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**OBJETIVOS**

Cumplimiento de los Objetivos planteados en la etapa final, o pendientes de cumplir. Recuerde que en esta sección debe referirse a objetivos desarrollados, NO listar actividades desarrolladas.

<table>
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<tr>
<th>Nº</th>
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<th>CUMPLIMIENTO</th>
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<td>1</td>
<td>To research a database of RNAs from the human bacterial flora</td>
<td>TOTAL</td>
<td>For this goal, we extracted all the bacterial RNAs sequences from both, the complete and the draft genomes available, including mRNA, tRNA, rRNA and other non-coding RNAs (?) like small regulatory RNAs. This information, for which one large database is attached to this report, has been classified in two parts, (A) all nucleotides sequences in “FASTA” format. And, (B) all information details about these sequences, like, source, taxonomy, length, and function.</td>
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<td>2</td>
<td>To search for candidates of miRNAs generated from the total bacterial RNAs sequences</td>
<td>TOTAL</td>
<td>For this aim and based on the RNAs databases generated in the previous goal, we used a bioinformatic approach to predict secondary structure for each bacterial RNA. We compared these results with pre-miRNA secondary structure prototype-model to generate a list of candidate miRNA.</td>
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<td>3</td>
<td>To find possible targets in the human mRNAs sequences that may bind these predicted bacterial miRNA</td>
<td>TOTAL</td>
<td>Our results suggest that 110 predicted bacterial RNAs derived from 37 different bacteria contain all the properties to generate miRNAs, which can be involved in 47 different types of human diseases</td>
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<td>4</td>
<td>To evaluate the significance of the bacterial miRNA prediction and determine their binding to human RNAs</td>
<td>TOTAL</td>
<td>This goal includes two parts: A) miRNA generated by bacterial RNAs: we transfected total bacterial RNAs and bacterial siRNA to human cells using lipofectamine (Invitrogen) and have checked their effect on human RNAs by Quantitative PCR (Real Time-PCR), and B) the analysis of the bacterial miRNAs binding to human RNA: by using the Northern blot technique, we have hybridized the predicted bacterial miRNAs with total human RNAs. The results show that the three miRNA can significantly regulate the expression of the respective target human gene.</td>
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Otro(s) aspecto(s) que Ud. considere importante(s) en la evaluación del cumplimiento de objetivos planteados en la propuesta original o en las modificaciones autorizadas por los Consejos.
This project proposes to use a bioinformatic approach to search and identify possible miRNAs generated from bacterial RNAs and to experimentally validate it. For this, we will perform the following studies:

**Goal 1: To research a database of RNAs from the human bacterial flora:**

For this goal, we extracted obtained all the bacterial genes sequences from the complete and the draft genomes available, included mRNA, tRNA, rRNA and other non coding RNAs like small regulatory RNAs. A database of bacterial RNA sequences was created using Bioperl scripts developed in-house. Genomic sequences and annotation for all bacteria from human flora was obtained from the GenBank database that is accessible through the Human Microbiome Project (HMP) website (www.hmpdacc.org/). This database included all available bacteria genomes from the human flora from different parts of the body, such as airways, blood, eye, gastrointestinal tract, heart, none, oral, skin and urogenital tract (438 genomes) [Figure 1]. The sequence sources of these bacteria are from different institutes like “Broad”, “WashU”, “JCVI” and others.

**Figure 1: genome of microbes from seven parts of the human body.**

In addition, we incorporated all bacterial genomes available from the NCBI database (http://www.ncbi.nlm.nih.gov/), which includes pathogenic bacteria (389 genomes). One big database is attached to this report, which is divided in two parts, (A) all nucleotides sequences in “FASTA” format. And, (B) all the detailed information about these sequences like source, taxonomy, length, and function. This database includes more than 2.5 million bacterial RNAs!! **This goal was accomplished.**

**Goal 2: Search for candidates of miRNAs generated from the total bacterial RNAs sequences:**
For this aim we used a bioinformatic approach to predict the secondary structure of each bacterial RNA utilizing the RNAs database generated in the previous goal. We compared those results with pre-miRNA secondary structure prototype-model to generate a list of candidate miRNAs. The prediction of the single stranded nucleic acid secondary structure was calculated using the "Mfold” program (http://frontend.bioinfo.rpi.edu/applications/mfold/) at optimal temperature (37°C). Each result was searched automatically using the in-house developed “Bioperl” scripts to find secondary structure [Figure 2] similar to pre miRNA, approximately 60–80 nucleotides long imperfect hairpin (Han, J., Lee, Y., Yeom, K.H, Kim, Y.K, Jin, H., and Kim, V.N. 2004. The Drosha-DGCR8 complex in primary microRNA processing. Genes. Dev. 18(24), 3016–27).

