity to Bcep0425 due to loss of both the L-rhamnose and α -glucose residues (Fig. 3.3). The PAO1 mutant sensitivity assays further confirmed the sugar adsorption assays that indicated that L-rhamnose and α -glucose were the sugar components involved in Bcep0425 adsorption.

Targeted mutagenesis of genes involved in the biosynthesis of the core oligosaccharide. The first Bcc genome to be sequenced was that of *B. cenocepacia* J2315, the reference strain for CF epidemiology (21). Unfortunately, J2315 has displayed major barriers to genetic manipulation and has resulted in the use of a proxy strain *B. cenocepacia* K56-2 for genetic experiments. K56-2 is clonally related to the sequence strain J2315 and is more permissive to gene transfer (21, 33). Ortega et al. (41) constructed a panel of isogenic insertion mutants, in strain K56-2, with various core oligosaccharide truncations to determine the genetic components involved in biosynthesis of the K56-2 LPS. They determined the structure of the core oligosaccharide for two of the LPS mutants, XOA3 and XOA7. The mutant XOA3 had an insertional mutation in the *wbxE*, which resulted in the production of a lipid A-core oligosaccharide and partial O-antigen component. The XOA7 mutant, which had an inactivated *waaL* gene, resulted in the loss of O-antigen and loss of the terminal L-

rhamnose, which resulted in a truncated core. An insertional mutation in *waaC* of the Bcc strain K56-2 resulted in the loss of O-antigen and outer core oligosaccharide surface expression (41).

Strain K56-2 was found to be sensitive to tailocin Bcep0425 and since it was genetically malleable, experiments to identify the receptor site were conducted using this strain. The allelic exchange and complementation system developed by Hamad et al. (41) was used to obtain deletions in genes in *B. cenocepacia* strain K-56, involved in LPS synthesis. Deletion of the *waaL*, an O-antigen ligase, predicted a loss of the O-antigen surface expression in K56-2, and indicates the role of the O-antigen in adsorption (83). Deletion of *waaC*, heptosyltransferase I, involved in lipid A-core biosynthesis would also identify the role of core moieties. We predicted that the *waaL* deletion would remain sensitive, whereas the *waaC* mutant would be insensitive due to the loss of L-rhamnose and α -glucose residues. The sensitivity of the wild type and LPS mutants was determined using the overlay spot assay.

Strains K56-2, J2315, and the K56-2 *waaL* deletion mutant (Δ *waaL*) all showed similar sensitivity to Bcep0425 (Fig. 3.4). This was because the L-rhamnose and α -glucose residues were accessible to the tailocin as shown in Fig. 3.4. When both residues were removed, Δ *waaC*, tailocin Bcep0425 is no longer able to adsorb to the bacterial surface and cells were not sensitive (Fig. 3.4).

Data obtained from targeted mutagenesis and sugar adsorption assays in *B. cenocepacia* K56-2, combined with sensitivity studies using PAO1 LPS mutants, support that L-rhamnose and α -glucose are the adsorption sites for tailocin Bcep0425.

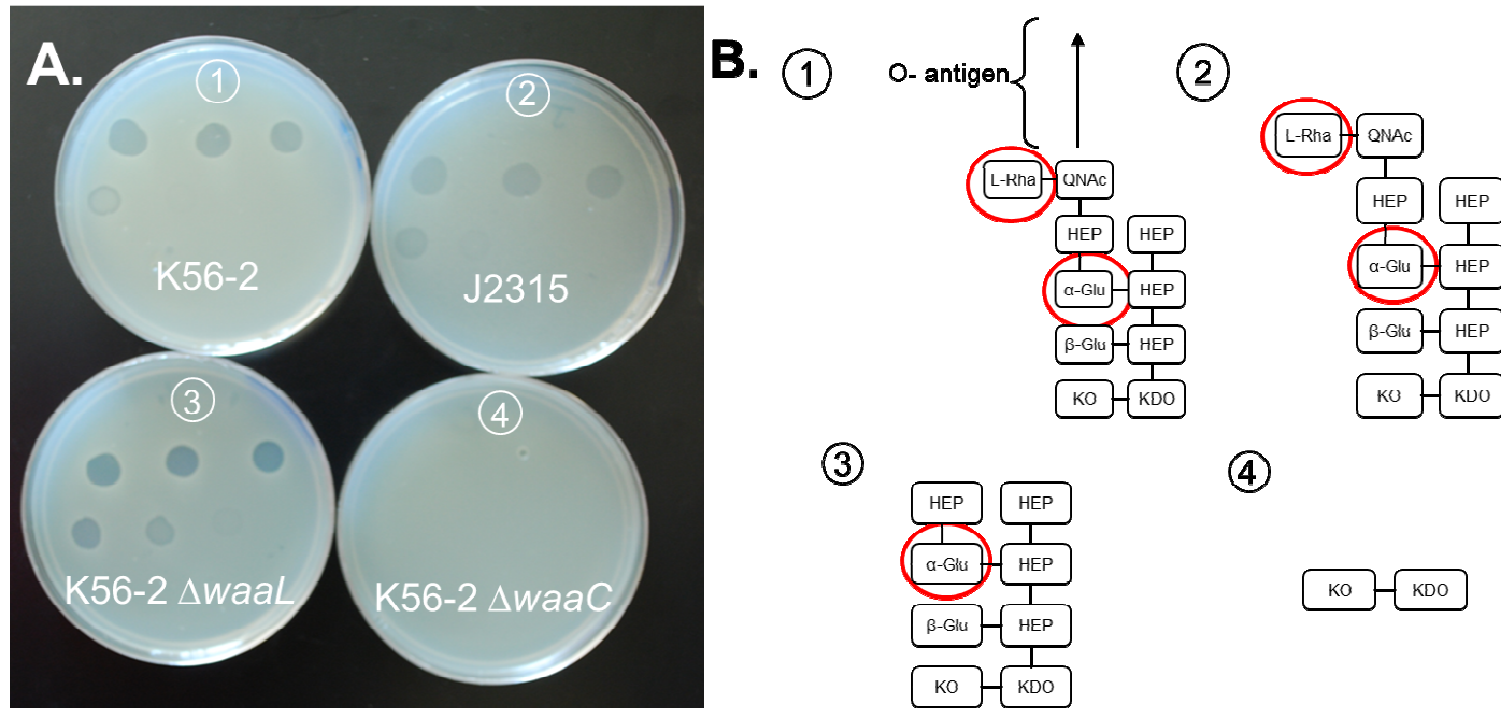


Fig. 3.4. Sensitivity of *Burkholderia* LPS mutants to tailocin Bcep0425 (A) Activity of serially diluted (1:10) tailocin Bcep0425 against: (1) K56-2; (2) J2315; (3) K56-2 $\Delta waaL$; or (4) K56-2 $\Delta waaC$. (B) Circled numbers indicated in Fig. 3.4A correspond to depicted LPS structure with same number. Residues involved in Bcep0425 adsorption are circled in red. HEP, heptose; KO, D-glycero- α -D-talo-oct-2-ulopyranolsylonic acid ; KDO, 2-keto-3 deoxyoctulosonic acid; QNAc, β -D-QuiNAc.

Complementation of LPS mutants. In *trans* complementation of K56-2 Δ *waaL* and K56-2 Δ *waaC* deletion mutants was achieved using plasmids pMo168::*waaL*-comp and pMo168::*waaC*-comp, respectively. LPS was extracted from K56-2-WT, J2315, K56-2 Δ *waaL*, K56-2 Δ *waaC*, K56-2::*waaL*-comp, and K56-2::*waaC*-comp and compared using SDS gel electrophoresis. The ladder-like banding pattern corresponded to the presence of polymeric O-antigen, as has previously been shown for strain K56-2 (72) (Fig. 3.5). In contrast, J2315 was devoid of an O-antigen (Fig. 3.5). K56-2 Δ *waaL* showed a defect in O-antigen production when compared to wild-type strain K56-2 (Fig. 3.5) and the lipid A-core region was truncated. The K56-2 Δ *waaC* mutant resulted in a severely truncated lipid-A-core region and the loss of an O-antigen (Fig. 3.5). Complementation of both the Δ *waaC* and Δ *waaL* resulted in restoration of O-antigen production and the lipid-A core regions were no longer truncated (Fig. 3.5).

The sensitivity of the wild type and LPS mutant complements was determined using the overlay spot assay. K56-2, K56-2::*waaL*-comp and K56-2::*waaC* were all sensitive to tailocin Bcep0425 (Fig. 3.6). Sensitivity was restored to K56-2::*waaC* – comp, indicative of a clean deletion of the *waaC* gene.

DISCUSSION

It was recently reported that approximately 68% of Bcc isolates produced bacteriocin-like toxins capable of inhibition (6). However, only a small fraction (16%) could be attributed to uncharacterized bacteriocin-like proteins. Previous studies of

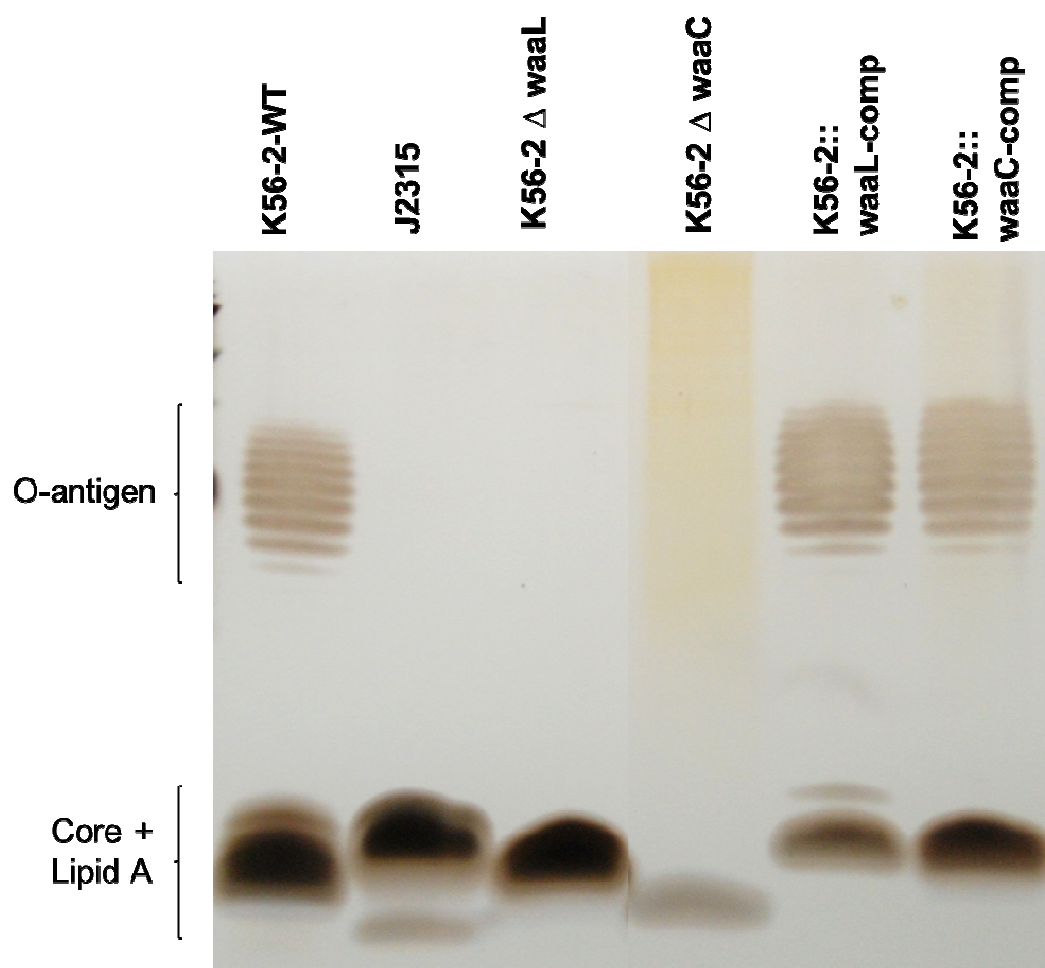


Fig. 3.5. Electrophoretic profiles of LPS extracted from J2315, K56-2 Δ *waaL*, K56-2 Δ *waaC*, K56-2:: *waaL*-comp, and K56-2:: *waaC*-comp in comparison to the profile of K56-2-WT. A total of 1.0 μ g LPS for each isolate was loaded on a 16.5% polyacrylamide gel in a Tricine-SDS system and developed by silver staining as described in Materials and Methods.

