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Moran Foundation
2004-2005 Progress Report

Project Title:

"Regulation of TNF-α Processing by the *Lactobacillus* Secretome"

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Award Period: August 1st, 2004 - June 30th, 2005

Hypothesis and Specific Aims

The overall hypothesis is that soluble bioactive proteins secreted by intestinal Lactobacillus *organisms regulate mucosal TNF-α processing in the intestine.* Probiotic mechanisms of action partly depend on the abilities of intestinal commensal bacteria such as *Lactobacillus reuteri* to modulate pro-inflammatory cytokine responses by the host's innate immune system. Current treatment strategies such as infliximab or anti-tumor necrosis factor-α (TNF-α) emphasize the therapeutic importance of counteracting pro-inflammatory cytokines in the treatment of IBD. Such local immunoregulation contributes to homeostasis in a healthy host and may account for predisposition to chronic colitis and inflammatory bowel disease (IBD) in susceptible individuals. Specific bacterial:enteric cross-talk mechanisms remain to be elucidated.

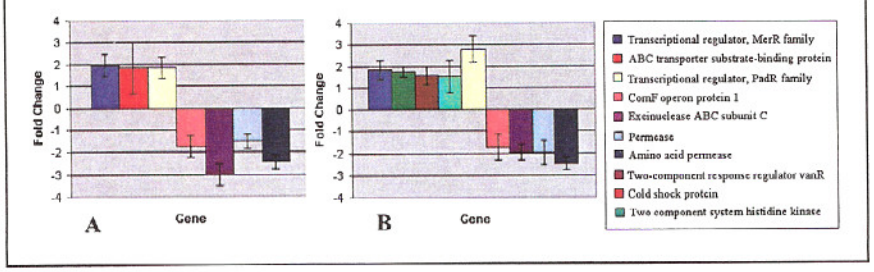
Aims

1. **Identify candidate anti-inflammatory proteins by comparative proteomics of the *Lactobacillus reuteri* secretome.** Comparative proteomic strategies will enable the identification of proteins secreted by TNF-α-inhibitory and absent in TNF-α-stimulatory *Lactobacillus* clones.
2. **Specify proteins secreted by *Lactobacillus* that regulate TNF-α processing by TNF-α convertase in murine macrophages.** Recombinant proteins will be tested for regulation of TNF-α convertase activity and up-regulation of macrophage TIMP-3 activity.

Research Accomplishments During Award Period

Comparative Gene Expression Profiling of *Lactobacillus reuteri* Strains

Figure 1. Candidate Genes are Up-Regulated in Probiotic *Lactobacillus*

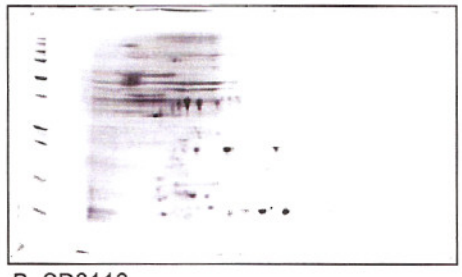


Prior support from the Moran Foundation had enabled our laboratory to design custom genomic microarrays so that candidate probiotic genes could be identified. Comparative gene expression profiling has been performed during the past year to help us identify genes that may regulate expression of secreted proteins or encode secreted proteins themselves. Preliminary data with *Lactobacillus reuteri* microarrays are shown in **Figure 1** and highlight several candidate probiotic genes. Differences in gene expression were compared between strains that were TNF-inhibitory and strains that lacked effects on TNF production by macrophages.

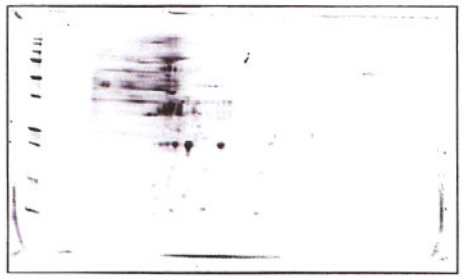
Mouse-derived TNF-α inhibitory Reuteri strains with probiotic activity *in vivo* were compared with mouse-derived Reuteri strains that lacked TNF-α inhibitory activity in mouse macrophages. Candidate probiotic genes were differentially expressed in probiotic TNF-α inhibitory Reuteri clones **(Figure 1)**. Of 1879 *L. reuteri* genes included in this second microarray version, **64 genes (3.4%)** were up-regulated in anti-inflammatory probiotic strains 1583 and 6798 when compared with either of two mouse-derived Reuteri strains lacking TNF-inhibitory activity (6799 and 6801). **Genes were differentially up-regulated if they were expressed at values of 1.5-fold or greater with a p value of 0.05.** Each strain comparison includes a minimum of three replicate experiments. Microarray data displayed normal distributions (normalized by total signal). Candidate quorum sensing genes including *luxS* were consistently up-regulated in the probiotic Reuteri strain 1583 **(Figure 1)** (A, 1583 vs. 6799; B, 1583 vs. 6801). Microarray data with selected genes has been confirmed by semi-quantitative RT-PCR.