**Figure 2:** Pre-miRNA secondary structure. Definition of pre-miRNA secondary structure (A) hairpin secondary structure with a minimum length of 19 nucleotides, and (B) hairpin secondary structure with a minimum length of 19 nucleotides with an internal loop of up to 25% nucleotides more than the total nucleotides of the complementary strand. The end loop length is unlimited.

For this heavy calculation we run, at the same time, 10 RNA molecules using a computer cluster with the LINUX system X64. Given that the average running time for calculating a single-molecule is 5 minutes, the whole calculation would take 2,500,000 RNAs X 5 min = more than 28 month!. To circumvent this problem, we designed a robot (BioPerl) that automatically checks for each RNA molecule before listing it, calculating the feasibility of its sleeping area sequence to be compatible with any single human cell target [Figure 3] (42,753 target genes of the human genome – cell RNAs and mitochondrial RNAs, from NCBI, GenBank database).

**Figure 3:** work steps to identify possible human mRNA sequence targets of bacterial RNAs.(note! The first search between bacterial RNA and the Human genome is a global comparison, the second one is to confirm the local comparison (~21 bases between miRNA and the possible targets).

In addition, this robot receive all the input files for each RNA molecule and separate only the necessary information for its secondary structure and it automatically illumined any garbage information (frees disk space above 90%), with that method we expect to finish this calculation in half of the time!. (This goal was accomplished)

**Goal 3:** To find possible targets in the human mRNA sequences that may bind these bacterial miRNA predictions:
As a natural extension of the above results we have chosen to deepen our study to find possible target genes in the case of infection of a human cell. To do this, we first introduced all the predicted results previously obtained, in an "UNAFold" program. This showed feasibility of over 3000 genes including secondary structure similar to pre-miRNA (data not attached), these were jointed into a new database and processed using "BioPerl" program and "BLAST" program to find any complementary sequence relationship between the miRNA prediction and any human mRNA included all mitochondrial mRNA obtained from the NCBI. A total of 548 of total miRNA secondary structure prediction derived from 122 different bacteria presented complementary site between miRNA sequence and human mRNA. All of those genes were added to a new database together with target genes data. The average quantity of miRNA forecasting per organism was 15 miRNA predictions [Figure 4, A]. Statistically, the length range of all miRNA predicted with known target is between 19 nucleotides up to 50 nucleotides, while 96.6% of this miRNA predictions showing length between 19-29 nucleotides [Figure 4, B].

![Figure 4: miRNA prediction yield and length. (A) This graph shows that 40 bacteria had only one miRNA prediction, the highest yield miRNA predictions (28 miRNA) was in Burkholderia cenocepacia J2315 bacteria and the average amount of total miRNA predictions per organism was 15. (B) The area (blue color) showing that most of miRNA prediction length (96.6%) is between 19-29 nucleotides.](image)

Additionally, we have searched all human mRNA targets obtained from our last database in the "OMIM" database (Online Mendelian Inheritance in Man) that catalogues all the known diseases with a genetic component and contains information of all the medical studies published for each human gene and diseases involved, including cases of gene mutations. After a search that included a total of 122 different bacteria including 548 of total miRNA secondary structure prediction, only 37 bacteria with a total of 68 miRNA predictions indicate genes target involvement in known human diseases such as leukemia and colon cancer. **This goal was accomplished.**

**Goal 4: Evaluate the affect significance of bacterial miRNA prediction and determine their binding to human RNAs.**

This goal includes two types of experiments: A) **Quantitative PCR** - we will transfrect bacterial RNAs to human cells using lipofectamine (Invitrogen) and check their effect (down or up regulation) on human mRNA by Quantitative PCR (Real Time-PCR). These experiments will be performed with selected predicted miRNA and will use total bacterial RNA, and B) **Northern blot** - the analysis of the bacterial miRNAs binding to human mRNA using the Northern blot technique, the idea is to see if the bacterial RNA had process to miRNA after transfrecting human cell by internal RISC mechanism. **This goal was accomplished.**

**Experimental result:** To confirm our prediction, specific assays were carried out with bacterial miRNAs, which are complementary to mRNA of the human genes PTPRJ (protein tyrosine phosphatase receptor type J); NFKB1 (Nuclear factor NF-kappa-B); and DEK oncogene variants 1 and 2. Human HEK 293 cells are transfrected with synthetic miRNA, derived from Burkholderia vietnamiensis G4, Burkholderia mallei, and Arcobacter butzleri, respectively. After 0,12, 24, and
48 hours, the cells are collected, total RNA is prepared and total cDNA is synthesized. Changes in the expression of human targets mRNAs (down-regulation) is measured by real time PCR using primers specific for the above mRNAs, in all three cases, the results indicated significant reduction in target gene expression significant expression.