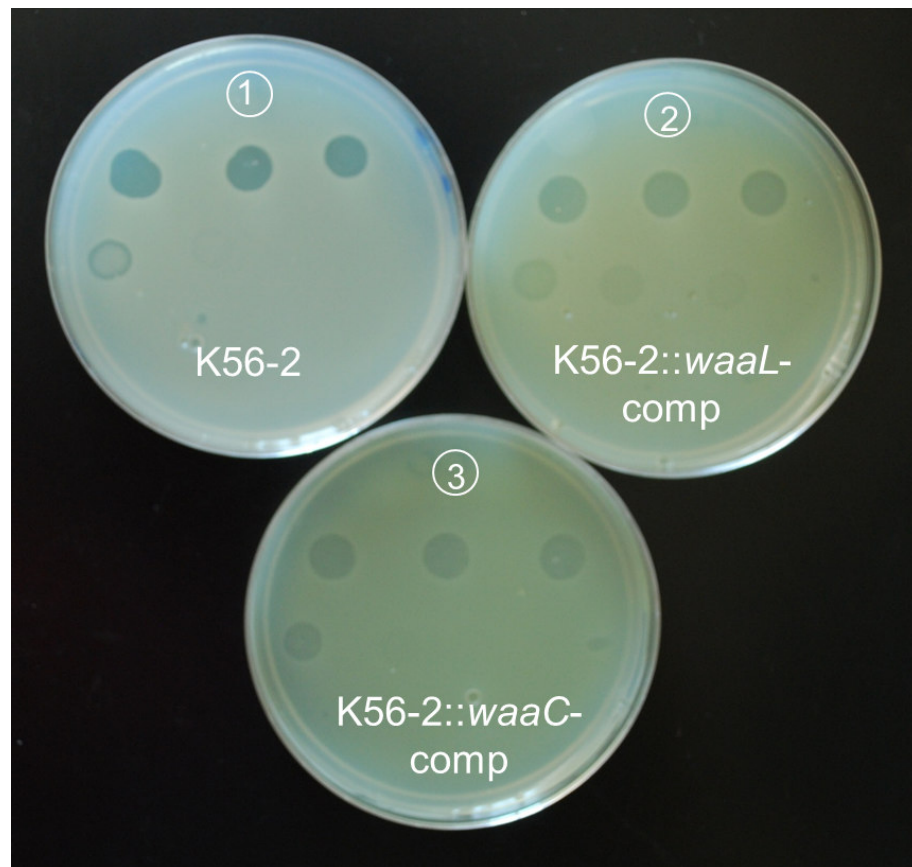


Fig. 3.6. Sensitivity of *Burkholderia* LPS mutant complements to tailocin Bcep0425
Activity of serially diluted (1:10) tailocin Bcep0425 against: (1) K56-2; (2) K56-2::waaL-comp ; or (3) K56-2::waaC-comp. Zones of growth inhibition are indicative of tailocin activity/sensitivity.

Gram-negative bacteriocins have described bacteriocins as having a narrow-spectrum. These bacteriocins were only active against members of the same species and displayed restricted levels of inhibition outside of the producing species (88, 89). Some bacteriocins have been shown to kill more broadly, and they have been implicated as a primary mechanism for mediating microbial diversity (57). We have isolated a phage-tail-like bacteriocin (tailocin), which exhibits broad host range activity against members of the Bcc. Even more striking is that tailocin Bcep0425 was also effective against bacteria from other genera, *P. aeruginosa* and *E. coli*. In all, over 70% of the Bcc strains tested showed sensitivity to Bcep0425, with 26/29 non-Bcc exhibiting sensitivity (Table 3.3). In addition, 50% of *P. aeruginosa* isolates tested showed sensitivity, as well as 1 of 2 *E. coli* tested. This is significant since most clinically relevant strains of the Bcc have demonstrated *in vitro* broad-spectrum antibiotic resistance. The use of tailocin Bcep0425 could provide an approach to treat infections caused by multi-drug-resistant strains of the Bcc.

A critical feature for pyocins/tailocins is their ability to recognize and bind to their bacterial host cell receptors. The LPS of Gram-negative bacteria is often an adsorption site for pyocins (35, 45). The Bcep0425 receptor site was determined to be part of the outer core component of the bacterial LPS. Both Bcc and *P. aeruginosa* contain rhamnose and glucose as major components of the core oligosaccharides (48, 74). Sugar inhibition assays revealed L-rhamnose and sugars containing an α -glucose linkage to be potentially involved in Bcep0425 adsorption (Fig. 3.2). Kohler et al. (58) recently proposed that LPS plays an essential role both as a protective shield and

receptor for R-pyocins in *P. aeruginosa*. The testing of the sensitivity of genetically defined LPS mutants of *P. aeruginosa* confirmed the role of LPS as a protective shield. PA01-WT showed minimal sensitivity to Bcep0425, but mutations in PA01 LPS exposed the Bcep0425 α -glucose and L-rhamnose receptor sites, resulting in sensitivity (Fig. 3.3). The testing of the sensitivity of genetically defined LPS mutants of *B. cenocepacia*, confirmed that L-rhamnose and α -glucose are the Bcep0425 receptor sites (Fig. 3.4). Loss of the L-rhamnose and α -glucose residues in the K56-2 $\Delta waaC$ mutant resulted in resistance to tailocin Bcep0425 (Fig. 3.4). In *trans* complementation of both the $\Delta waaC$ and $\Delta waaL$ resulted in restoration of O-antigen production and the lipid-A core regions (Fig. 3.5) and sensitivity to tailocin Bcep0425 was restored to the $\Delta waaC$ mutant complement.

Recently, there has been interest in R-pyocins as specific bactericidal agents. Currently R-type pyocins are being reengineered to generate novel bactericidal protein complexes (95, 119). The therapeutic efficacy of engineered R-pyocins has been demonstrated exogenously by using food products contaminated by *E. coli* O157:H7 and *in vivo* in a mouse model of *P. aeruginosa* peritonitis (95, 96). Future studies will focus on the therapeutic efficacy of Bcep0425 against Bcc infections.

CHAPTER IV

IDENTIFICATION OF COMPONENTS INVOLVED IN CONTROLLING PRODUCTION OF BCEP0425 AFTER INDUCTION

INTRODUCTION

The production of pyocins in *Pseudomonas aeruginosa* is inducible by treatments that cause DNA damage (93). It was first thought that pyocin production was regulated by a system similar to the SOS system in *Escherichia coli*. In this system the cleavage of the LexA repressor catalyzed by activated RecA, resulted in the activation of various SOS genes and colicin genes (115). It was later shown that pyocin synthesis is dependent on the RecA and that the LexA repressor is not involved in pyocin production (75, 101). Using *P. aeruginosa* PAO1 derivative strains defective in the synthesis of R2, F2, and S2 pyocins, together with complementation tests, later allowed for the identification of two regulatory genes involved in pyocin production, *prtN* and *prtR*, (75, 81). The *prtN* and *prtR* were identified upstream of the R2-F2 pyocin genes in PAO1 (81).

According to Matsui et al. (75), the *prtN* encodes a novel type of positive regulator composed of 104 amino acids. The PrtN protein binds to conserved P boxes, which are regulatory sequences located approximately 60-100 bp upstream of the ribosomal binding site with a consensus motif of ATTGnn(n)GTnn(n), and activate the transcription of pyocin genes. The PrtR protein (256 amino acids), which shows striking

similarity to the cI repressor of $\Phi 80$ phage, presents a site of cleavage for activated RecA. The *prtN* promoter activity is repressed until the cleavage of PrtR by an activated RecA. According to Matsui et al. (75), all three genes are required for the inducible production of pyocins, since a mutation of any of the three genes results in the inability of the pyocin genes to be induced above basal levels.

This study reports on the identification of a putative positive transcriptional regulator, *bctN*, and a cI-like repressor, *bctR*, upstream of the tailocin Bcep0425 structural genes and lysis cassette. Deletion mutants of *bctN*, *bctR*, and *recA* constructed in BC0425 confirm that all three genes are required for the induction of tailocin Bcep0425.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this chapter are listed in Table 4.1. The complex medium TN broth (TNB) was used for routine maintenance of cultures (42). Solid medium, TNA, was identical except it lacked KNO_3 and was supplemented with 20 g/L agar. For soft agar overlays, TN medium was amended with 7.5 g agar/L (TNSA). Luria-Bertani (LB) medium was used in mating experiments (11). For counter-selection, co-integrants were grown in Yeast-Tryptone (YT) broth, which was made by dissolving 10 g of tryptone and 10 g of yeast per liter of water. Sucrose was added to YT broth at a final concentration of 15% (wt/vol) and supplemented with 20 g/L agar (YTSA). *B.*