Figure 2. 2-D Gels

A. Concentrated Media



B. SD2112



B. MM2-3

Key point: Candidate probiotic genes have been identified in *L. reuteri* based on differential gene expression profiling in *L. reuteri*. Candidates will be targeted for insertional mutagenesis in order to determine the presence of master regulatory genes for probiosis.

Comparative Proteomics by Two-Dimensional Gel Electrophoresis

Lactobacillus conditioned media with known TNF-α inhibitory activity will be subjected to two-dimensional SDS-PAGE studies. We focused these preliminary studies on human-derived *Lactobacillus reuteri* strains with or without TNF-inhibitory activity. The strains with potent TNF-α-inhibitory activity, MM4-1 and MM2-3, were compared to strains that lacked TNF-α-inhibitory activity (SD2112 and CF48).

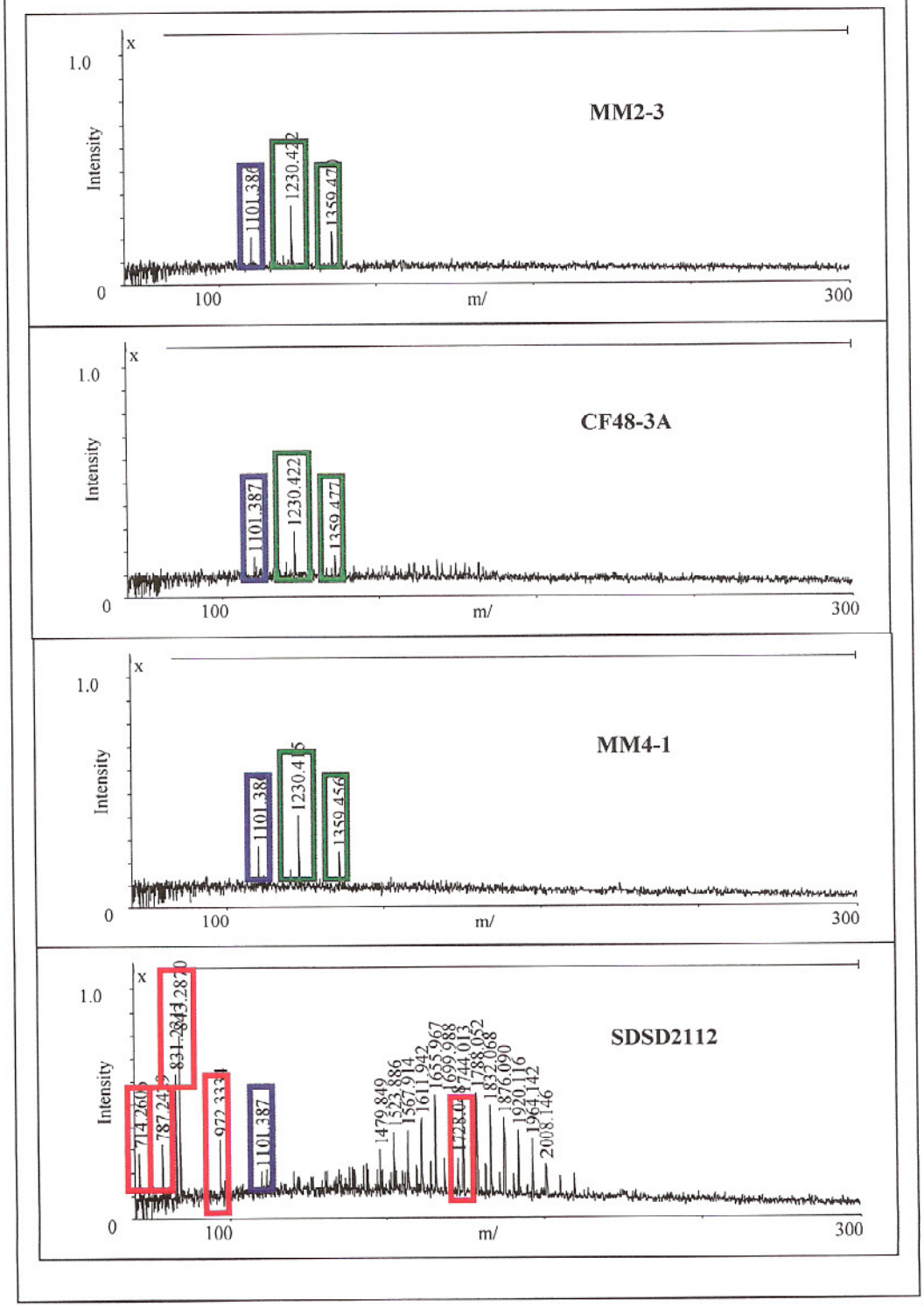
Total protein concentrations were measured by the BCA Protein assay (Pierce, Rockford, IL) and absorbance spectrophotometry (ND 1000, Nanodrop Technologies). Briefly, proteins were separated in the first dimension by isoelectric focusing in a Protein IEF Cell system (Bio-Rad Laboratories, Hercules, CA) using immobilized pH gradient (IPG) gel strips (Bio-Rad) (pH ranges 4-7; 5-8). Proteins were stained with SYPRO fluorescent dyes to maximize detection and resolution, in collaboration with the Proteomics Core of the UTMB-BioMolecular Resource Facility directed by A. Kurosky. Digital images of two-dimensional gel electrophoresis profiles were captured with a deeply-cooled, high resolution CCD camera **(Figure 2)**. Two-dimensional fluorescent protein profiles were compared between *Lactobacillus* clones that inhibit pro-inflammatory cytokines versus isolates that have TNF-α-stimulatory effects.

