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創傷弧菌未知毒力因素之探索

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行政院國家科學委員會補助專題研究計畫成果報告

創傷弧菌未知毒力因素之探討

Exploration of unidentified virulence factors of *Vibrio vulnificus*

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計畫主持人：何漣漪
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- 國際合作研究計畫國外研究報告書一份

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一、中文摘要

於本研究中我們嘗試利用微陣列，以比較基因體學和基因表現圖譜分析法來進行創傷弧菌致病機轉之基因體學研究。我們製備了一個包含衍生自可能參與莢膜多醣體合成及其表現可能受鐵離子調控之開放閱讀架的探針之微陣列。之後，我們分別以由不同創傷弧菌菌株抽取出的基因體DNA和由菌株及其突變株抽取出的RNA作為標的基因，以進行比較基因體學和基因表現圖譜分析。我們在本次比較基因體學的前驅試驗當中，找到了一個YJ016菌株中參與莢膜合成的一個ORF，並進一步找到包含此ORF的一個與莢膜合成相關的區域。本研究結果顯示此兩方法可用以鑑識新的毒力基因。

關鍵詞：創傷弧菌、比較基因體學、基因表現圖譜分析、微陣列

二、緣由與目的

V. vulnificus is a marine bacterium causing severe wound infection and fulminant primary septicemia via gastrointestinal tract infection in humans, particularly in those with liver cirrhosis or immunocompromised conditions (1, 2, 3). A few virulence factors, such as the capsule (4) and iron-acquisition ability (5, 6), have been identified. However, the mechanism for its invasion from local infection site to the bloodstream and the genes involved in this process remain largely unknown.

We have used several strategies for identification of genes that are important for this function, including the computational search for homologues of known virulence genes of other bacteria and complementation of a spontaneous avirulent mutant. Several novel virulence factors, such as the RTX toxin and TolC, and regulators of virulence, like the quorum-sensing signaling system, were thus identified. We have demonstrated that a regulator, LuxO, of the quorum-sensing system is required for the expression of virulence in mice (7). It would be crucial to know the target genes regulated by LuxO in order to understand the virulence mechanism of this organism.

DNA microarray has been used in a variety of techniques for the identification of
virulence genes (8, 9). In the comparative genomic studies, the genes of a virulent strain, whose genome sequence is known, that are absent from an avirulent strain can be identified on a DNA microarray hybridized with a mixture of genomic DNAs from both strains. In the gene expression profile analysis, the genes differentially expressed in the virulent strain and avirulent strains are identified on a DNA microarray hybridized with a mixture of cDNAs prepared from the total RNA extracted from both strains. Therefore, either method is useful for the identification of potential virulence genes. The importance of the genes identified by these methods is further determined by the isolation and characterization of specific gene-knockout mutants.

The whole genome sequence of *V. vulnificus* strains YJ016, a clinical isolate that is highly virulent in mice, has been completed recently (10). This lends us an opportunity to perform the genomic studies. For example, to identify the target genes regulated by LuxO, a gene expression profile analysis can be conducted with YJ016 and its ∆luxO mutant. In this study, we compared different probe preparations and have found the optimal conditions for these two techniques in the genomic studies on *V. vulnificus*. In addition, by the comparative genomics, we have identified a gene cluster for the synthesis of capsular polysaccharide in strain YJ016.

三、結果與討論

From the completed genome sequence of *V. vulnificus* YJ016, it is known that this strain contains a large chromosome of 3,377 kbp, a small chromosome of 1,857 kbp, and a plasmid of 48.5 kbp. The numbers of ORFs predicted are 3,262, 1,697, and 69, respectively, for the large chromosome, small chromosome and plasmid. For the pilot study, we chose 12 ORFs potentially involved in the biosynthesis of capsular polysaccharide (CPS) and 29 ORFs that may be regulated by iron, according to the annotation data. The reason why we made such a choice was because both properties have been shown to be related to the bacterial virulence in mice.

Two types of probes were prepared: one was derived from the shotgun libraries used in the determination of genome sequence; the other was ORF-specific. To prepare the first type of probes, 2 to 3 overlapping clones that contained the tested ORFs were selected, and the probes were produced from each clone by PCR with the universal primers. To prepare the second type of probes, the primers were designed according to the nucleotide sequence of each ORF, and the probes were produced by PCR with these primers. Both types of probes were added an amine group at the 5’ end of one of the primer pair used in each reaction to achieve a better linkage of the denatured probe with the glass slide. One hundred and thirteen PCR products, including 66 (24 derived from CPS synthesis genes and 42 derived from iron-regulated genes) amplified from the shot gun libraries, 41 ORF-specific ones, and 6 controls (2 from 23S rDNA, 2 from lacZ, and 2 from hlyA) were prepared for each strand of the PCR products. Therefore, there were 226 single-stranded probes in total. The probes were denatured and dotted onto a 3D-link™ activated glass slide with a DNA microarrayer. The glass slides with the DNA microarray were then used in two experiments.