Table. 4.1. Bacterial strains and plasmids used in Chapter IV

Strain	Relevant Characteristics	Reference or Source
<i>Burkholderia cenocepacia</i>		
BC0425	Producer of Bcep0425	J. LiPuma
PC184	Midwest epidemic lineage	J. LiPuma
BC0425Δ <i>bctN</i> 2	BC0425, deleted <i>bctN</i> gene	This study
BC0425Δ <i>bctR</i>	BC0425, deleted <i>bctR</i> gene	This study
BC0425Δ <i>recA</i>	BC0425, deleted <i>recA</i> gene	This study
<i>Escherichia coli</i>		
DH5αMCR	Cloning strain, F ⁻ <i>endA</i> 1 <i>hsdR</i> 17 (r _k ⁻ , m _k ⁺) <i>supE</i> 44 <i>thi</i> -1 λ ⁻ <i>recA</i> 1 <i>gyrA</i> 96 <i>relA</i> 1 <i>deoR</i> Δ(<i>lacZYA-argF</i>)- U169 Φ80d <i>lacZ</i> ΔM15	Invitrogen
Plasmids		
pRK2013	Tra ⁺ Mob ⁺ ColE1 replicon, Km ^r	Laboratory stock (30)
pMo130	Suicide vector for allelic exchange in <i>Burkholderia</i> ; ColE1 <i>ori</i> , RK2 <i>oriT</i> , <i>xylE</i> , <i>sacB</i> , Km ^R	M. Voskuil (41)
pMo168	Replicative vector for <i>Burkholderia</i> ; <i>ori</i> pBBR1, <i>mob</i> ⁺ , <i>xylE</i> , Km ^R	M. Voskuil (41)
pMo130:: <i>bctN</i> 2	pM0130 with <i>bctN</i> upstream and downstream fragments	This study
pMo130:: <i>bctR</i> 2	pM0130 with <i>bctR</i> upstream and downstream fragments	This study
pMo130:: <i>recA</i>	pM0130 with <i>recA</i> upstream and downstream fragments	This study
pMo168:: <i>bctN</i> -comp	<i>bctN</i> gene cloned into pMo168	This study
pMo168:: <i>bctR</i> -comp	<i>bctR</i> gene cloned into pMo168	This study
pMo168:: <i>recA</i> -comp	<i>recA</i> gene cloned into pMo168	This study
Km ^R = resistance to kanamycin. + and - = positive and negative for phenotype, respectively		

cenoepectia and *E. coli* strains were grown at 37°C. Antibiotics were added to the media at the following concentrations: 600 µg/ml kanamycin (Km) and 20 µg/ml tetracycline (Tc) for *B. cenoepectia*; 30 µg/ml Km for *E. coli*.

DNA manipulations. Genomic DNA was extracted using a DNeasy kit (Qiagen), small-scale plasmid preparation was accomplished using the Miniprep kit (Qiagen), and gel extraction and PCR product purification were conducted using Quiaquick gel extraction kit and Quiaquick PCR purification kit, respectively (Qiagen). All restriction endonucleases, *Taq* DNA polymerase and T4 DNA ligase were purchased from New England Biolabs and were used according to manufacturer protocols. Oligonucleotide primers were synthesized by Integrated DNA Technologies (IDT). DNA sequence analyses were conducted by the Institute of Developmental and Molecular Biology, Gene Technologies Laboratory at Texas A&M University.

Plasmid construction for allelic exchange. Deletion of *bctN*, *bctR*, and *recA* genes of BC0425 were constructed using the system developed by Hamad et al. (41). PCR primers for regions flanking *bctN* were designed based on the annotated sequence of BC0425. To construct plasmid pMo130::*bctN2* used to delete *bctN* from BC0425, a 1.0 kb fragment upstream of *bctN* was amplified with primers PrtN2-up-F and PrtN2-up-R (Table 4.2) and cloned into multiple cloning site (MCS) 1 of plasmid pMo130, digested with *NheI* and *BglII*, to obtain plasmid pMo130::prtN2-up. The 1.0 kb fragment downstream of *bctN* was amplified using primers prtN2-down-F and prtN2-down-R (Table 4.2). The PCR product was cloned into pMo130:: prtN2-up, digested with *BglII* and *HindIII*, to obtain pMo130:: *bctN2*.

Table 4.2. Primers used in Chapter IV

Primer	Relevant Characteristics	Reference
prtN2-up-F	5'-GTGG <u>GCTAGC</u> AGATCGAGCGGTTGCGCTGCG-3'	This study
prtN2-up-R	5'-CTGAGATCTCGCGCCGATCGTCCCGTTGTC-3'	This study
prtN2-down-F	5'-GTCAGATCTGCGGGCGTCAGCAATCGAGCT-3'	This study
prtN2-down-R	5'-GTGAAGCTTAGCTGTTCGATCGGCTGGGTCG-3'	This study
prtR-up-F	5'-GAGGCTAGCCGCCGATACGAAGGGGCGGAG-3'	This study
prtR-up-R	5'-CGCAGATCTACCCATTAACAATTGACGCGCC-3'	This study
prtR2-down-F	5'-GCAGATCTGAAGTAACGCATCCGCGGGGTG-3'	This study
prtR2-down-R	5'-GTGAAGCTTAGCGCGACAGCTACACGTCGG-3'	This study
recA-up-F	5'-GTGGCTAGCTGACCGGATGGATCTGGCGCG-3'	This study
recA-up-R	5'-CTCAGATCTCAGCGTCTGTTGGAGGCGCGC-3'	This study
recA-down-F	5'-GTGAGATCTGAGTGATGGTTGCGCGCCGAG-3'	This study
recA-down-R	5'-GTGAAGCTTACGGGCTCGGATAGCGGCATG-3'	This study
bctN-comp-F	5'-CACGGATCCTGACTGAGTTTGCGGAGACC-3'	This study
bctN-comp-R	5'-GAGTCTAGAGCGGGAGATCAGTGTGGGGG-3'	This study
bctR-comp-F	5'-CAGCTGCAGACGGCGCGTCAATTGTTAATGG-3'	This study
bctR-comp-R	5'-CTCGGATCCCACCCCGCGGATGCGTTACTTC-3'	This study
recA-comp-F	5'-CTCGGATCCGGAAGATAGCAAGAAGGGCTC-3'	This study
recA-comp-R	5'-CTCTCTAGACTCGGCGCGCAACCATCACTC -3'	This study

Added restriction site is underlined

PCR primers for regions flanking *bctR* were designed based on the annotated sequence of BC0425. To construct plasmid pMo130::*bctR2* used to delete *bctR* from BC0425, a 1.0 kb fragment upstream of *bctR* was amplified with primers prtR-up-F and prtR-up-R (Table 4.2) and cloned into MCS1 of plasmid pMo130, digested with *NheI* and *BglII*, to obtain plasmid pMo130::*bctR2*-up. The 1.0 kb fragment downstream of *bctR* was amplified using primers prtR2-down-F and prtR2-down-R (Table 4.2). The PCR product was cloned into pMo130::*bctR2*-up, digested with *BglII* and *HindIII*, to obtain pMo130::*bctR2*.

PCR primers for regions flanking *recA* were designed based on the annotated sequence of BC0425. To construct plasmid pMo130::*recA* used to delete *recA* from BC0425, a 1.0 kb fragment upstream of *recA* was amplified with primers recA-up-F and recA2-up-R (Table 4.2) and cloned into MCS1 of plasmid pMo130, digested with *NheI* and *BglII*, to obtain plasmid pMo130::*recA*-up. The 1.0 kb fragment downstream of *recA* was amplified using primers recA-down-F and recA-down-R (Table 4.2). The PCR product was cloned into pMo130::*recA*-up, digested with *BglII* and *HindIII*, to obtain pMo130::*recA*.

PCR reactions were conducted for 30 cycles and parameters as follows: denaturation at 95°C for 1 min; annealing at 64°C, 63°C, 64°C, 63°C, 64°C, 63°C for primer sets prtN2-up, prtN2-down, prtR-up, prtR2-down, recA-up, and recA-down, respectively; and extension at 72°C for 1 min. All constructed plasmids were confirmed by restriction endonuclease digestion. All plasmid constructs were performed using *E. coli* DH5 α MCR as a host. Plasmid constructs were introduced by chemical

transformation (92), followed by selection on LB agar amended with 30 µg/ml Km at 30°C.

Site-directed mutagenesis. Plasmids pMo130::*bctN2*, pMo130::*bctR2*, and pMo130::*recA* were individually introduced into *B. cenocepacia* BC0425 by conjugation using triparental matings with pRK2013 as the mobilizing plasmid. Donor, mobilizer, and recipient suspensions were made in LB broth from cultures grown on solid media under selective conditions, as appropriate for 18 h. Bacterial suspensions were adjusted spectrophotometrically ($A_{600} = 0.5$), mixed at an equal ratio (1:1:1), and transferred to a positively charged sterile membrane layered on a 100 x 15 mm LB agar petri dish. Following an 18 h incubation period at 37°C, the cells from the mating and respective controls were washed twice in phosphate buffer (0.125 M, pH 7.1) by centrifugation (12,096 X *g* for 10 min at 5°C). The bacterial pellets were suspended in phosphate buffer and dilution plated to LB agar plates amended with 600 µg/ml Km and 20 µg/ml Tc for selection of single crossover events. Tranconjugants were sprayed with 0.45 M pyrocatechol to identify colonies in which single crossover events had occurred. Co-integrates exhibited a yellow color because, *xylE* encodes for catechol 2,3-dioxygenase, which catalyzes the ring cleavage of pyrocatechol and converts the colorless substrate to an intensely yellow hydroxymuconic semialdehyde. Single yellow colonies were grown in YT broth medium for five hours and then plated to YTSA to select for resolved co-integrants. The colonies were once again sprayed with pyrocatechol. Presumptive resolved co-integrants should exhibit a white phenotype. To

confirm gene deletion, co-integrants were analyzed by PCR using multiple primer combinations, both internal and external to the target gene deletion.

Complementation experiments. Plasmids pMo168::*bctN*-comp, pMo168::*bctR*-comp and pMo168::*recA*-comp were individually introduced into strain BC0425 Δ *bctN*, BC0425 Δ *bctR* and BC0425 Δ *recA*, respectively, by conjugation using triparental matings with pRK2013 as the mobilizing plasmid. The *bctN* gene was amplified using primers bctN-comp-F and bctN-comp-R (Table 4.2) and cloned into MCS2 of plasmid pMo168, digested with *Bam*HI and *Xba*I, to obtain plasmid pMo168::*bctN*-comp. Triparental matings (1:1:1) with selection on LB media supplemented with 600 μ g/ml Km and 20 μ g/ml Tc were performed as described above to mobilize pMo168::*bctN*-comp into BC0425 Δ *bctN*2. Transconjugants were sprayed with 0.45 M pyrocatechol to identify colonies which contained the pMo168 derivative plasmid.

The *bctR* was amplified using primers bctR-comp-F and bctR-comp-R (Table 4.2) and cloned into MCS2 of plasmid pMo168, digested with *Bam*HI and *Pst*I, to obtain plasmid pMo168::*bctR*-comp. Triparental matings (1:1:1) with selection on LB media supplemented with 600 μ g/ml and 20 μ g/ml Tc were performed as described above to mobilize pMo168::*bctR*-comp into BC0425 Δ *bctR*2. Transconjugants were sprayed with 0.45 M pyrocatechol to identify colonies which contained the pMo168 derivative plasmid.