Figure 2 indicates that the strains have very similar two-dimensional electrophoresis profiles. The images were collected at the UTMB-BioMolecular Resource Facility directed by Dr. Alexander Kurosky. Since no obvious differences were detected, we are now performing more

detailed bio-informatics and imaging studies to detect differences in these complex profiles. We hope to identify subtle differences that may represent key components of the *L. reuteri* secretome.

Multistage Mass Spectrometry (MS-MS)-based Studies of *L. reuteri* Secretome

Figure 3. MS-MS Profiles



In parallel with two-dimensional electrophoresis studies of conditioned media, we have pursued the isolation and identification of small secreted proteins or peptides by MS-MS approaches. In collaboration with Dr. Markus Kalkum at the Beckman Research Institute (City of Hope, CA), we generated comparative MMS profiles of human-derived *L. reuteri* strains **(Figure 3)**. Again, we compared strains with potent TNF-α-inhibitory activity, MM4-1 and MM2-3, to strains that lacked TNF-α-inhibitory activity (SD2112 and CF48).

Lactobacillus conditioned media with known TNF-α inhibitory activity were filtered (0.4 μM filter) and desalted using spin columns (Pierce). Conditioned media was lyophilized and submitted for MS-MS studies. Acetonitrile were removed by evaporation, and fractions were lyophilized. Fractions were shipped to Dr. Markus Kalkum at the City of Hope for MS-MS experiments as part of a formal scientific collaboration. Results were obtained and analyzed by both groups.

We have identified peptides that are differentially expressed and contain poly-glutamate tracts. We are currently trying to identify genes that may encode such peptides in the immunomodulatory *L. reuteri* strains. In the figure, we have indicated peaks that are common to all strains and different or unique to individual strains. The data shown in **Figure 3** represent the first clean set of data with MS-MS studies of *Lactobacillus* conditioned media. Additional experiments are planned so that results may be repeated and individual secreted proteins or peptides may be identified.

This project is still active.

Relevant Abstracts and Publications During Award Period

Abstracts

1. Jones S, Dillon MG, Whitehead KJ, Britton RA, and **Versalovic J**. Expression of *luxS* and Auto-Inducer 2 (AI-2) activity in Probiotic *Lactobacillus reuteri.* American Society for Microbiology Conference on Beneficial Microbes 2005 Annual Meeting, Lake Tahoe, NV. 04/05.

Manuscripts

1. Huang Y, Peña JA, **Versalovic J**. *Lactobacillus*-Mediated Antagonism of Lipopolysaccharide- or *Clostridium difficile* Toxin A-Stimulated Interleukin-8 Production by Human Intestinal Epithelial Cells. Submitted for publication.

2. Dobrogosz WJ and **Versalovic J**. Intestinal Immune Homeostasis: Role of *Lactobacillus reuteri* - An Immunobiotic Species. Submitted for publication.

Submitted manuscripts and meeting posters have acknowledged the support of the Moran Foundation.