FUNCTIONAL CHARACTERIZATION OF SPURT USING TRANSGENIC MOUSE MODEL

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BS, Vilnius University, 1995

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Submitted to the Graduate Faculty of

Department of Environmental and Occupational Health

Graduate School of Public Health in partial fulfillment

Of the requirements for the degree of

Master of Science

University of Pittsburgh

2005
UNIVERSITY OF PITTSBURGH

Graduate School of Public Health

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06/10/2005

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Abstract

The respiratory tract is the target of multiple infectious agents. Because the lungs are continually exposed to infectious pathogens in inspired air, natural defense mechanisms have devolved to prevent infection. These defense mechanisms coordinate with each other to provide efficient protection against infection. As a result, pulmonary infections can be viewed not just as a consequence of exposure to a virulent pathogen but as a result of a breakdown of natural host defenses. SPLUNC1 or SPURT (secretory protein in upper respiratory tracts) is small, secreted protein that is expressed in epithelial areas of the nose, mouth, pharynx and lungs. It may be involved in host defense because it is highly homologous to bactericidal/permeability-increasing protein (BPI); a protein that mediates LPS related bacteria killing.

In this study, we established a constitutively overexpress CCSP-spurt transgenic mouse model to examine the biological function of spurt. We compared mRNA expression of CCSP-spurt in transgenic mice and their transgenic negative littermates. We determined that mRNA expression of CCSP-spurt was elevated in transgenic mice. The tissue distribution of overexpressed spurt in CCSP-spurt mice was confirmed to be only at trachea and lung and exist in no other tissues. Bronchoalveolar lavage (BAL) fluid from unchallenged CCSP-spurt mice
had higher *spurt* concentration as was determined by ELISA and western blot. BAL from unchallenged CCSP-spurt mice also exhibited antibacterial activity. Furthermore, CCSP-spurt mice display enhanced bacterial clearance than wild-type mice after both groups of mice were challenged with aerosolized gram negative microorganisms *Pseudomonas aeruginosa* (PAO1) infection. BAL of CCSP-spurt mice had lower levels of proinflammatory cytokines than BAL of their wild-type littermates after the challenge with aerosolized *P. aeruginosa*.

Results from our studies suggest that *spurt* is a novel BPI-like antibacterial protein that may play a critical role in airway specific innate immunity. Further studies of this protein might have potential public health significance in providing better understanding of natural defense processes in respiratory tract.
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1. INTRODUCTION

The respiratory tract is the target of multiple infectious agents. Because the lungs are continually exposed to infectious pathogens in inspired air, natural defense mechanisms have evolved to prevent infection, which include mucociliary transport, innate immunity, cell-mediated immunity, and antibody production. These defense mechanisms coordinate with each other to provide efficient protection against infection. As a result, pulmonary infections can be viewed not just as a consequence of exposure to a virulent pathogen but as a result of a breakdown of natural host defenses.

Upper respiratory tract infections affect the airways in the nose, ears, and throat. They can be caused by viruses, bacteria, or other microscopic organisms. Acute pharyngitis accounts for 1% to 2% of all visits to outpatient and emergency departments, resulting in 7 million annual visits by adults alone [1]. It is estimated that 31 million to 35 million US citizens have sinus disease [2]. About 12 million individuals are diagnosed with acute tracheobronchitis annually, accounting for one-third of patients presenting with acute cough [3]. The estimated economic impact of non-influenza-related upper respiratory tract infections is $40 billion annually [4]. However most of the upper respiratory tract infections are common and not serious. Those infections are minimized by an efficient mucociliary escalator clearance mechanism, which is supported by antibacterial substances within the upper respiratory tract.

Lower respiratory tract infections are the major cause of death in the world and the major cause of death due to infectious diseases in the United States. Lower respiratory tract infection
(LRTI) describes a range of symptoms and signs, varying in severity from non-pneumonic LRTI in the young healthy adult through to pneumonia or life-threatening exacerbation in a patient with severe disabling chronic obstructive pulmonary disease (COPD). LRTI is a particularly common and serious illness in the elderly. In a population-based retrospective cohort study in Rochester, Minnesota, USA, approximately one in 18 residents older than 65 years experienced one or more episodes of bronchitis or pneumonia over one year, with an overall 30-day mortality of 10.7%\[^{[5]}\].

Pneumonia is a leading cause of death in the world and the sixth most common cause of death in the United States. It is the number one cause of death from infectious diseases in the United States\[^{[6]}\]. The overall rates of death due to pneumonia and influenza have increased by 59% from 1979 through 1994 and by 22% when adjusted for age greater than 65 years\[^{[7]}\].

Community-acquired pneumonia (CAP) is one of the most common infectious diseases addressed by clinicians. Community-acquired pneumonia is caused most commonly by bacteria that traditionally have been divided into 2 groups, typical and atypical. Typical organisms include *S. pneumoniae* (pneumococcus) and *Haemophilus* and *Staphylococcus* species. Atypical refers to pneumonia caused by *Legionella, Mycoplasma*, and *Chlamydia* species. Less frequent, but still important, causative pathogens in CAP include *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*\[^{[8]}\]. CAP is an important cause of mortality and morbidity worldwide. Every year in the United States, there are from 5 million to 10 million cases of CAP leading to as many as 1.1 million hospitalizations and 45,000 deaths. It costs about $7,500 to manage a single in-hospital case of CAP, an amount that is more than 20-fold higher than the cost of outpatient treatment ($150 to $350)\[^{[7]}\]. The mortality rate is less than 1% for persons with CAP who do not require hospitalization; however, the mortality rate averages from
12% to 14% among hospitalized patients with CAP. Among patients who are admitted to the intensive care unit (ICU), or who are bacteremic, or who are admitted from a nursing home, the mortality rate averages from 30% to 40% \[^6\].

In 2002, 11.2 million U.S. adults aged 18 years and older were estimated to have Chronic Obstructive Pulmonary Disease (COPD) \[^9\]. Bacteria are isolated from between 40-60% of acute exacerbations of COPD. Three bacterial species account for most isolates: *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Moraxella cattarhalis*. *Haemophilus parainfluenzae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Enterobacteriaciae* are encountered less frequently. *Haemophilus influenzae* is present in about 50% of culture positive sputa in most clinical trials. Variation in incidence between trials may be accounted for by patient selection criteria, previous antibiotic exposure and sputum culture techniques \[^10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23\].