The *recA* gene was amplified using primers recA-comp-F and recA-comp-R (Table 4.2) and cloned into MCS2 of plasmid pMo168, digested with *Bam*HI and *Xba*I,

to obtain plasmid pMo168::*recA*-comp. Triparental matings (1:1:1) with selection on LB media supplemented with 600 µg/ml Km and 20 µg/ml Tc were performed as described above to mobilize pMo168::*recA*-comp into BC0425 Δ *recA*. Transconjugants were sprayed with 0.45 M pyrocatechol to identify colonies that contained the pMo168 derivative plasmid.

PCR reactions were conducted for 30 cycles and parameters as follows: denaturation at 95°C for 1 min; annealing at 62°C, 64°C, and 62°C for primer sets *bctN*-comp, *bctR*-comp, and *recA*-comp, respectively; and extension at 72°C for 1 min. All constructed plasmids were confirmed by restriction endonuclease digestion. All plasmid constructs were performed using *E. coli* DH5 α MCR as a host. Plasmid constructs were introduced by chemical transformation, followed by selection on LB agar amended with 30 µg/ml Km at 30°C.

Growth curve experiments. Deletion mutants, BC0425 Δ *bctN*, BC0425 Δ *bctR*, BC0425 Δ *recA* and BC0425-WT were UV induced and their growth followed by recording the optical density at 600 nm (OD₆₀₀) to observe for lysis, if any. Suspensions were made in 20 ml TN broth from cultures grown on TNA under selective conditions, as appropriate for 18 h at 37°C. Bacterial suspensions were adjusted spectrophotometrically (A₆₀₀= 0.08-0.1) and shaken at 170-200 rpm at 37 °C. Once cultures attained an OD₆₀₀= 0.5, they were centrifuged at 7741 X *g* for 10 min at 4 °C and the pellet was resuspended in 10 ml of 0.85% NaCl. Centrifugation was repeated and pellets were resuspended in 10 ml of 0.85% NaCl. The 10 ml suspensions were then aliquoted to individual sterile petri plates and UV irradiated (400µw/cm²/sec) for 7 sec.

UV irradiated cells were transferred to a 500 ml Erlenmeyer flask with 10 ml 2X TN broth and 30 ml TN broth and grown at 37 ° C (180 rpm) with the OD₆₀₀ sampled and recorded at 60 min intervals.

Tailocin activity quantification. Deletion mutants, BC0425 Δ *bctN*, BC0425 Δ *bctR*, BC0425 Δ *recA* and BC0425-WT were UV induced as described above.

Suspensions were made in 20 ml TN broth from cultures grown on solid media under selective conditions, as appropriate for 18 h. Bacterial suspensions were adjusted spectrophotometrically (A_{600} = 0.08-0.1) and shaken at 170-200 rpm at 37 °C. Once cultures obtained an A_{600} = 0.5, they were centrifuged (7741 X g) for 10 min at 4 °C and the pellet was resuspended in 10 ml of 0.85% NaCl. Centrifugation was repeated and pellets were resuspended in 10 ml of 0.85% NaCl. The 10 ml suspensions were then aliquoted to individual petri plates and UV irradiated (400 μ w/cm²/sec) for 7 sec. UV irradiated cells were combined in a 500 ml flask with 10 ml 2X TN broth and 30 ml TN broth and incubated at 37 ° C (200 rpm). After 4 h, the cultures were centrifuged and the supernatant filter-sterilized. The supernatant was assayed for tailocin activity using the overlay spot assay and a plate assay.

The overlay spot assay was used to assay for tailocin activity as described in Chapter II. Serial dilutions (1:10) of preparations were spotted (10 μ l) onto TNSA seeded with *B. cenocepacia* PC184, allowed to dry and incubated at 37°C. After 18h plates were accessed for zones of inhibition. Zones of inhibition were indicative of tailocin activity.

For the tailocin plate assay, colonies were short-streaked onto TNA contained in a glass petri plate and incubated at 37°C. After 18 h plates were exposed to chloroform vapor by adding 2 ml of chloroform to the lid of the plate and inverting the plate on top of the lid for 20 min and then ventilated for an additional 30 min to remove vapors. The plates were then overlayed with TNSA, seeded with *B. cenocepacia* strain PC184, allowed to harden, and incubated at 37°C. After 18 h plates were evaluated for zones of inhibition.

Transmission Electron Microscopy (TEM). TEM was performed using high titer lysates, with 10^{11} tailocin/ml spotted onto 400 mesh carbon-coated copper grids and negatively stained with 2% (wt/vol) uranyl acetate. The samples were visualized with a JEOL 1200EX at an accelerated voltage of 100 kV.

RESULTS

Identification of transcriptional regulators. Studies of PAO1 strains defective in the synthesis of R2, F2, and S2 pyocins, together with complementation tests, allowed two regulatory genes, *priN* and *priR*, to be identified upstream of the R2-F2 pyocin genes in PAO1 (75). Based on the genetic organization of R2-pyocins of *P. aeruginosa*, identification of potential transcriptional regulators was conducted by analyzing the Bcep0425 tailocin cluster for genes located upstream of the tailocin structural genes and those which were transcribed in the opposite direction. Based on this criteria, two

putative genes, *bctN* and *bctR*, were identified and their homology to known repressors and transcriptional activators was determined (Fig. 4.1 A).

The predicted *bctN* gene product was composed of 98 amino acid residues and did not exhibit any significant homology to any known protein sequences (Fig. 4.1 C). This was consistent with the identified *priN* gene of *P. aeruginosa* strain PAO1. The *bctN* showed homology to hypothetical proteins found in other *Burkholderia* strains (Fig. 4.1 C). The predicted *bctR* gene product was composed of 163 amino acid residues and contained a helix-turn-helix DNA-binding domain, which is a characteristic of known repressors (2, 15, 76). BctR showed significant homology to known transcriptional regulators found within *Burkholderia* and *Pseudomonas* strains, such as *B. glumae* (Bg), *B. multivorans* CGD1 (Bm) and *P. fluorescens* (Pf) (Fig. 4.1 B).

Targeted mutagenesis of putative transcriptional regulators. Using the allelic exchange system developed by Hamad et al. (41), deletion mutants were constructed of the putative regulatory genes, *bctN* and *bctR*, of BC0425. The *recA*, which is necessary for pyocin production, was identified in the BC0425 sequence based on homology to *B. cenocepacia* J2315, and a deletion mutant of this gene was also constructed to determine its effect on tailocin production and lysis.

BC0425-WT, BC0425 Δ *bctN*, BC0425 Δ *bctR*, and BC0425 Δ *recA* were UV induced and their optical densities monitored to determine how each deletion would affect lysis. Lysis of BC0425-WT coincides with a sudden drop in OD₆₀₀ and release of tailocin to its surroundings, as determined by the overlay spot assay. Lysis of Δ *bctR* and Δ *bctN* mutants was delayed by 60 min as compared to BC0425-WT (Fig. 4.2) with

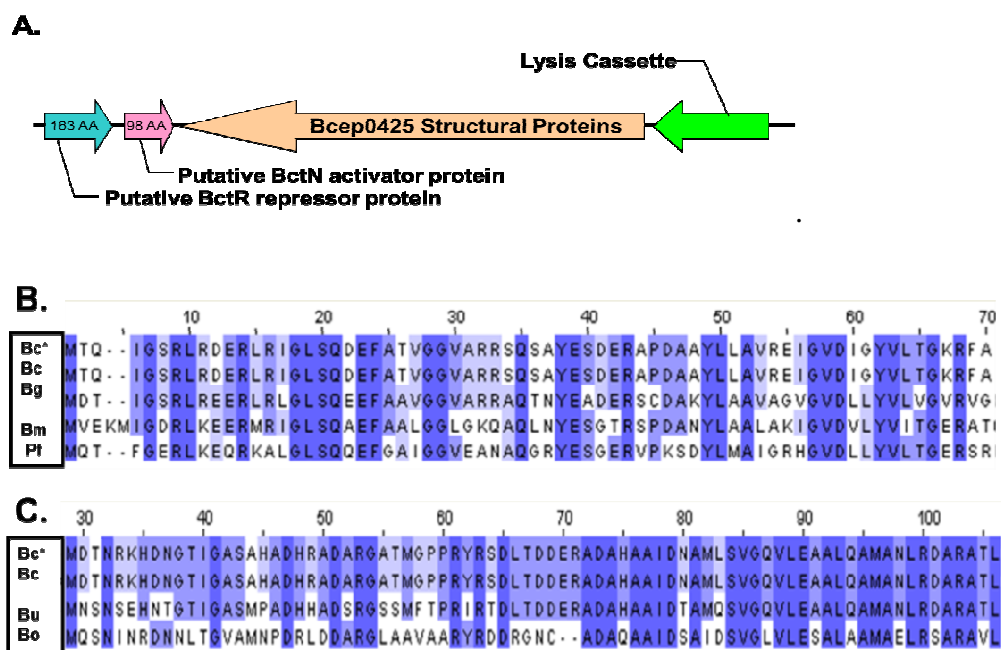


Fig. 4.1. Identification of Bcep0425 transcriptional regulators, *bctN* and *bctR*. (A) Transcriptional direction of putative *bctR* and *bctN* relative to Bcep0425 structural proteins and lysis cassette. Genes are not drawn to scale. (B) Amino acid sequence alignment comparison of Bcep0425 (Bc*) BctR to transcriptional regulatory proteins. N-terminal amino acid sequence of BctR exhibits homology to N-terminal parts of a hypothetical phage protein of *B. cenocepacia* J2315 (Bc), a transcriptional regulator from the XRE family in *B. glumae* BGR1 (Bg), a helix-turn-helix domain protein in *B. multivorans* CGD1 (Bm) and a Cro/CI family transcriptional regulator protein in *P. fluorescens* (Pf). (C) Amino acid sequence alignment comparison of Bcep0425 (Bc*) BctN to hypothetical proteins. N-terminal amino acid sequence of BctN exhibits homology to N-terminal parts of a hypothetical phage protein of *B. cenocepacia* J2315 (Bc), a hypothetical protein of *B. ubonensis* Bu (Bu), and a hypothetical protein of *B. oklahomensis* (Bo).