In one experiment, the genomic DNAs were extracted from strain YJ016, and other three translucent (supposedly acapsular) environmental strains. The DNAs were then labeled with Cy3 or Cy5 fluorescent dye by random priming with the Klenow fragment. The labeled genomic DNAs from YJ016 and one of the translucent strains (20 µg of each) were mixed and added onto the DNA microarray. After hybridization between the targets and the probes, the fluorescence on the glass slide was detected by a fluorometer, and the cy3:cy5 ratios were calculated with GenePix v4.0. Each reaction was triplicated, and the results were very similar (One of the reactions, comparative genomic analysis between YJ016 and CG061 is shown in Fig. 1). By comparative genomics, ORF VV0361 was found to be absent from all three acapsular strains. This ORF was located in a
region (Fig. 2) that contained ORFs with high sequence homology with the genes involved in biosynthesis of the type 1 CPS in *E. coli*.

Fig. 1 Comparative genomic analysis with DNA microarray. The probes were duplicated on the glass slide. The target DNA prepared from strain YJ016ΔlacZ was labeled with Cy3 (red) and that prepared from CG061 was labeled with Cy5 (green). (1) Three probes derived from overlapping shotgun clones that all contained ORF VV0361. (2) ORF VV0361-specific probe. (3) lacZ-specific probe used as a control.

By detecting the ORFs of this region in a collection of encapsulated and acapsular environmental *V. vulnificus* strains by southern hybridization, we found that ORFs VV337 to VV340 may be required for CPS synthesis, and VV0363 to VV0366 may be important for bacterial resistance to human serum killing effect (data not shown).

In the other experiment, the total RNA was extracted from strain YJ016 and a Δfur isogenic mutant, which lacks the repressor (Fur) for the iron-regulated genes and the expression of these genes are therefore expected to be upregulated. The cDNAs were then synthesized from total RNA by reverse transcription with a random primer and labeled with Cy3- or Cy5-dUTP. The cDNAs from these two strains that are labeled with different fluorescent dyes were mixed in equal amounts and then added to the DNA microarray. After hybridization, the fluorescence of each spot was detected and the Cy3:Cy5 ratio was calculated as described above. Our data indicated that, as expected, the genes involved in synthesis of the siderophores, their receptors, or heme receptor gave rise to a Cy3 (labeled cDNA of Δfur) to Cy5 (labeled cDNA of YJ016) ratio of higher than 3 (data not shown).

Our results indicate that DNA microarray can be used to search in a genome-wide manner for genes in *V. vulnificus* that are present in one strain but not in another. It can also be used to identify the genes that are differentially expressed under different conditions or in different strains. Both have potential applications in researches on the virulence mechanism. Furthermore, by comparing the results obtained from DNA microarrays with different types of probe, we found that the ORF-specific probes are superior to those prepared from the shotgun library for the following reasons. First, the probes derived from the clones in a shotgun library usually contain multiple ORFs and, therefore, the reaction of an ORF may be masked by other ORFs contained in the same probe. Second, preparation of shotgun library-derived probes is much more time-consuming, because the clones need to be organized in order, and the plasmid has to be extracted from each clone to be used as the template in PCR. All of these drawbacks were not found with the ORF-specific probes. We suggest that although preparing the ORF-specific probes is more expensive, this type of probes is preferable, particularly in gene expression.
profile analysis.

In the future, we would like to use DNA microarray to identify the genes that are not expressed in the \( \Delta \text{luxO} \) mutant and a mutant strain, NY303, which contains an unknown mutation that results in loss of cytotoxicity and virulence for mice. The importance of such genes in virulence can then be tested by isolation and characterization of the specific gene-knockout mutants.

四、計畫成果自評

The purpose of the three-year research project that ended by July of this year was to identify novel virulence genes by genomic studies. Several strategies have been employed, and a few genes that encode a novel cytotoxin, a protein transporter, and a regulator, have been found to be involved in \( V. vulnificus \) virulence in mice. Still some other techniques that can be used for a genome-wide search of the potential virulence genes, such as signature-tagged mutagenesis, have been developed. However, we have not succeeded in adopting these techniques in our studies on \( V. vulnificus \). We thought that DNA microarray, a technique that has been established in some laboratories in Taiwan, would be a useful tool for our purpose. We started with a small-scale pilot study to evaluate the feasibility of this in our studies, and we obtained a courageous result.

We went on to define the border of the deletion in the acapsular environmental strains, hoping that might be able to identify a region involved in the biosynthesis of CPS. Indeed, we found a gene cluster (containing 32 putative ORFs) in strain YJ016, in which two genes have been shown to be required for formation of capsule. This part of work has been finished in our laboratory by JF Wang and published as a Master’s thesis (11), and a manuscript on this work is in preparation.

With adequate research grant, we would be able to construct a genome-wide scale of DNA microarray and commence a genome-wide search of novel virulence genes by gene expression profile analysis or comparative genomics. By characterizing the mutants knocked out in these genes, we will be able to delineate the pathogenesis of infectious disease caused by \( V. vulnificus \).