In general, timely recognition of bacterial products is critical for human survival. The best studied constituent of gram negative bacteria is lipopolysaccharide (LPS). LPS is a potent activator of innate immune responses that result in the production of pro- and anti-inflammatory mediators from myeloid lineages and other cell types. In humans, numbers of proteins are important in mediating signals from LPS. Two of those are bactericidal/permeability-increasing protein (BPI) and LPS-binding protein (LBP). These proteins are structurally related, however their functions are antagonistic. LBP has proinflammatory action and BPI has more direct bactericidal action \[^24\]. SPLUNC1 or SPURT (secretory protein in upper respiratory tracts) is a small, secreted protein that is expressed in epithelial areas of the nose, mouth, pharynx and lungs \[^25\]. In this study we used genetically modified mouse model to do a functional characterization of SPLUNC1 (SPURT).
Gram-negative bacteria possess an outer membrane composed of lipopolysaccharide (LPS; endotoxin), which protects the microorganism from harsh environmental extremes. LPS contains 3 distinct components: (1) the O-polysaccharide chain, which confers strain specificity; (2) the deep-core region, which links the O-polysaccharide chain to the third component; and (3) lipid A. The deep-core/lipid A regions are evolutionarily conserved throughout many genera of gram-negative bacteria. The recognition of and response against this glycolipid by the host will provide protection against a wide range of microorganisms. The innate immune system enables the host to mount an immediate response to variety of invading pathogens. To recognize the presence of pathogens, innate immune cells express receptors that identify highly conserved pathogen-associated molecular structures. Endotoxin is a pathogen-associated molecular pattern molecule for the pattern recognition receptor Toll-like receptor 4 (TLR4), which is found on many cells of the innate immune system, including macrophages \[26, 27\]. LBP and BPI are naturally occurring LPS binding proteins that bind LPS from the outer envelope of Gram-negative bacteria and are structurally similar. BPI is a 55kD mammalian host defense protein found in the primary granules of polymorphonuclear neutrophils. It binds to the lipid A moiety of gram-negative bacteria and kills them by depolarizing the inner membrane \[28\]. BPI acts as an intracellular anti-bacterial protein. Based on amino acid sequence comparison, BPI belongs to the same family of proteins as the human LPS binding protein (LBP), the cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP).
PLUNC (palate lung and nasal epithelium clone) is a family of proteins. The exact function of the PLUNC proteins is unknown. PLUNC was identified while studying gene expression in the embryonic murine palate. The new gene codes for a small, 26.7-kDa protein and was expressed around developing palate and quite strongly in the nasal septum and nasal conchae as well as in trachea and main stem bronchi of adult mice. LeClair et al. showed that PLUNC expression in the mouse upper airway is largely postnatal. PLUNC is expressed as early as e14.5, is maintained during the latter stages of development and persist until adulthood. Growing number of related mammalian homologues of PLUNC are being identified. So far the homologues of PLUNC were found in such species as mouse, human, rat, cow and pigs.

The PLUNC family of genes can be divided into two subgroups: the "Short" PLUNC genes (SPLUNCs) code for proteins of ~250 residues; while the "Long" PLUNC genes (LPLUNCs) code for ~480 residue proteins. In humans, the PLUNC family of proteins are located in a 300Kb cluster on chromosome 20. PLUNC gene cluster identified in humans corresponds to clusters on mouse chromosome 2 and rat chromosome 3. Figure 1 shows the human PLUNC gene cluster on chromosome 20q11.21. The pale red box indicates the position of a pseudogene for the human orthologue of rodent LPLUNC5 and the blue arrows indicate the direction of transcription.

![Figure 1](image-url)  
**Figure 1** The human PLUNC locus.
While the levels of amino acid identity are very low, typically 15%- 30% \(^{[37]}\), between proteins of the PLUNC and BPI/ LBP/ CETP/ PLTP families, all proteins share a similar predicted fold, a single predicted disulphide bond, and a conserved exon structure. It shows that PLUNC genes are clearly evolutionary related to each other and to BPI/LBP/CETP/PLTP families. The LPLUNC proteins are have homology to both N- and C—terminal domains of BPI and are predicted to be structurally equivalent to whole BPI (red boxes Figure 1), while the SPLUNC proteins are equivalent to the N-terminal LPS binding domain alone (blue box Figure 1). Within the PLUNC family the sequence identity is rather low, ranging typically from 16% to 28% with LPLUNC3 and LPLUNC4 sharing higher pairwise identity of 37% \(^{[24]}\). The low level of identity tends to support the hypothesis that those proteins might function in host defense. Among mammals there is big diversity among short PLUNC proteins, which might suggest that short genes evolved separately by duplication within certain mammalian lineages \(^{[37]}\).

The product of SPLUNC1 (also called LUNX, SPURT) gene was predicted to have 256 amino acids and to be rich in leucine \(^{[40]}\). The expression pattern of SPLUNC1 seems to be common to all mammalian species examined so far. SPLUNC1 is expressed in the upper airways, the salivary gland, the oropharynx, the trachea. It is also a major secreted protein in the upper respiratory tract \(^{[38]}\) and was also detected in human saliva \(^{[39]}\). Some studies have shown that SPLUNC1 is present in submucosal glands and in some cells of the tracheal epithelium \(^{[40]}\). The sites of SPLUNC1 expression correspond to locations were significant pathogenic loads are encountered and were bacterial sensing and/or neutralizing proteins might be required. Those sites are constantly exposed to bacteria, either by virtue of new exposures or by the resident microbial communities, however infections are rare. Thus epithelial expressed PLUNC might play a role in controlling microbial attacks within physiological limits by neutralizing pathogens,
diminishing their ability to grow or silencing released signaling molecules. Ghafouri et al. showed that PLUNC binds LPS in vitro \[41\]. Based on that SPLUNC1 could be good candidate member of the innate immune system.