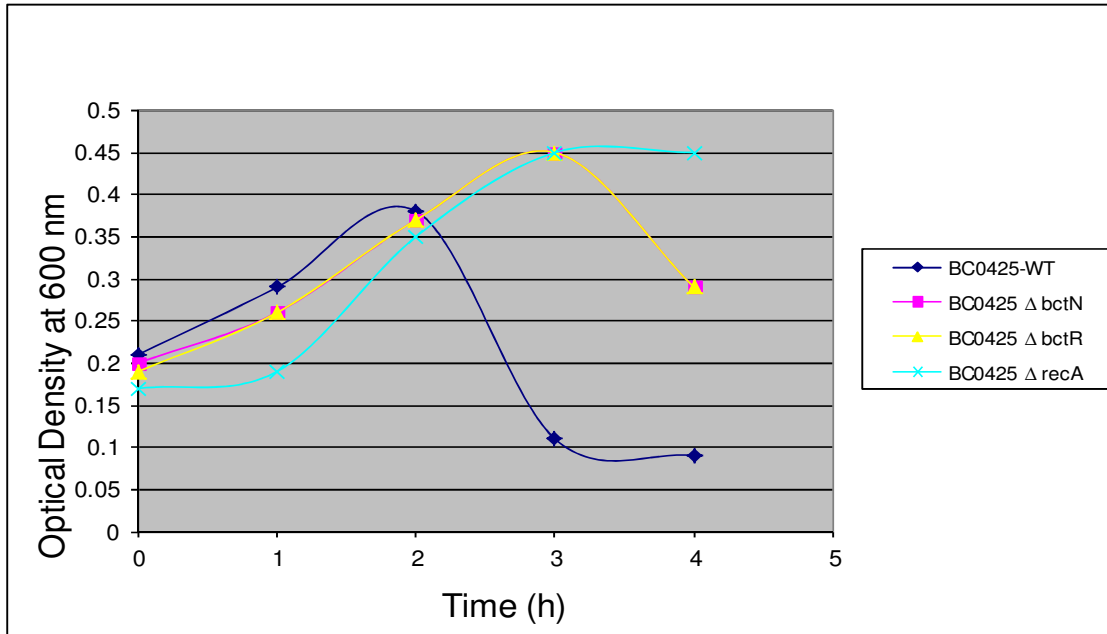


Fig. 4.2. Growth curve of BC0425 and *bctR*, *bctN*, and *recA* mutants post-UV induction. Lysis of BC0425-WT coincides with a sudden drop in OD₆₀₀. The OD₆₀₀ was sampled and recorded at 60 min intervals. Results are representative of duplicate experiments.

lysis not observed until 3 h post-UV induction as compared to BC0425-WT, which occurred 2 h post-induction (Fig. 4.2). Lysis for the $\Delta bctR$ and $\Delta bctN$ mutants was not expected, since both were hypothesized to be involved in tailocin induction and production. The observed lysis at 3 h post-UV induction for the $\Delta bctR$ and $\Delta bctN$ mutants is proposed to have occurred as a result of a temperate phage (designated Bcep315; no host identified to date) that was identified in UV induced lysates of strain BC0425 by electron microscopy (Fig.4.3). Lysis was not observed for the BC0425 $\Delta recA$ mutant (Fig. 4.2). This was the same observation made for R-pyocins where the *recA* has been shown to be essential for both phage and pyocin production.

Tailocin activity of *bctR*, *bctN*, and *recA* mutants. Regulation of tailocin Bcep0425 was thought to be similar to that of the R2-type pyocins produced by PAO1. BC0425-wild type (WT), BC0425 $\Delta bctN$, BC0425 $\Delta bctR$, and BC0425 $\Delta recA$ were UV induced and the lysates from cultures were assayed for tailocin activity using both the plate assay and overlay spot assay. When detecting tailocin activity, the plate assay was found to be more sensitive than the overlay spot assay. The supernatant of UV induced BC0425-WT exhibited tailocin activity of 10^{12} tailocins/ ml and the plate assay showed a small zone of inhibition, which indicates a positive score for tailocin activity (Table 4.3). If Bcep0425 was regulated similarly to pyocins, then the deletion of the putative activator gene (*brtN*) should result in no tailocin production in UV induced cultures. In Table 4.3, no tailocin activity was observed in BC0425 $\Delta bctN$ lysates using either the plate assay or overlay spot assay. This was expected since the PrtN was no longer able to activate the tailocin genes. A similar result was observed with the induced

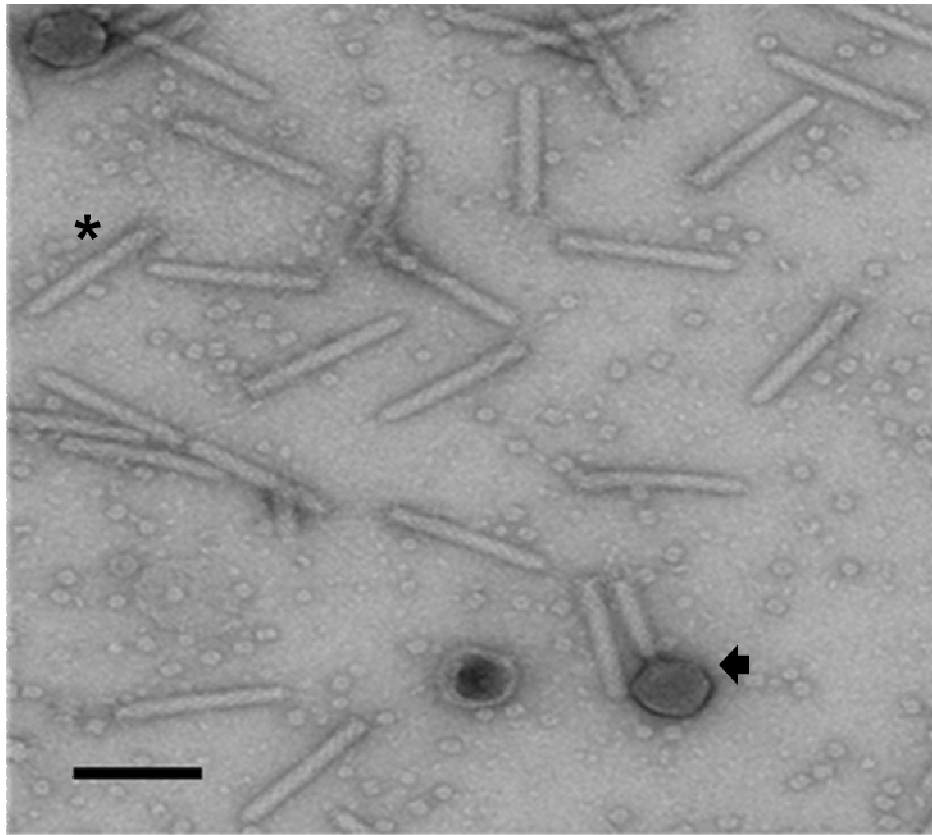


Fig. 4.3. Electron micrograph of lysate from UV treated BC0425. Tailocin Bcep0425 can be observed along with temperate phage Bcep315 produced by BC0425. The solid arrow and asterisk indicates phage Bcep315 and tailocin Bcep0425, respectively. Sample was stained with 2% (wt/vol) aqueous uranyl acetate. Bar = 100 nm

Table 4.3. Tailocin activity of *bctR*, *bctN*, and *recA* mutants

Isolate	Overlay Spot assay (# of tailocin)	Plate Assay
BC0425 Δ <i>bctN2</i>	0	-
BC0425 Δ <i>bctR2</i>	0	+
BC0425 Δ <i>recA</i>	0	-
BC0425-WT	10^{12}	+

+/- indicates tailocin activity

culture of the BC0425 Δ *recA* strain (Table 4.3). Tailocin activity was also not observed using either of the plate assay or overlay spot assay. The deletion of the putative repressor (*brtR*) should have resulted in constitutive tailocin production, since BctR is no longer repressing transcription of the *bctN* activator gene. This was not observed in BC0425 Δ *bctR* induced lysates. No tailocin activity was detected using the overlay spot assay, but was detected using the more sensitive plate assay (Table 4.3). These results indicate that the BctR may not only be acting as a repressor, but an activator of tailocin genes as well.

Complementation of *bctR*, *bctN*, and *recA* mutants. In *trans* complementation of BC0425 Δ *bctR*, BC0425 Δ *bctN* and BC0425 Δ *recA* deletion mutants was achieved using plasmids pMo168-*bctR*-comp, pMo168-*bctN*-comp and pMo168-*recA*-comp, respectively. BC0425-WT, BC0425 Δ *bctN*-comp, BC0425 Δ *bctR*-comp, and BC0425 Δ *recA*-comp were UV induced and the lysates from cultures were assayed for tailocin activity using both the plate and overlay spot assays. In *trans* complementation of individual mutants restored Bcep0425 tailocin activity to the BC0425 Δ *bctN*, BC0425 Δ *bctR*, and BC0425 Δ *recA* mutants (Table 4.4).

DISCUSSION

The SOS regulatory system of *E. coli* is a set of responses to agents that threaten the cell by disrupting the structure or synthesis of its DNA (69, 115). The SOS regulatory system is controlled by the action of the LexA repressor, which regulates a set

Table 4.4. Tailocin activity of *bctR*, *bctN*, and *recA* complements

Isolate	Overlay Spot Assay (# of tailocin)	Plate Assay
BC0425 Δ <i>bctN</i>- comp	10^{10}	+
BC0425 Δ <i>bctR</i>- comp	10^9	+
BC0425 Δ <i>recA</i>- comp	10^{11}	+
BC0425-WT	10^{12}	+

+/- indicates tailocin activity

of ~20 genes during normal growth, and the RecA protein, which is required for inactivation of the LexA repressor after treatments that damage DNA (i.e. UV irradiation) (69, 115). RecA is required for the autodigestion of the LexA repressor (67). LexA repressor is inactivated by specific cleavage of an Ala-Gly bond (44, 68, 70). Phage repressor inactivation is similarly mediated by cleavage at an Ala-Gly bond near the center of the polypeptide chain by activated RecA (23, 85). The production of pyocins in *P. aeruginosa* is inducible by treatments that cause DNA damage and synthesis of pyocins is effectively dependent on the *recA* gene (93). The LexA repressor does not seem to be involved in pyocin synthesis unlike the *prtN* activator gene and *prtR* repressor gene identified by Matsui et al. (75). In this Chapter we provided evidence for the involvement of RecA and two putative transcriptional regulators, BctN and BctR, in the production of tailocin Bcep0425.

The deletion of *recA* and *bctN* in BC0425 did not result in tailocin production, since each was expected to be required for the activation of tailocin Bcep0425 genes, based on observed results with pyocins R2 and F2 in *P. aeruginosa* PA01 (Table 4.3). Based on the regulation of pyocins in *P. aeruginosa*, the deletion of the putative repressor (*brtR*) should have resulted in constitutive tailocin production, since BctR is no longer repressing transcription of the *bctN* activator gene. This was not observed for the BC0425 Δ *bctR* mutant (Table 4.3). In fact, we observed the opposite effect and minimal amounts of tailocin were detected.

There are three characteristic features of known repressor proteins involved in the SOS regulation of genes (i) The N-terminal sequence contains a helix-turn-helix

domain necessary for binding DNA (ii) The presence of an Ala-Gly (Cys-Gly in the $\Phi 80$ cI repressor) to be cleaved by the activated RecA protein (28, 44, 94) and (iii) A Gly-Asn-Ser-Met sequence that is conserved among phage and LexA repressors and proposed to be involved in the cleavage of these repressors (102). The BctR contains two of the three characteristic features of known repressors. The BctR contains a helix-turn-helix domain and contains an Ala-Gly sequence at residue 76 (Fig. 4.1). This provides evidence that BctR may be acting as a repressor.