A first assay was carried out with a putative miRNA from *Burkholderia vietnamiensis G4* in modifying the amounts of the protein tyrosine phosphatase receptor type J (PTPRJ) transcript variant 2 mRNA in human cells [Figure 5]. The results show that putative miRNA sequence generated from *Burkholderia vietnamiensis G4* bacteria, down-regulation between tow up three times its target PTPRJ gene expression. The protein encoded by this gene is a member of the protein tyrosine phosphatase (PTP) family. PTPs are known to be signaling molecules that regulate a variety of cellular processes, including cell growth, differentiation, mitotic cycle, and oncogenic transformation. The protein PTPRJ, is a tumor suppressor gene that has been implicated in a range of cancers, including colon cancer and breast cancer. We suggest that reducing PTPRJ mRNA expression by putative bacterial miRNA may increase the risk as creating tumors cells.

![Figure 5: Changes in the synthesis of human protein tyrosine phosphatase receptor type J (PTPRJ) mRNA, at different times, at 3 different Burkholderia vietnamiensis G4 miRNA concentrations.](image)

A second assay was carried out with a putative miRNA from *Burkholderia mallei* in modifying the amounts of the NFkB1 mRNA in human cells [Figure 6]. The results show that our prediction putative miRNA sequence generated from *Burkholderia mallei* bacteria, can down-regulation and reduce up to five times its target NFkB1 mRNA. NFkB1 is part of NFkB complex (nuclear factor kappa-light-chain-enhancer of activated B cells), activated NFkB complex translocates into the nucleus and binds DNA at kappa-B-binding motifs. NFkB1 gene mutation have show causes to second rheumatoid arthritis. In additionally, NFkB complex is involved in cellular responses to stimuli such as stress, and bacterial or viral antigen. Given the above, we can suggest that disruption of this system facility pathogen penetration and general defense suppression.

![Figure 6: Changes in the synthesis of NFkB1 mRNA, at different times, at 3 different Burkholderia mallei predicted miRNA concentrations.](image)
Another assay was carried out with a putative miRNA from *Arcobacter butzleri* in modifying the amounts of the DEK oncogene mRNA in human cells [Figure 7]. The results show that putative miRNA sequence generated from *Arcobacter butzleri* bacteria, can reduce strongly its target DEK mRNA amount. DEK is a highly abundant, evolutionarily conserved and ubiquitous nuclear protein that can be regulated at the level of transcription and post-translational modifications. Evidence suggests that it may function as a nuclear architectural protein. Many studies support distinct intracellular functions for DEK in DNA replication, positive and negative regulation of gene transcription, histone acetylation, mRNA splicing and nucleosome assembly. Several reports have already suggested that DEK may modulate genome stability. Undoubtedly, the silencing of this protein can cause abundant mutations and damage in the cell.

**Figure 7**: Changes in the synthesis of human DEK oncogene mRNA of variants 1 and 2 at different times, at 3 different *Arcobacter butzleri* miRNA concentrations

In additionally, using Northern blot technique we detect (only in *Burkholderia vietnamiensis G4 miRNA prediction*) that total bacterial RNA had process to miRNA after transfecting into human cell by internal RISC mechanism. The results clearly showed that human cells infected with total RNAs can down regulated the expression of differences genes involved in diverse diseases, starting 24 hours after the infection.

**Figure 8**: miRNA generated from total RNA of *Burkholderia vietnamiensis G4*, by the internal human cell mechanism (FT293). Amount of miRNA increased inversely to bacterial mRNA degradation.

Our results show that sequences derived from the bacterial RNA can act down regulating of specific human target genes. This regulation may affect processes such as metabolism, cell division, etc, of the human cell which may be causes of different diseases. The results of this research indicate the possibility of studying in more detail the molecular basis of bacterial pathogenicity.
OTHER ACHIEVEMENTS OF THE PROJECT:
- Research visit(s) to other institution(s).
- Outreach activities related to the project’s main topic.
- Any other contribution, not addressed elsewhere, that you consider important.