Ghafouri et al. showed that PLUNC can be a marker of chemically induced airway irritation. During the study two forms of PLUNC protein in nasal lavage fluid with molecular mass of 24.8kDa and 25.1kDa were found. The proportion of two forms in healthy adults was about 70/30, however in smokers the proportion was altered to 50/50 \[42\]. In another study Ghafouri et al showed decreased PLUNC1 levels in nasal lavage fluid from epoxy workers with upper respiratory symptoms after long term exposure to organic acid anhydrides when compared to healthy controls. SPLUNC1 levels in exposed individuals were 2-6 times lower than in controls \[43\]. Human SPLUNC1 was proposed as a marker of micromethastasis in non-small cell lung carcinoma (NSCLC). It is found in all the lung carcinoma cell lines, and it is differentially expressed in lymph nodes of cancer patients \[44\]. SPLUNC1 has been shown to be a sensitive marker for the detection of NSCLC in peripheral blood samples \[45\]. SPLUNC1 was also expressed in head and squamous cell carcinoma \[46\] and in nasopharyngeal cancer \[47\]. Bingle et al showed that SPLUNC1 is commonly expressed in adenocarcinomas, muco-epidermoid carcinoma, bronchio-alveolar carcinoma \[48\]. Up-regulated SPLUNC1 levels were also noted after invasive surgery \[32\] and in chronic obstructive pulmonary disease (COPD) \[38\]. Di et al examined sputum of normal subjects and of patients with COPD. The results showed that PLUNC1 amounts were higher in samples obtained from COPD patients when compared with normal subjects \[38\].
3. MATERIALS AND METHODS

3.1. TRANSGENIC MICE GENERATION

The expression of CCSP-<i>spurt</i> transgenic mice were under the transcriptional control of the mouse CCSP promoter. Human <i>spurt</i> cDNA (0.77 kb), coding for the active, mature SPURT protein, was generated by RT-PCR from total RNA isolated from airway epithelial cells. Protein translational start codon and 12 nucleotides of the 5' untranslated region flanking it were incorporated into the forward PCR cloning primer. The primer sequences (5' to 3') used were: forward primer, ATA AGA ATG CGG CCG CCT AAG AGC AAA GAT GTT TC, and reverse primer, ATA AGA ATG CGG CCG CAC CTT GAT GAC AAA CTG. The PCR product was gel purified and cloned using the pCR2.1-TOPO cloning system (Invitrogen, Carlsbad, CA) and sequenced. The insert was subcloned into the Not I site of the 1<sup>st</sup> exon of a mouse Clara cell secretory protein (mCCSP) 9.6 kb genomic sequence in a pUC19 construct vector. The construct was verified by restriction enzyme digestion and sequencing. The CCSP-<i>spurt</i> DNA fragment was then isolated from the plasmid by digestion with Sph I, and used for generation of transgenic mice by microinjection into mouse oocytes. Transgenic founder mice were identified by Southern analysis, and the results also confirmed by PCR genotyping as described below. Genotyping was done by PCR using primers specific for transgene constructs as follows. CCSP transgene primers: forward primer in mCCSP promoter: 5’-GTT GGC AAG TCT ACA GTT
GC-3’, reverse primer in CCSP 1st intron region: 5’-GAA AGA GAC CCT GGG CAC TCA-3’, and forward primer of *spurt* coding region: 5’-GAC GTC AGT GAT TCC TGG CC-3’.

### 3.2. RNA ISOLATION AND RT-PCR ANALYSIS

Total RNA was isolated from lungs and trachea of wild type and transgenic mice. RT-PCR analyses of Spurt were performed with the following primers: Oligo-2 (5’- GAC GGT ACC GAG ACC TTG ATG ACA AAC TG -3’ and Oligo-3 (5’- TAG AAT TCA GTT TGG AGG CCT GCC -3’). The following program was used: denaturation at 94°C for 2min, 35 cycles 94°C for 30 sec, 55°C for 30sec, 68°C for 1min. The PCR program was concluded by an extension at 68°C for 7min. 8microliters of the amplification product was applied to 1% agarose gel and visualized after electrophoresis by ethidium bromide staining.

### 3.3. REAL TIME RT-PCR ANALYSIS

Total RNA was isolated from lungs of wild type and transgenic mice. Real time RT-PCR analyses of Spurt were performed with the following primers and probes: forward 5’-TTCAGGGCAACGTGTGCC-3’, reverse 5’-TAGTCCGTGGATCAGCATGTTAACA-3’, probe 5’- CTGGTCAATGAGGT TCTCAGAGGCTTGG-3’ were used to determine human Spurt. Another set of primers and specific probe were used to determine mouse mPlunc: forward 5’- TGG GAT TCT CAG CGG TTT GGA TGT -3’, reverse 5’- TCA GCC AAG ATA GCC TTC CCT -3’, probe 5’- /56-FAM/CAC CCT GGT GCA CAA CAT TGC TGA AT/36-
The following program was used: 95°C for 12min, 40 cycles 95°C for 15 sec, 60°C for 1min.

3.4. DEVELOPMENT OF THREE ANTIBODY SANDWICH ELISA

Polystyrene 96 well microtiter plates (Costar, Corning Inc.) were coated with 100 µl of 3 µg/ml antibodies against spurt in 50mM carbonate buffer. Four different antibodies were used: rabbit anti Plunc antibody (University of Miami School of Medicine, Miami, Florida), Ab1, Ab2, Ab3. Ab1 (S84-I99), Ab2 (A165-C180), Ab3 (Leu73-Gly86) were created by injecting rabbits with peptides that targets specific regions of spurt protein. Plates were kept at 37°C in humid chamber overnight. Prior to use, plates were washed and wells were blocked by addition of 200µL of blocking buffer (PBS – 1% BSA) and kept at 37°C for one hour. Calibration curve was prepared using recombinant human PLUNC (rhPLUNC, R&D Systems, Minneapolis, MN). Five different concentrations of recombinant protein were prepared (5ng/well; 10ng/well, 25ng/well, 50ng/well, 100ng/well) and 100µl of solution was loaded into the well. Calibration curves were run in duplicates. Plates were incubated at 37°C for 1hr. Afterwards a goat anti-human Plunc (R&D Systems, Minneapolis, MN) diluted 1:400 in blocking buffer was added and plates were incubated for 1hr at 37°C in humid chamber. After one hour, a mouse anti goat antibody (Pierce Biotechnology, Inc.) was added (1:10000). Plates were incubated at 37°C for 1hr. In order to develop the plate 100µl of 1-Step Turbo TMB-ELISA (Pierce Biotechnology, Inc.) was added to each well. Plate was incubated at room temperature for 30 min. In order to stop the reaction 100µl of 1N HCl was added to each well. The absorbance was measured at 450nm. Obtained
curves were evaluated and one with the best sensitivity and longest linear range was selected to use in future experiments.