Repressors of transcription have been recognized as central to the regulation of gene expression in prokaryotes. Their function was initially thought to be limited specifically to the turn-off of transcription. This view has changed since it became known that the λ repressor could serve as an activator and the cAMP receptor protein, a known activator, could serve as a repressor of transcription (73, 86, 106). Our results indicate that the putative repressor, BctR, may not only be acting to repress *bctN*, but may be involved in the activation of tailocin genes as well.

The results presented in this study were not consistent with the regulation model proposed for the pyocins of *P. aeruginosa*, where BctR acts to repress *bctN*, which in turn activates pyocin genes. Future studies will focus on determining the exact role of *recA*, *bctN* and *bctR* in the induction process of tailocin Bcep0425 genes.

CHAPTER V

CONCLUSION

The *Burkholderia cepacia* complex (Bcc) comprises a group of Gram negative, phenotypically similar species (21, 109). Bcc organisms are extremely diverse and versatile in their metabolic capabilities. While Bcc bacteria can be involved in beneficial interactions with plants, they are also considered opportunistic pathogens in immunocompromised patients, specifically those with cystic fibrosis (9, 34, 63). Essentially all Bcc isolates demonstrate *in vitro* broad-spectrum antibiotic resistance. In fact, many clinical isolates are resistant to all currently available antibiotics, rendering antibiotic therapy ineffective. With the emergence of multi-drug resistant bacteria, the potential use of phage-tail-like high molecular weight bacteriocins, or "tailocins", as alternative anti-bacterial agents against bacterial infections is being reexamined.

In this study, we have identified a broad host range tailocin from *B. cenocepacia* strain BC0425, designated Bcep0425. We have determined the genetic organization of the Bcep0425 tailocin cluster and have identified the major structural proteins and lysis cassette (Chapter II). We have reported for the first time genes involved in replication and integration that are associated with a pyocin/tailocin gene cluster. This provides evidence that tailocin Bcep0425 is a defective phage. The identified gene products of the Bcep0425 tailocin cluster showed homology to *Burkholderia* phage KL3 (Chapter II). Tailocin Bcep0425 and phage KL3 are also organized similarly, except Bcep0425 does not contain genes involved in capsid formation or DNA modification. The

significant homology and similar gene order between Bcep0425 and phage KL3 provides evidence that Bcep0425 may be a derivative of phage KL3 or related P2-like phage. The presence of a lysis gene cassette similar to those for bacteriophages is also supporting evidence that tailocin Bcep0425 is a defective phage (Chapter II). The function of the Bcep0425 holin, along with the antiholin and endolysin, were confirmed using mutational analysis. Upon examination of the lysis cassette a new class of holin, which contains 4 TMDs with a N-in and C-in topology, was identified (Class IV).

Tailocin Bcep0425 was shown to inhibit 70% of *Burkholderia* isolates tested and strains of *Pseudomonas aeruginosa* and *Escherichia coli* (Chapter III). The Bcep0425 receptor site was determined to be part of the outer core component of the bacterial LPS (Chapter III). The testing of the sensitivity of genetically defined LPS mutants, of *P. aeruginosa* and *B. cenocepacia*, confirmed that L-rhamnose and α -glucose are the Bcep0425 receptor sites (Chapter III).

In Chapter IV we provide evidence for the involvement of RecA and two novel transcriptional regulators, BctN and BctR, in the production of tailocin Bcep0425. The results presented in Chapter IV were not consistent with the regulation model proposed for the pyocins of *P. aeruginosa*, where BctR acts to repress *bctN*, which in turn activates pyocin genes. Our results indicated that the putative repressor, BctR, may not only be acting to repress *bctN*, but may also be involved in the activation of tailocin genes as well. Future studies will focus on determining the exact role of *recA*, *bctN* and *bctR* in the induction process of tailocin Bcep0425 genes.

REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**:3389-3402.
2. **Anderson, W. F., D. H. Ohlendorf, Y. Takeda, and B. W. Matthews.** 1981. Structure of the *cro* repressor from bacteriophage lambda and its interaction with DNA. *Nature* **290**:754-758.
3. **Apicella, M. A., J. M. Griffiss, and H. Schneider.** 1994. Isolation and characterization of lipopolysaccharides, lipooligosaccharides, and lipid A. *Meth. Enzymol.* **235**:242-252.
4. **Aravind, L., D. D. Leipe, and E. V. Koonin.** 1998. Toprim--a conserved catalytic domain in type IA and II topoisomerases, DnaG-type primases, OLD family nucleases and RecR proteins. *Nucleic Acids Res.* **26**:4205-4213.
5. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G., Seidman, J. A. Smith, K. Struhl** (ed). 1987. *Current protocols in molecular biology*, John Wiley & Sons, Inc., New York.
6. **Bakkal, S., S. M. Robinson, C. L. Ordonez, D. A. Waltz, and M. A. Riley.** 2010. Role of bacteriocins in mediating interactions of bacterial isolates taken from cystic fibrosis patients. *Microbiol.* **156**:2058-2067.
7. **Barik, S., and D. M. Munnecke.** 1982. Enzymatic hydrolysis of concentrated diazinon in soil. *Bulln. Environ. Contam. Toxicol.* **29**:235-239.
8. **Bernhardt, T. G., W. D. Roof, and R. Young.** 2000. Genetic evidence that the bacteriophage phi X174 lysis protein inhibits cell wall synthesis. *Proc. Natl. Acad. Sci. USA* **97**:4297-4302.
9. **Berriatua, E., I. Ziluaga, C. Miguel-Virto, P. Uribarren, R. Juste, S. Laevens, P. Vandamme, and J. R. Govan.** 2001. Outbreak of subclinical

mastitis in a flock of dairy sheep associated with *Burkholderia cepacia* complex infection. *J. Clin. Microbiol.* **39**:990-994.

10. **Berry, J., C. Savva, A. Holzenburg, and R. Young.** 2010. The lambda spanin components Rz and Rz1 undergo tertiary and quaternary rearrangements upon complex formation. *Prot. Sci.* **19**:1967-1977.
11. **Bertani, G.** 1951. Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *J. Bacteriol.* **62**:293-300.
12. **Besemer, J., and M. Borodovsky.** 1999. Heuristic approach to deriving models for gene finding. *Nucleic Acids Res.* **27**:3911-3920.
13. **Boeckmann, B., A. Bairoch, R. Apweiler, M. C. Blatter, A. Estreicher, E. Gasteiger, M. J. Martin, K. Michoud, C. O'Donovan, I. Phan, S. Pilbout, and M. Schneider.** 2003. *Nucleic Acids Res.* **31**:365-370.
14. **Bradley, D. E.** 1967. Ultrastructure of bacteriophage and bacteriocins. *Bacteriol Rev* **31**:230-314.
15. **Brennan, R. G., and B. W. Matthews.** 1989. Structural basis of DNA-protein recognition. *Trends Biochem. Sci.* **14**:286-290.
16. **Burrows, L. L., D. F. Charter, and J. S. Lam.** 1996. Molecular characterization of the *Pseudomonas aeruginosa* serotype O5 (PAO1) B-band lipopolysaccharide gene cluster. *Mol. Microbiol.* **22**:481-495.
17. **Caroff, M., and D. Karibian.** 2003. Structure of bacterial lipopolysaccharides. *Carbohydr. Res.* **338**:2431-2447.
18. **Carver, T. J., K. M. Rutherford, M. Berriman, M. A. Rajandream, B. G. Barrell, and J. Parkhill.** 2005. ACT: the Artemis Comparison Tool. *Bioinformatics* **21**:3422-3423.
19. **Chatterjee, S. N., and M. Maiti.** 1984. Vibriophages and vibriocins: physical, chemical, and biological properties. *Adv. Virus Res.* **29**:263-312.

20. **Coenye, T., and P. Vandamme.** 2003. Diversity and significance of *Burkholderia* species occupying diverse ecological niches. *Environ. Microbiol.* **5**:719-729.
21. **Coenye, T., P. Vandamme, J. R. Govan, and J. J. LiPuma.** 2001. Taxonomy and identification of the *Burkholderia cepacia* complex. *J. Clin. Microbiol.* **39**:3427-3436.
22. **Cox, A. D., and S. G. Wilkinson.** 1991. Ionizing groups in lipopolysaccharides of *Pseudomonas cepacia* in relation to antibiotic resistance. *Mol. Microbiol.* **5**:641-646.
23. **Craig, N. L., and J. W. Roberts.** 1980. *E. coli recA* protein-directed cleavage of phage lambda repressor requires polynucleotide. *Nature* **283**:26-30.
24. **Dawes, J.** 1975. Characterisation of the bacteriophage T4 receptor site. *Nature* **256**:127-128.
25. **Dinwiddie, R.** 2000. Pathogenesis of lung disease in cystic fibrosis. *Respiration* **67**:3-8.
26. **Doermann, A. H.** 1952. The intracellular growth of bacteriophages. I. Liberation of intracellular bacteriophage T4 by premature lysis with another phage or with cyanide. *J. Gen. Physiol.* **35**:645-656.
27. **Dykes, G. A.** 1995. Bacteriocins - Ecological and Evolutionary Significance. *Trends Ecol. Evol.* **10**:186-189.
28. **Eguchi, Y., T. Ogawa, and H. Ogawa.** 1988. Two forms of RecA-single-stranded DNA-adenosine 5'-O-(3-thiotriphosphate) complexes with different activities for cleavage of phage phi 80 cI repressor. *J. Mol. Biol.* **204**:61-67.
29. **Esakowitz, L., S. D. Cook, J. Adams, D. Doyle, K. W. Grossart, P. Macpherson, and R. M. McFadzean.** 1987. Rhino-orbital-cerebral mucormycosis--a clinico-pathological report of two cases. *Scot. Med. J.* **32**:180-182.

30. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proc. Natl. Acad. Sci. USA* **76**:1648-1652.
31. **Foster, S. J.** 1991. Cloning, expression, sequence analysis and biochemical characterization of an autolytic amidase of *Bacillus subtilis* 168 trpC2. *J. Gen. Microbiol.* **137**:1987-1998.
32. **Gamsjaeger, R., C. K. Liew, F. E. Loughlin, M. Crossley, and J. P. Mackay.** 2007. Sticky fingers: zinc-fingers as protein-recognition motifs. *Trends Biochem. Sci.* **32**:63-70.
33. **Garrett, J., R. Fusselman, J. Hise, L. Chiou, D. Smith-Grillo, J. Schulz, and R. Young.** 1981. Cell lysis by induction of cloned lambda lysis genes. *Molecular & general genetics : Mol. Gen. Genet.* **182**:326-331.
34. **Gonzalez, C. F., E. A. Pettit, V. A. Valadez, and E. M. Provin.** 1997. Mobilization, cloning, and sequence determination of a plasmid-encoded polygalacturonase from a phytopathogenic *Burkholderia (Pseudomonas) cepacia*. *Mol. Plant-Microbe Interac.* **10**:840-851.
35. **Govan, J. R.** 1974. Studies on the pyocins of *Pseudomonas aeruginosa*: morphology and mode of action of contractile pyocins. *J. Gen. Microbiol.* **80**:1-15.
36. **Grambow, N. J., N. K. Birkeland, D. L. Anders, and G. E. Christie.** 1990. Deletion analysis of a bacteriophage P2 late promoter. *Gene* **95**:9-15.
37. **Haas, H., T. Sacks, and N. Saltz.** 1974. Protective effect of pyocin against lethal *Pseudomonas aeruginosa* infections in mice. *J. Infect. Dis.* **129**:470-472.
38. **Haggard-Ljungquist, E., C. Halling, and R. Calendar.** 1992. DNA sequences of the tail fiber genes of bacteriophage P2: evidence for horizontal transfer of tail fiber genes among unrelated bacteriophages. *J. Bacteriol.* **174**:1462-1477.
39. **Hall, T. M.** 2005. Multiple modes of RNA recognition by zinc finger proteins. *Curr. Opin. Struct. Biol.* **15**:367-373.