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1) Congress EMBO, Switzerland, May, 2011.
   I had presented the poster “Bacterial RNAs and host human cells interactions” showing my bioinformatic pipeline developed to discover possible miRNAs generated from bacterial RNAs that may have the potential to regulate gene expression of the host human cell, in case of infection. This work got special attention.

2) Collaboration with Dr. Daniel Paredes-Sabja y Juan Pablo Morales.
   I established a collaboration with the Laboratory of Dr. Daniel Paredes-Sabja and Juan Pablo Morales Montenegro from Universidad Andres Bello, Santiago. They are working with several pathogenic bacteria and the main subject of this collaboration is to obtain images of the bacterial RNAs movement from the bacteria to other cells. So far, we have obtained images showing fluorescent bacterial RNAs go out from the bacteria to the external environment, and now we are trying to see it inside human cells after the infection.

   I presented the poster "Genetic infection - case study of microRNA in bacterial infection" showing the bioinformatic pipeline developed to discover possible miRNAs generated from bacterial RNAs that may have the potential to regulate gene expression of the infected host human cells. This work got special attention from "NAVAL Medical Research Center".

4) Meeting "ISCB Latin America 2012 - Conference on Bioinformatics", Santiago, Chile.
   The poster “Bacterial RNAs and host human cells interactions” presented showed the bioinformatic pipeline developed to discover possible miRNAs generated from bacterial RNAs that may have the potential to regulate gene expression of the infected host human cells.
5) **Mathematical and Computational Medicine Conference, Mexico, Diciembre 2012.**
I have showed poster indicated the bioinformatic algorithm developed to discover possible miRNAs generated from bacterial RNAs that may have the potential to regulate gene expression of the infected host human cells.

6) "**Bio-IT conference & expo**. Boston, USA, April 2013.** I had presented the poster "**Bacterial RNAs and host human cells interactions**" showing the "wet" results base my bioinformatic pipeline developed to discover possible miRNAs generated from bacterial RNAs that may have the potential to regulate gene expression of the host human cell, in case of infection.
En este último año de su postdoctorado sobre "Bacterial RNA and host human cell interaction", Amir Shmaryahu, ha completado el objetivo 1 del proyecto.

Completada la evaluación experimental de los predicciones, buscando efectos de regulación de la expresión genómica generada por los micro ARN bacteriano seleccionados. Se diseñó y normalizó exitosamente un experimento vía transfección de ARN bacteriano para ver los posibles efectos en el blanco humano.

Estos experimentos incluyen: A) transfección de total ARN de 3 bacterias seleccionadas, *Arocobacter butzleri RM4018, Burkholderia vietnamiensis G4 y Chromobacterium violaceum* para evaluar los efectos en el blanco humano que relacionan en diferentes enfermedades. B) transfección de siRNA de 7 bacterias seleccionadas. 5 de los 7 miRNA he mostraron efecto significativo a su genes blancos que pueden afectar diversas enfermedades tales como cáncer de colon, diabetes, artritis, y leucemia.

La hipótesis de este proyecto junto con los resultados se van a publicar en una revista ISI próximamente (el paper está en revisión).

Durante estos 3 años Amir ha desarrollado un trabajo de alta calidad e interés en un tema muy novedoso, estableciendo colaboraciones con investigadores de la UNAB dispuestos demostrar experimentalmente el movimiento del ARN entre células. Los resultados obtenidos muestran la migración de ARN intracelular de bacteria hacia el medio ambiente externo.


Firma Investigador(a) Patrocinante

Fecha: 10 de Septiembre 2013
PRODUCTOS

ARTÍCULOS
Para trabajos en Prensa/ Aceptados/Enviados adjunte copia de carta de aceptación o de recepción.

Nº : 1
Autor (a)(es/as) : Amir Shmaryahu Margarita Carrasco and Pablo D.T. Valenzuela
Nombre Completo de la Revista : Journal of Microbiology
Título (Idioma original) : Prediction of bacterial microRNAs and 1 possible targets in human cell transcriptome.
Indexación : ISI
ISSN : 1976-3794
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OTRAS PUBLICACIONES / PRODUCTOS

Sin información ingresada.

CONGRESOS

Nº : 1
Autor (a)(es/as) : Amir S. and Pablo D.T.V.
Título (Idioma original) : Bacterial RNAs and host human cells interactions
Nombre del Congreso : Mathematical and Computational Medicine Conference
País : MEXICO
Ciudad : Xcaret
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