Specificity was tested by coating multiwell plates with unrelated antibodies. Specifically plates were coated with 100 µl of 3 µg/ml antibodies against PBEF-a, TTF-1, Intergrin α6 and with normal rabbit serum in 50mM carbonate buffer. Plates were kept at 37°C in humid chamber overnight. Prior to use, plates were washed and wells were blocked by addition of 200µL of blocking buffer (PBS – 1% BSA) and kept at 37°C for one hour. Five different concentrations of recombinant protein (rhPLUNC, R&D Systems, Minneapolis, MN) were prepared (5ng/well; 10ng/well, 25ng/well, 50ng/well, 100ng/well) and 100µl of solution was loaded into the well. Samples were loaded in duplicates and experiment was continued as described above. Obtained results showed no nonspecific binding.

3.5. THREE ANTIBODY SANDWICH ELISA

Polystyrene microtiter plates (Costar, Corning Inc.) were coated with 6µg/mL (0.6 µg/well) rabbit anti-spurt antibody (Ab2) in 50mM carbonate buffer. Plates were kept at 37°C in humid chamber overnight. Prior to use, plates were washed and wells were blocked by addition of 200µL of blocking buffer (PBS – 1% BSA) and kept at 37°C for one hour. Calibration curve was prepared using recombinant human PLUNC (rhPLUNC, R&D Systems, Minneapolis, MN) as was described above. 30µl of sample BAL was added to the wells and diluted up to 100µl with blocking buffer. Samples were added in duplicates. Plates were incubated at 37°C for 1hr. Afterwards a goat anti-human Plunc (R&D Systems, Minneapolis, MN) diluted 1:400 in blocking buffer was added and plates were incubated for 1hr at 37°C in humid chamber. After
one hour, a mouse anti goat antibody (Pierce Biotechnology, Inc.) was added (1:10000). Plates were incubated at 37°C for 1hr. In order to develop the plate 100µl of 1-Step Turbo TMB-ELISA (Pierce Biotechnology, Inc.) was added to each well. Plate was incubated at room temperature for 30 min. In order to stop the reaction 100µl of 1N HCl was added to each well. The absorbance was measured at 450nm.

3.6. SDS-PAGE

BAL, lung homogenate and rhPLUNC (R&D Systems, Minneapolis, MN) were diluted with sample loading buffer. rhPLUNC was used as a control. Samples were heated for 5 min at 95 °C, and then applied to the wells of a gradient gel (NuPAGE 12% Bis-Tris Gel, Invitrogen). 20µg of protein was added to each lane when BAL or lung homogenate was loaded into the gel. 40µg/lane of protein was loaded for the control. Electrophoresis was performed at 100 volts for 10min and continued at 200 volts for 30 min.

3.7. WESTERN BLOT ANALYSIS

For Western blots the proteins were electroblotted onto nitrocellulose membrane (Pierce Biotechnology, Inc.) in transfer buffer containing 25mM Tris base, 0.2M glycine, 20% methanol (pH~8.5). Membranes were blocked with 5% non fat dry milk in Tris-buffered saline with 0.1% Tween-20 (TBST) for 1 h. Membranes were washed with TBST 3x for 5min and then incubated
with goat anti-human PLUNC antibody (R&D Systems, Minneapolis, MN) (diluted into blocking solution, 1:1000) overnight in 4°C. After washing with TTBS 3x for 5 min, the second antibody was added and membranes were incubated for 1 h (mouse anti goat-HRP antibody (Pierce Biotechnology, Inc.), diluted 1:10000). Final 3x 5min washes with TBST were performed. Membrane was developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Inc.).

3.8. BAL TESTING FOR ANTIBACTERIAL ACTIVITY

*Pseudomonas aeruginosa* was grown in LB solution for overnight. Before using it to make serial dilutions, OD of bacterial stock solution was measured and adjusted to ~0.74. After that adjusted bacterial stock was used for serial dilutions. Five 1:10 dilutions were performed using 500µl of bacterial stock solution (OD_{470} ~0.740; approximately 2*10^{11} bacteria) in 4500µl PBS (10^{-1} to 10^{-5}). Dilutions were performed right before the use. 30ul of PBS, transgenic and control mice BAL was loaded into the wells of multiwell plate. Samples were loaded in duplicates. Plates were kept on ice while serial dilutions of bacteria were performed. Subsequently 30µl of 10^{-5} bacterial solution was added to the wells. Samples were mixed and incubated for two hours with shaking. After incubation plates were placed on ice to slow down further bacterial growth. 50µl of bacteria and BAL mix was plated on LB agar plate. After plating agar plates were inverted and placed into 37°C incubator. Colonies were enumerated after 24h.
3.9. **NEUTRALIZATION OF ANTIBACTERIAL ACTIVITY BY ANTI-SPURT ANTIBODY**

*Pseudomonas aeruginosa* was grown in LB solution overnight. Before use OD of bacterial stock solution was measured and adjusted to ~0.74. After that adjusted bacterial stock was used for serial dilutions. Ten 1:10 dilutions were performed using 500µl of bacterial stock solution (OD$_{470}$ ~0.74; approximately $2\times10^{11}$ bacteria) in 4500µl PBS ($10^{-1}$ to $10^{-10}$). Dilutions were performed right before the use. Only three bacterial dilutions ($10^{-8}$ to $10^{-10}$) were used for the experiment. Mice BAL were preincubated with slight agitation for one hour at RT with normal rabbit serum (NRS) or with anti Plunc antibody (University of Miami School of Medicine, Miami, Florida). 3µl of NRS or anti Plunc antibody per 20µl of BAL was used. After incubation 20µl BAL plus NRS or anti Plunc antibody was loaded into the wells of multiwell plate. Plate was kept on ice while serial dilutions of bacteria were performed. Subsequently 20µl of appropriate bacterial solution was added to the wells. Samples were incubated for two hours with shaking. After incubation plate was placed on ice to slow down further bacterial growth. LB agar plate was divided into 3 sections and 10µl of each dilution were plated 3 times. After liquid soaked into the agar plates were inverted and placed into 37°C incubator. Colonies were enumerated after 24h.