40. **Halling, C., and R. Calendar.** 1990. Bacteriophage P2 ogr and P4 delta genes act independently and are essential for P4 multiplication. *J. Bacteriol.* **172**:3549-3558.
41. **Hamad, M. A., S. L. Zajdowicz, R. K. Holmes, and M. I. Voskuil.** 2009. An allelic exchange system for compliant genetic manipulation of the select agents *Burkholderia pseudomallei* and *Burkholderia mallei*. *Gene* **430**:123-131.
42. **Hansen, J. B., and R. H. Olsen.** 1978. Isolation of large bacterial plasmids and characterization of the P2 incompatibility group plasmids pMG1 and pMG5. *J. Bacteriol.* **135**:227-238.
43. **Holden, M. T., H. M. Seth-Smith, L. C. Crossman, M. Sebahia, S. D. Bentley, A. M. Cerdono-Tarraga, N. R. Thomson, N. Bason, M. A. Quail, S. Sharp, I. Cherevach, C. Churcher, I. Goodhead, H. Hauser, N. Holroyd, K. Mungall, P. Scott, D. Walker, B. White, H. Rose, P. Iversen, D. Mil-Homens, E. P. Rocha, A. M. Fialho, A. Baldwin, C. Dowson, B. G. Barrell, J. R. Govan, P. Vandamme, C. A. Hart, E. Mahenthiralingam, and J. Parkhill.** 2009. The genome of *Burkholderia cenocepacia* J2315, an epidemic pathogen of cystic fibrosis patients. *J. Bacteriol.* **191**:261-277.
44. **Horii, T., T. Ogawa, T. Nakatani, T. Hase, H. Matsubara, and H. Ogawa.** 1981. Regulation of SOS functions: purification of *E. coli* LexA protein and determination of its specific site cleaved by the RecA protein. *Cell* **27**:515-522.
45. **Ikeda, K.** 1973. [Receptor of pyocin R (author's transl)]. Tanpakushitsu kakusan koso. Prot., Nucl. Acid, Enz. **18**:603-610.
46. **Imada, M., and A. Tsugita.** 1971. Amino-acid sequence of lambda phage endolysin. *Nature: New Biol.* **233**:230-231.
47. **Ishii, S. I., Y. Nishi, and F. Egami.** 1965. The fine structure of a pyocin. *J. Mol. Biol.* **13**:428-431.
48. **Isshiki, Y., K. Kawahara, and U. Zahringer.** 1998. Isolation and characterisation of disodium (4-amino-4-deoxy-beta-L- arabinopyranosyl)-(1-->8)-(D-glycero-alpha-D-talo-oct-2-ulopyranosylona te)- (2-->4)-(methyl 3-

deoxy-D-manno-oct-2-ulopyranosid)onate from the lipopolysaccharide of *Burkholderia cepacia*. Carbohydr. Res. **313**:21-27.

49. **Jacob, F.** 1954. Induced biosynthesis and mode of action of a pyocine, antibiotic produced by *Pseudomonas aeruginosa*. Ann. Inst. Pasteur **86**:149-160.
50. **Jarrell, K., and A. M. Kropinski.** 1977. The chemical composition of the lipopolysaccharide from *Pseudomonas aeruginosa* strain PAO and a spontaneously derived rough mutant. Microbios **19**:103-116.
51. **Josslin, R.** 1970. The lysis mechanism of phage T4: mutants affecting lysis. Virology **40**:719-726.
52. **Kageyama, M.** 1979. Structure and function of pyocins. Prot. Nucl. Acid Enz. **24**:871-880.
53. **Kageyama, M.** 1964. Studies of a Pyocin. I. Physical and chemical properties. J. Biochem. **55**:49-53.
54. **Kageyama, M., K. Ikeda, and F. Egami.** 1964. Studies of a pyocin III. Biological properties of the pyocin. J. Biochem. **55**:59-64.
55. **Kato, N., N. Kido, M. Ohta, and S. Naito.** 1985. Adjuvant activity of *Klebsiella* O3 lipopolysaccharide: inhibition of the adjuvant activity by concanavalin A. Microbiol. Immunol. **29**:205-211.
56. **Kedzierska, S., A. Wawrzynow, and A. Taylor.** 1996. The *RzI* gene product of bacteriophage lambda is a lipoprotein localized in the outer membrane of *Escherichia coli*. Gene **168**:1-8.
57. **Kerr, B., M. A. Riley, M. W. Feldman, and B. J. Bohannan.** 2002. Local dispersal promotes biodiversity in a real-life game of rock-paper-scissors. Nature **418**:171-174.

58. **Kohler, T., V. Donner, and C. van Delden.** 2010. Lipopolysaccharide as shield and receptor for R-pyocin-mediated killing in *Pseudomonas aeruginosa*. *J. Bacteriol.* **192**:1921-1928.
59. **Krogh, S., S. T. Jorgensen, and K. M. Devine.** 1998. Lysis genes of the *Bacillus subtilis* defective prophage PBSX. *J. Bacteriol.* **180**:2110-2117.
60. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680-685.
61. **Lee, T. C., and G. E. Christie.** 1990. Purification and properties of the bacteriophage P2 ogr gene product. A prokaryotic zinc-binding transcriptional activator. *J. Biol. Chem.* **265**:7472-7477.
62. **Lengyel, J. A., R. N. Goldstein, M. Marsh, and R. Calendar.** 1974. Structure of the bacteriophage P2 tail. *Virology* **62**:161-174.
63. **LiPuma, J. J.** 1998. *Burkholderia cepacia* epidemiology and pathogenesis: implications for infection control. *Curr. Opin. Pulm. Med.* **4**:337-341.
64. **LiPuma, J. J.** 1998. *Burkholderia cepacia*. Management issues and new insights. *Clin. Chest Med.* **19**:473-486, vi.
65. **LiPuma, J. J.** 2003. *Burkholderia cepacia* complex as human pathogens. *J. Nematol.* **35**:212-217.
66. **Lipuma, J. J.** 2010. The changing microbial epidemiology in cystic fibrosis. *Clin. Microbiol. Rev.* **23**:299-323.
67. **Little, J. W.** 1984. Autodigestion of *lexA* and phage lambda repressors. *Proc. Natl. Acad. Sci. USA.* **81**:1375-1379.
68. **Little, J. W., S. H. Edmiston, L. Z. Pacelli, and D. W. Mount.** 1980. Cleavage of the *Escherichia coli* *lexA* protein by the *recA* protease. *Proc. Natl. Acad. Sci. USA.* **77**:3225-3229.

69. **Little, J. W., and D. W. Mount.** 1982. The SOS regulatory system of *Escherichia coli*. *Cell* **29**:11-22.
70. **Little, J. W., D. W. Mount, and C. R. Yanisch-Perron.** 1981. Purified *lexA* protein is a repressor of the *recA* and *lexA* genes. *Proc. Natl. Acad. Sci. USA*. **78**:4199-4203.
71. **Lynch, K. H., P. Stothard, and J. J. Dennis.** 2010. Genomic analysis and relatedness of P2-like phages of the *Burkholderia cepacia* complex. *BMC Genomics* **11**:599.
72. **Mahenthiralingam, E., T. Coenye, J. W. Chung, D. P. Speert, J. R. Govan, P. Taylor, and P. Vandamme.** 2000. Diagnostically and experimentally useful panel of strains from the *Burkholderia cepacia* complex. *J. Clin. Microbiol.* **38**:910-913.
73. **Malan, T. P., and W. R. McClure.** 1984. Dual promoter control of the *Escherichia coli* lactose operon. *Cell* **39**:173-180.
74. **Manniello, J. M., H. Heymann, and F. W. Adair.** 1979. Isolation of atypical lipopolysaccharides from purified cell walls of *Pseudomonas cepacia*. *J. Gen. Microbiol.* **112**:397-400.
75. **Matsui, H., Y. Sano, H. Ishihara, and T. Shinomiya.** 1993. Regulation of pyocin genes in *Pseudomonas aeruginosa* by positive (*prtN*) and negative (*prtR*) regulatory genes. *J. Bacteriol.* **175**:1257-1263.
76. **Matthews, B. W., D. H. Ohlendorf, W. F. Anderson, and Y. Takeda.** 1982. Structure of the DNA-binding region of *lac* repressor inferred from its homology with *cro* repressor. *Proc. Natl. Acad. Sci. USA*. **79**:1428-1432.
77. **Merrikin, D. J., and C. S. Terry.** 1972. Use of pyocin 78-C2 in the treatment of *Pseudomonas aeruginosa* infection in mice. *Appl. Microbiol.* **23**:164-165.
78. **Michel-Briand, Y., and C. Baysse.** 2002. The pyocins of *Pseudomonas aeruginosa*. *Biochimie* **84**:499-510.

79. **Moore, J. E., and J. S. Elborn.** 2001. *Burkholderia cepacia* and cystic fibrosis--50 years on. Commun. Dis. Pub. Health **4**:114-116.
80. **Moore, R. A., and R. E. Hancock.** 1986. Involvement of outer membrane of *Pseudomonas cepacia* in aminoglycoside and polymyxin resistance. Antimicrob. Agents Chemother. **30**:923-926.
81. **Nakayama, K., K. Takashima, H. Ishihara, T. Shinomiya, M. Kageyama, S. Kanaya, M. Ohnishi, T. Murata, H. Mori, and T. Hayashi.** 2000. The R-type pyocin of *Pseudomonas aeruginosa* is related to P2 phage, and the F-type is related to lambda phage. Mol. Microbiol. **38**:213-231.
82. **Ohsumi, M., T. Shinomiya, and M. Kageyama.** 1980. Comparative study on R-type pyocins of *Pseudomonas aeruginosa*. J. Biochem. **87**:1119-1125.
83. **Ortega, X., A. Silipo, M. S. Saldias, C. C. Bates, A. Molinaro, and M. A. Valvano.** 2009. Biosynthesis and structure of the *Burkholderia cenocepacia* K56-2 lipopolysaccharide core oligosaccharide: truncation of the core oligosaccharide leads to increased binding and sensitivity to polymyxin B. The J. Biol. Chem. **284**:21738-21751.
84. **Pabo, C. O., R. T. Sauer, J. M. Sturtevant, and M. Ptashne.** 1979. The lambda repressor contains two domains. Proc. Natl. Acad. Sci. USA. **76**:1608-1612.
85. **Phizicky, E. M., and J. W. Roberts.** 1980. Kinetics of RecA protein-directed inactivation of repressors of phage lambda and phage P22. J. Mol. Biol. **139**:319-328.
86. **Ptashne, M., A. Jeffrey, A. D. Johnson, R. Maurer, B. J. Meyer, C. O. Pabo, T. M. Roberts, and R. T. Sauer.** 1980. How the lambda repressor and cro work. Cell **19**:1-11.
87. **Reader, R. W., and L. Siminovitch.** 1971. Lysis defective mutants of bacteriophage lambda: on the role of the S function in lysis. Virology **43**:623-637.