3.10. **BACTERIAL CULTURE**

*P. aeruginosa* was a generous gift from Chad Steele (Department of Pediatrics, Division of Pulmonology Children's Hospital of Pittsburgh, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania). For each experiment, *P. aeruginosa* was inoculated from fresh culture
plate, and then incubated overnight at 37°C in a shaking incubator. Then bacterial culture was centrifuged and pellet resuspended in 10ml of phosphate-buffered saline (PBS) to approximately 1*10^{10} CFU*ml^{-1}.

3.11. MICE EXPOSURE TO PSEUDOMONAS AERUGINOSA

Wild type and transgenic mice were exposed to aerosolized *p. aeruginosa* for 45min using the Inhalation Exposure System (Glas-Col, model A42X). Mice were placed in a compartmentalized mesh basket (five chambers, each with a capacity for 20 mice). Parameters for the standard aerosol exposure cycle were 45min for nebulization, 15min for cloud decay, 15min for decontamination (UV irradiation). For determination of initial bacterial deposition in the lungs, animals were sacrificed immediately after the standard exposure cycle.

3.12. BRONCHOALVEOLAR LAVAGE (BAL)

At each time point, 4 and 24 hours after aerosol exposure, 5 to 6 transgenic mice and non-transgenic control mice were anesthetized with 2.5% Avertin (2% 2,2,2-tribromoethanol 2% tert-amyl alcohol, 0.9% NaCl). The diaphragmatic lobe was tied off and the rest of the lung was lavaged 2 times through a tracheal cannula with total of 1 ml of sterile PBS. BAL was collected and kept on ice. Furthermore, lung was lavaged with five more separate 1ml volumes of sterile PBS. The aliquots from individual mice were combined, centrifuged at 300 x g. After centrifugation the supernatants were removed and cell pellets were resuspended in PBS. The
cells were counted in a hemacytometer in order to obtain cell counts. Differentials were performed on slides prepared in a Shandon Cytospin (ThermoShandon, Pittsburgh, PA) and stained with a modified Wright-Giemsa technique (Diff-Quik, Dade Behring, Dudingen, Switzerland).

3.13. BACTERIAL ENUMERATION

After excision diaphramatic lobe of the lung was placed into eppendorf tube containing 0.4ml of sterile PBS and kept on ice. Later on lung was homogenized and additional 0.6ml of sterile PBS was added to homogenized mixture. Six 1:10 dilutions were performed using 50µl of lung homogenate in 450ul PBS (10\(^{-1}\) to 10\(^{-6}\)). LB agar plate was divided into 6 sections and 10µl of each dilution were plated 3 times. Plates were left on the counter top till the liquid soaked into the plate. Then plates were inverted and placed into 37\(^\circ\)C incubator. Colonies were enumerated after 24h.

3.14. HISTOLOGY

Lungs were inflated in situ with 4% paraformaldehyde, then removed and stored at 4\(^\circ\)C in the same fixative. The tissue was embedded in paraffin, sectioned, and stained with hematoxylin and eosin.
3.15. IMMUNOHISTOCHEMISTRY

Immunohistochemical procedures were performed on treated paraffin sections. Rehydrated slides were quenched in 3% H$_2$O$_2$ for 10 min. Antigen retrieval was performed by boiling slides for 20 minutes in 10 mM citrate buffer (pH 6.0) following which sections were washed, blocked (0.03% casein in 0.05% PBS/Tween-20) for an hour and incubated with anti 1:200 anti human PLUNC (R&D Systems, Minneapolis, MN) antibody overnight at 4°C. A mouse anti goat antibody (Pierce Biotechnology, Inc.) was used as a second antibody. A serum to from preimmune rabbit was used as a control. Slides were stained with Metal Enhanced DAB solution (Pierce Biotechnology, Inc.) and counterstained with Mayer’s hematoxylin solution (Sigma-Aldrich Co.).

3.16. BIO-PLEX CYTOKINE ASSAY

Commercially available mouse Cytokine 18-Plex Panel assay (Bio-Rad Inc., cat#171F11181) was used to analyze BAL. In short, anti- cytokine beads were added to the wells. Standard curve was prepared following kit instructions. 50µl of each standard and 50µl of sample was added to appropriate wells. Plate was incubated with shaking at room temperature. Plate was washed 3x with Bio-Plex wash buffer and 25µl of detection antibody was added afterwards. After incubation plates was washed again and Streptavidin-Pe solution was added to each well. Plate was incubated for 10min with shaking. After incubation streptavidin–Pe solution was removed and wells were washed 3x again with wash buffer. Lastly, beads were resuspended in Bio-Plex assay buffer A, plate was shaken for 30sec and afterwards read by machine.
4. RESULTS

4.1. ESTABLISHMENT OF TRANSGENIC MOUSE THAT OVER-EXPRESS HUMAN SPURT PROTEIN

Our laboratory has previously identified an airway specific gene, *spurt*, from cDNA microarray analysis of primary human airway epithelium exposed to retinoic acid. Gene expression of *spurt* is higher in sputum and tissue samples obtained from patients with chronic obstructive lung disease (COPD) \[^{38}\]. We propose that SPURT may be involved in host defense because it is highly homologous to bactericidal/ permeability-increasing protein (BPI) which mediates LPS related bacteria killing.

To test the antimicrobial activity of SPURT in vivo, we generated a constitutively overexpressing CCSP-*spurt* transgenic mouse line in FVB/n strain (Figure 2A). The CCSP-*spurt* mice were generated by targeting expression of the human *spurt* cDNA (coding region) to the mouse respiratory epithelium under the direction of the 9.6-kb mouse CCSP promoter. We used both RT-PCR and real time RT-PCR analyses to confirm elevated mRNA expression of CCSP-*spurt* when compared with their transgenic negative littermates. The tissue distribution of over-expressed Hs. *spurt* in CCSP-*spurt* mice was confirmed to be only at trachea and lung and exist in no other tissues examined (Figure 2B). Results of real time RT-PCR of tissues from a transgenic mouse from mouse founder line 60583 using primer and probe set specifically designed for human *spurt* are shown in Figure 2C. DDCt was calculated and normalized to
mouse 18S. The mouse homologue of *spurt* is named *plunc* ( palate, lung, and nasal epithelium clone) and express exclusively in the mouse upper airways that similar to what was observed in human airways. To distinguish the expression levels of transgenic human *spurt* from the endogenous mouse *plunc*, all primers and probes used in our experiments are specifically designed and unique to only amplify gene product of either human *spurt* or mouse *plunc*.

![Diagram of CCSP-spurt Transgenic Mice](image)

**Figure 2  Generation and Assessment of CCSP-spurt Transgenic Mice**

The over-expression of *spurt* in mouse airways was further confirmed by immunohistochemical staining using an anti-*spurt* antibody. The significant increase of SPURT staining in CCSP-*spurt* transgenic mice as compared to wild type littermate was observed in airway surface epithelium (Figure 3). The expression of mouse *plunc* normally does not extend from upper airway region to lower airway area but in our case we observed increased staining
intensity from significantly higher expression of SPURT in the distal airway epithelium of transgenic mice when comparing with their wild type littermates. The result indicated successful expression of transgenic *spurt* under CCSP promoter that drives the over-expression of *spurt* to where CCSP normally expresses (Figure 3).

![Figure 3](image)

**Figure 3** Over-expressed SPURT is observed in CCSP-*spurt* mice airway epithelium

### 4.2. ENHANCED ANTIBACTERIAL ACTIVITY USING BAL FROM CCSP-SPURT MICE

SPURT is a secretory protein and our previous data demonstrated that the gene product of *spurt* (SPURT) is secreted onto the apical side of primary human airway epithelial cultures and is present in clinical sputum samples. To evaluate whether over-expressed gene product of *spurt* in
CCSP-spurt transgenic mice can be detected as a secretory protein in mouse airways, we examined the expression level of SPURT in bronchoalveolar lavage (BAL) fluids and lung homogenate from transgene negative (Tg\(^-\)) and transgene positive (Tg\(^+\)) littermates from line 60583. Samples were probed with a polyclonal antibody to the spurt. Mouse CCSP in BALF and GAPDH in lung homogenate were used as internal control for normalization of mice. The BAL fluids (BALF) obtained from CCSP-spurt transgenic mice showed much greater SPURT expression than their wildtype (Wt) littermates when examined by Western blot analyses. Since our anti-spurt antibody recognizes active form of both human spurt and mouse plunc, secreted proteins of both over-expressed human SPURT protein and endogenous mouse PLUNC protein were detected in our Western analyses (Figure 4A).

Two antibody sandwich ELISA was also used to compare SPURT levels in BAL from transgenic and wild type littermates. Figure 4B shows the dramatic increase of secreted SPURT in BAL from transgenic mice. Results showed about ~5 fold increase in concentration of the potential functional SPURT protein.

We then examined whether the BALF from unchallenged CCSP-spurt transgenic mice that overexpress SPURT exhibited antibacterial bio-activity. We established a biological assay in vitro to assess colony formation unit (CFU) using BALF. After optimization of experimental assay conditions, we mixed equal volumes of BALF with serial diluted P. aeruginosa (PAO1) and incubated mixtures for 2 hours at 225rpm before plating onto LB agar in triplicate. Plates were incubated at 37\(^\circ\)C for 24 hours before counting for CFU. Numbers of CFU counts using BALF from CCSP-spurt mice were consistently 50% to 60% lower than BALF from wild-type mice (n=7) (Figure 4C).
Figure 4 Enhanced antibacterial activity using BALF from CCSP-spurt transgenic mice.
To further confirm the decreased CFUs in CCSP-spurt BALF were specifically due to increased SPURT overexpression, we used anti-plunc (spurt) antibody to neutralize the functional SPURT (spurt protein) in BALF. Normal rabbit sera (NRS) or anti-plunc rabbit antisera were pre-incubated with BALF (1:10 ratio) from both Wt (n=8) and CCSP-spurt mice (n=8) at 25°C for 1 hour before BALF from mice were incubated with P. aeruginosa at 37°C and followed up with plating in triplicate. BALF obtained from CCSP-spurt had only 51% of CFU counts compared to BALF from wild-type mice.

![Figure 5](image)

**Figure 5** Neutralization of antibacterial activity by anti-plunc (spurt) antibody.

Adding anti-plunc antisera into BALF of CCSP-spurt mice increased the average CFU counts significantly to more than twice the average CFU counts from Wt BALF with NRS and about 4x CFU counts from CCSP-spurt with NRS. Interestingly, adding anti-plunc antisera into BALF of Wt mice also increased the average CFU counts when compared with Wt+NRS (Figure...
5). The increased CFU counts after adding anti-\textit{plunc} antibody may represent the antibacterial activity contributed by endogenous mouse \textit{plunc}. There was no statistically significance when we compared the average CFU counts from both groups of \textit{Wt} and \textit{Tg} mice after preincubation of BALF with \textit{plunc} antisera (Figure 5).

4.3. \textbf{INCREASED ANTIBACTERIAL ACTIVITY OF CCSP-SPURT MICE AFTER AEROSOLIZED PSEUDOMONAS AERUGINOSA EXPOSURE}

To examine the feasibility and to establish a mouse model for our proposed studies in this application regarding bacterial infection, both wild-type littermates and CCSP-\textit{spurt} mice were aerosolized with or without \textit{P. aeruginosa} (PAO1) bacteria. Administration of bacteria into the respiratory tract of mice was accomplished by aerosolization of bacteria in a whole-animal chamber for 45 min, as previously described \cite{49}. Four hours and twenty-four hours after infection, right lungs were homogenized in 1ml of sterile PBS, serially diluted, and quantitatively cultured on LB agar. Colonies were counted after 24h incubation at 37°C. \(10^{10}\)/ml of bacteria (\textit{P. aeruginosa}, PAO1) aerosolized for 45 min is an applicable concentration to induce acute mouse airway infection. These conditions resulted in an average of \(\sim 2 \times 10^6\) CFU/lung (\(\sim 5 \times 10^5\)/ diaphragmatic lobe of lung) at the time point immediately after exposure. (n=5~6 for each experimental group)

We observed differences in the outcome between transgenic CCSP-\textit{spurt} group and \textit{Wt} group of mice after inhalation of \textit{P. aeruginosa}. Transgenic CCSP-\textit{spurt} mice displayed more active and less lethargic behavior than their wild-type littermates at four hours following aerosolized inoculum of \textit{P. aeruginosa}. Both CCSP-\textit{spurt} and wild type control groups of mice
effected more than 4-fold reduction in the number of bacteria in their lungs with signs of illness largely resolved by 24 hours and no sign of in respiratory distress was observed. No mice (control or CCSP-spurt) died after inoculum in our repeated experiments (n=5). *P. aeruginosa* replicated more in the lungs of *Wt* mice and that resulted in greater numbers of bacteria recovered from their lungs (Figure 6).