88. **Riley, M. A., and J. E. Wertz.** 2002. Bacteriocin diversity: ecological and evolutionary perspectives. *Biochimie* **84**:357-364.
89. **Riley, M. A., and J. E. Wertz.** 2002. Bacteriocins: evolution, ecology, and application. *Ann. Rev. Microbiol.* **56**:117-137.
90. **Rocchetta, H. L., L. L. Burrows, J. C. Pacan, and J. S. Lam.** 1998. Three rhamnosyltransferases responsible for assembly of the A-band D-rhamnan polysaccharide in *Pseudomonas aeruginosa*: a fourth transferase, WbpL, is required for the initiation of both A-band and B-band lipopolysaccharide synthesis. *Mol. Microbiol.* **28**:1103-1119.
91. **Rocchetta, H. L., J. C. Pacan, and J. S. Lam.** 1998. Synthesis of the A-band polysaccharide sugar D-rhamnose requires Rmd and WbpW: identification of multiple AlgA homologues, WbpW and ORF488, in *Pseudomonas aeruginosa*. *Mol. Microbiol.* **29**:1419-1434.
92. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning : a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
93. **Sano, Y., and M. Kageyama.** 1987. The sequence and function of the recA gene and its protein in *Pseudomonas aeruginosa* PAO. *Mol. Gen. Genet.* **208**:412-419.
94. **Sauer, R. T., M. J. Ross, and M. Ptashne.** 1982. Cleavage of the lambda and P22 repressors by recA protein. *J. Biol. Chem.* **257**:4458-4462.
95. **Scholl, D., M. Cooley, S. R. Williams, D. Gebhart, D. Martin, A. Bates, and R. Mandrell.** 2009. An engineered R-type pyocin is a highly specific and sensitive bactericidal agent for the food-borne pathogen *Escherichia coli* O157:H7. *Antimicrob. Agents Chemother.* **53**:3074-3080.
96. **Scholl, D., and D. W. Martin, Jr.** 2008. Antibacterial efficacy of R-type pyocins towards *Pseudomonas aeruginosa* in a murine peritonitis model. *Antimicrob. Agents Chemother.* **52**:1647-1652.

97. **Shaw, D., I. R. Poxton, and J. R. Govan.** 1995. Biological activity of *Burkholderia (Pseudomonas) cepacia* lipopolysaccharide. FEMS Immunol. Med. Microbiol. **11**:99-106.
98. **Shimizu, Y., T. Kumazaki, and S. I. Ishii.** 1982. Specific cleavage at fibers of a bacteriophage-tail-like bacteriocin, pyocin R1, by successive treatment with organomercurial compounds and trypsin. J. Virology **44**:692-695.
99. **Shimomura, H., M. Matsuura, S. Saito, Y. Hirai, Y. Isshiki, and K. Kawahara.** 2001. Lipopolysaccharide of *Burkholderia cepacia* and its unique character to stimulate murine macrophages with relative lack of interleukin-1 β -inducing ability. Infect. Immun. **69**:3663-3669.
100. **Shinomiya, T., and S. Shiga.** 1979. Bactericidal activity of the tail of *Pseudomonas aeruginosa* bacteriophage PS17. J. Virology **32**:958-967.
101. **Shinomiya, T., S. Shiga, and M. Kageyama.** 1983. Genetic determinant of pyocin R2 in *Pseudomonas aeruginosa* PAO. I. Localization of the pyocin R2 gene cluster between the trpCD and trpE genes. Mol. Gen. Genet. **189**:375-381.
102. **Slilaty, S. N., and J. W. Little.** 1987. Lysine-156 and serine-119 are required for LexA repressor cleavage: a possible mechanism. Proc. Natl. Acad. Sci. USA **84**:3987-3991.
103. **Sokol, P. A., P. Darling, D. E. Woods, E. Mahenthiralingam, and C. Kooi.** 1999. Role of ornibactin biosynthesis in the virulence of *Burkholderia cepacia*: characterization of pvdA, the gene encoding L-ornithine N(5)-oxygenase. Infect. Immun. **67**:4443-4455.
104. **Summer, E. J., J. Berry, T. A. Tran, L. Niu, D. K. Struck, and R. Young.** 2007. Rz/Rz1 lysis gene equivalents in phages of gram-negative hosts. J. Mol. Biol. **373**:1098-1112.
105. **Takeda, Y., and M. Kageyama.** 1975. Subunit arrangement in the extended sheath of pyocin R. J. Biochem. **77**:679-684.

106. **Taniguchi, T., M. O'Neill, and B. de Crombrughe.** 1979. Interaction site of *Escherichia coli* cyclic AMP receptor protein on DNA of galactose operon promoters. *Proc. Natl. Acad. Sci. USA.* **76**:5090-5094.
107. **Tsai, C. M., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal. Biochem.* **119**:115-119.
108. **Uratani, Y., and T. Hoshino.** 1984. Pyocin R1 inhibits active transport in *Pseudomonas aeruginosa* and depolarizes membrane potential. *J. Bacteriol.* **157**:632-636.
109. **Vanlaere, E., T. Coenye, E. Samyn, C. Van den Plas, J. Govan, F. De Baets, K. De Boeck, C. Knoop, and P. Vandamme.** 2005. A novel strategy for the isolation and identification of environmental *Burkholderia cepacia* complex bacteria. *FEMS Microbiol. Lett.* **249**:303-307.
110. **Ventura, M., C. Canchaya, D. Pridmore, B. Berger, and H. Brussow.** 2003. Integration and distribution of *Lactobacillus johnsonii* prophages. *J. Bacteriol.* **185**:4603-4608.
111. **Vinion-Dubiel, A. D., and J. B. Goldberg.** 2003. Lipopolysaccharide of *Burkholderia cepacia* complex. *J. Endotox. Res.* **9**:201-213.
112. **Vinogradov, E. V., K. Bock, B. O. Petersen, O. Holst, and H. Brade.** 1997. The structure of the carbohydrate backbone of the lipopolysaccharide from *Acinetobacter* strain ATCC 17905. *Eur. J. Biochem.* **243**:122-127.
113. **Vinogradov, E. V., B. Lindner, N. A. Kocharova, S. N. Senchenkova, A. S. Shashkov, Y. A. Knirel, O. Holst, T. A. Gremyakova, R. Z. Shaikhutdinova, and A. P. Anisimov.** 2002. The core structure of the lipopolysaccharide from the causative agent of plague, *Yersinia pestis*. *Carbohydr. Res.* **337**:775-777.
114. **Vinogradov, E. V., R. Pantophlet, S. R. Haseley, L. Brade, O. Holst, and H. Brade.** 1997. Structural and serological characterisation of the O-specific polysaccharide from lipopolysaccharide of *Acinetobacter calcoaceticus* strain 7 (DNA group 1). *Eur. J. Biochem.* **243**:167-173.

115. **Walker, G. C.** 1984. Mutagenesis and inducible responses to deoxyribonucleic acid damage in *Escherichia coli*. *Microbiol. Rev.* **48**:60-93.
116. **Wang, I. N.** 2006. Lysis timing and bacteriophage fitness. *Genetics* **172**:17-26.
117. **Wang, I. N., D. L. Smith, and R. Young.** 2000. Holins: the protein clocks of bacteriophage infections. *Ann. Rev. Microbiol* **54**:799-825.
118. **Westphal, O., and Jann, K.** 1965. in *Methods in Carbohydrate Chemistry* p. 83-91. In R. L. Whistler (ed.), vol. V. Academic Press, New York.
119. **Williams, S. R., D. Gebhart, D. W. Martin, and D. Scholl.** 2008. Retargeting R-type pyocins to generate novel bactericidal protein complexes. *Appl. Environ. Microbiol.* **74**:3868-3876.
120. **Young, I., I. Wang, and W. D. Roof.** 2000. Phages will out: strategies of host cell lysis. *Trends Microbiol.* **8**:120-128.
121. **Young, R.** 1992. Bacteriophage lysis: mechanism and regulation. *Microbiol. Rev.* **56**:430-481.
122. **Young, R.** 2002. Bacteriophage holins: deadly diversity. *J. Mol. Microbiol. Biotechnol.* **4**:21-36.
123. **Young, R., and U. Blasi.** 1995. Holins: form and function in bacteriophage lysis. *FEMS Microbiol. Rev.* **17**:191-205.
124. **Young, R., J. Way, S. Way, J. Yin, and M. Syvanen.** 1979. Transposition mutagenesis of bacteriophage lambda: a new gene affecting cell lysis. *J. Mol. Biol.* **132**:307-322.
125. **Yui-Furihata, C.** 1972. Structure of pyocin R. II. Subunits of sheath. *J. Biochem.* **72**:1-10.

126. **Zhang, N., and R. Young.** 1999. Complementation and characterization of the nested Rz and Rz1 reading frames in the genome of bacteriophage lambda. *Mol. Gen. Genet.* **262**:659-667.
127. **Ziermann, R., B. Bartlett, R. Calendar, and G. E. Christie.** 1994. Functions involved in bacteriophage P2-induced host cell lysis and identification of a new tail gene. *J. Bacteriol.* **176**:4974-4984.

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Duarte, I., G. Wang, R. F. Young, J. J. LiPuma, C. F. Gonzalez. A broad host range tailocin from *Burkholderia*. American Society for Microbiology Annual Meeting, New Orleans, LA. May 21-24, 2011.

Duarte, I., G. Wang, R. F. Young, J. J. LiPuma, C. F. Gonzalez. A broad host range tailocin from *Burkholderia*. American Phytopathological Society Annual Meeting, Charlotte, North Carolina. August 7-11, 2010.

Duarte, I., G. Wang, C. F. Gonzalez. Characterization of a broad host range tailocin from *Burkholderia*. MANRRS 25th Annual Career Fair and Training Conference, Orlando, Florida. March 25-March 28, 2010.

Duarte, I., G. Wang, R. F. Young, J. J. LiPuma, C. F. Gonzalez. Characterization of a broad host range tailocin from *Burkholderia*. American Phytopathological Society Annual Meeting, Portland, Oregon. August 1-5, 2009.