![Figure 6](image)

**Figure 6** CCSP-spurt mice are more resistant to lung infection with *P. aeruginosa*.

### 4.4. DECREASED NEUTROPHILS INFILTRATION IN LUNGS OF CCSP-SPURT MICE AFTER AEROSOLIZED PSEUDOMONAS AERUGINOSA EXPOSURE

After bacterial exposure, we observed rapid production of proinflammatory cytokines and recruitment of neutrophils in response to *P. aeruginosa* challenge. Although the average cell differential counts of inflammatory cells in BAL were not statistically different between wild
type and CCSP-*spurt* transgenic mice, control group of wild type mice were consistently having a greater number of neutrophils in their BAL than in *Tg* positive mice in both 4h and 24h after bacterial exposure (Figure 7).

![Graph of Total number of neutrophils in mice BAL after exposure to *p.aeruginosa*](image)

**Figure 7** Total cell numbers of neutrophils were less in CCSP-*spurt* mice BAL than wild-type littermates.

Before, the bacterial exposure, the BAL cell populations of uninfected CCSP-*spurt* and control wild type mice did not differ in either total cell number (2 x 10^5/lung) or differential composition (>99% macrophages, data not shown). By 4h after aerosolized *p. aeruginosa* exposure, neutrophils accounted for more than 90% of BAL cells in both groups of mice. Since the total inflammatory cells in BAL were higher in wild type group of control mice, the total cell numbers of neutrophils would subsequently be lower in CCSP-*spurt* transgenic mice than wild type mice. Total protein concentrations in BALF were not of statistical significance at time
points of 4h and 24h after bacteria instillation and suggested that the exposure did not significantly change lung permeability (data not shown). Additionally, we did not notice any significant difference in regards to Lactate dehydrogenase (LDH) activity in BALF at all time points examined (data not shown). These results suggest that there was no detectable acute lung injury after infection during the experimental time intervals.

Wild-type and CCSP-spurt mice without P. aeruginosa exposure were used as a control and sacrificed to harvest lung tissues. The lung tissues of uninfected wild-type and CCSP-spurt mice were both histologically normal (Figure 8).

![Figure 8](image)

**Figure 8** Histological view of wild type and CCSP-spurt transgenic mice before *P. aeruginosa* exposure.

However, the histological patterns of lung inflammation and injury after inhalation of *P. aeruginosa* differed noticeably between CCSP-spurt and wild type control mice. At 4h after aerosolized *Pseudomonas aeruginosa* exposure, the lungs of control mice revealed
predominantly perivascular but also peribronchial and alveolar neutrophilic infiltrates associated with prominent edema and hemorrhage while Tg-spurt mice expressed much less severe neutrophilic infiltrates and alveolar hemorrhage (Figure 9).

Figure 9 Decreased neutrophils infiltration and lung injury in CCSP-spurt mice than wild type mice 4h after *P. aeruginosa* exposure.

4.5. **DECREASED PRO-INFLAMMATORY CYTOKINE PRODUCTION IN CCSP-SPURT MICE AFTER AEROSOLIZED PSEUDOMONAS AERUGINOSA EXPOSURE**

To evaluate whether pro-inflammatory cytokine production changed after *Pseudomonas aeruginosa* exposure, BALF were collected from mice of each group after bacterial challenge. Levels of multiple cytokines (pg/ml) were measured in BAL specimens by Bio-plex assay. Cytokine productions were measured in unchallenged specimens as well as at 1h, 4h and 24h
after bacterial exposure. First columns (Figure 10) in each exposure group (yellow bars) represent wild type control mice, second correspond to transgenic mice (green bars).

Figure 10  Decreased pro-inflammatory cytokine production in CCSP-spurt mice.
Of the multiple cytokines examined, tumor necrosis factor alpha (TNFα) increased rapidly (within 1 hour) after PAO1 bacteria instillation. Interestingly, pro-inflammatory cytokines such as IL-1β, IL-6, IL-17 and GM-CSF were all lower in CCSP-spurt mice than Wt mice at 4h and 24h after bacterial challenge (Figure 10). We did not observe significant difference between CCSP-spurt and wild type mice in regards to the production of RANTES.
5. DISCUSSION

Effective host defense against microbial invasion requires an innate immune system whose response is both rapid and independent of prior exposure\textsuperscript{[50]}. There are a variety of proteins that participate in innate immune response. SPURT, also named SPLUNC1, seems to be one of them. It is a secreted protein that is expressed in the secretory ducts and submucosal glands of tracheobronchial tissues\textsuperscript{[24]}. It is structurally related to bactericidal/permeability-increasing protein (BPI) and lipopolysaccharide binding protein (LBP), which have been demonstrated to be important to the host defense against gram-negative bacteria. SPURT/SPLUNC1 belongs to the short-peptide subfamily of PLUNC family proteins and has homology only to the N-terminal domains of BPI that has previously been shown to have direct bactericidal activity. Therefore, SPURT/SPLUNC1 may play a similar role to BPI in the innate immune response against bacteria. However, this will represent a unique mechanism of antimicrobial activity originated from airway epithelium since SPURT/SPLUNC1 only expresses in airway epithelial cells.

In this work, a newly established transgenic mouse model that over-expresses human \textit{spurt} gene has been described. Our results showed that \textit{spurt} was specifically expressed only in the trachea and lung of CCSP-\textit{spurt} transgenic mice as its tissue expression was driven by a mouse CCSP promoter. We have also confirmed the much higher mRNA expression level of \textit{spurt} message in CCSP-\textit{spurt} transgenic mice than their wild type littermates by a real time RT-PCR assay that specifically amplified human \textit{spurt} but not the endogenous mouse \textit{plunc} gene. Mature and secretory SPURT protein was also detected in mice BALF by both Western blot
analyses and ELISA. Protein levels of SPURT were much higher in transgenic mice BALF when compared to their wild type littermates. We subsequently used these newly established CCSP-spurt transgenic mice to evaluate effects of SPURT on bacterial clearance and inflammation.

Despite sequence similarity and functional suggestion by bioinformatic searches, there was little direct evidence that SPURT/SPLUNC1 protein does have antimicrobial functions and play a role in host defense against pathogens. Parotid secretory protein (PSP), another protein member in the PLUNC gene family, has been shown to bind to bacterial membranes\(^{[51]}\). SPLUNC1 has also been suggested to bind directly to \textit{Escherichia coli} lipopolysaccharide\(^{[41]}\) and is present in the antimicrobial fraction of nasal secretions\(^{[52]}\). Using a bio-activity assay to test antimicrobial functions of secretory proteins in BALF against bacteria, our results showed that there was a significant decrease in CFU counts of \textit{P. aeruginosa} in co-incubating with BALF from CCSP-spurt transgenic mice than with BALF from wild type littermates and these data strongly suggested that over-expression of SPURT in mouse airways may increase anti-microbial activity of mice.

To further prove that the increased anti-microbial activity by CCSP-spurt transgenic mice BALF actually happens in live mouse airways, we adapted a bacterial exposure model by aerosolizing \textit{P. aeruginosa} into mouse airways. The bacteria-containing mist was aerosolized, inhaled and bacteria were subsequently deposited into mouse airways. Similar to the results from \textit{in vitro} bio-activity assays, decreased CFU counts were consistently observed in the lung homogenates from transgenic mice than their wild type littermates after aerosolized \textit{p. aeruginosa} exposure. Additionally, there was significant difference observed in pathological examination of respiratory tracts between transgenic and wild type animals after aerosolized
exposure to *P. aeruginosa*. There was much more noticeable damage to the lung tissues in wild type animals comparing with their transgenic counterparts. Neutrophil infiltration into lung tissue was much less in CCSP-*spurt* transgenic mice than from wild type littermates. Furthermore, the total cell counts of neutrophils in BAL were also constantly lower in transgenic mice. Taken together, our data suggest there is a lower degree of neutrophils recruitment and subsequent lung inflammation in CCSP-*spurt* transgenic mice than their wild type littermates. This observation suggests that overexpressing of *spurt* may provide an airway epithelial cell specific protection against opportunistic pathogens such as *P. aeruginosa*.

Cytokines are frequently involved in normal regulation of all physiological processes. The role that cytokines play in the regulation and modulation of immunological and inflammatory processes is striking. Lung is particularly dependent on tightly regulated immunological and inflammatory processes since it is exposed to a large variety of infectious agents as well as a diverse group of noxious gases and particulates during the process of gas exchange. Lung defends itself from these injurious agents by deploying cytokine-regulated host defense mechanisms. The use of a transgenic mouse model for *in vivo* studies provided an opportunity to identify potential changes in cytokines poroduction before and after aerosolized infection of *P. aeruginosa*. Pro-inflammatory cytokines such as IL-1β, IL-6, IL-17 and TNF-α were almost non detectable prior to the bacterial exposure but significantly increased after the challenge. Usually following the recognition of microbial products, TLR-mediated signaling results in the production of TNF-α and IL-1β\(^{53, 54, 55}\). Neutrophil recruitment is dependent on the orchestrated generation of these two early-response cytokines. These substances usually play beneficial roles in the body's defense systems. However, in some instances, they may be released in overly-abundant amounts and cause too much inflammation in the lung. The
exacerbated inflammatory response could even contribute to inflammation and dysfunction in other organs, such as the kidneys and liver, if these inflammatory mediators are overly released into the bloodstream. Therefore, a well-regulated release of pro-inflammatory mediators is critical to an effective host defense. In our studies, the enhanced lung clearance of inhaled bacteria also correlated with decreased measurement of pro-inflammatory cytokines (including IL-1β, IL-6, IL-17 and GM-CSF) in BALF from CCSP-*spurt* mice than wild type mice at both 4h and 24h time points after bacterial challenge. Another pro-inflammatory chemokine, KC, has been shown to substantially attenuate the accumulation of neutrophils in the lungs after the intratracheal administration of LPS [56]. We also observed significant lower levels of KC secretion in BALF from CCSP-*spurt* mice when compared to wild type littermates after *P. aeruginosa* exposure. The lower amount of KC in BALF of CCSP-*spurt* mice might account for less neutrophil infiltration and resulted in lesser damage to the lung in transgenic mice as compared to their wild type littermates.

Several potential mechanisms may help to explain the enhanced protection against bacteria that was observed in CCSP-*spurt* transgenic mice than wild type mice. The first maybe a direct bacterial killing function of SPURT that could effectively decrease available amount of bacteria and only the surviving bacteria can form the colonies in mice lungs. The second possible way is through a bacterial-static mechanism so that SPURT binds to bacteria and prevent efficient propagation of bacteria so host lung will have a longer reaction time to clear inhaled bacteria. There is also a possibility that the better bacterial clearance observed in CCSP-SPURT transgenic mice was due to increased interaction of spurt to other antimicrobial proteins and peptides such as defensin and subsequently potentiate antibacterial activity in transgenic mice.
6. CONCLUSIONS

In conclusion, we confirmed in this study the antibacterial activity of spurt using both in vitro and in vivo approaches. We also demonstrated that CCSP-spurt transgenic mice exhibited significantly better resistance to P. aeruginosa infection. However, more studies need to be done in order to fully understand the function of SPURT and mechanisms of how bacteria were effectively decreased in CCSP-spurt transgenic mice. Given the emergence of highly resistant bacterial pathogens and the increasing population of immunocompromised hosts, the treatment of bacterial infection has and will continue to be quite difficult. A better understanding of airway epithelial cells specific and initiated host defense may provide an alternative approach to efficiently combat airway bacterial infection. This alternative approach could even be tested in future pharmaceutical use in creating new antimicrobial drugs or treating patients with recombinant SPURT/SPLUNC1 proteins that naturally exist in airways.
BIBLIOGRAPHY


