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Original article

DNA microarray analysis of the heat- and cold-shock stimulons in *Yersinia pestis*

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Abstract

DNA microarray was used as a tool to define the heat- and cold-shock stimulons in *Yersinia pestis*. Heat shock dramatically enhanced the transcription of genes encoding major heat-shock proteins (MHSPs) that are important for cell survival against the heat. Many other genes were also greatly up-regulated, but their roles in heat-shock response need to be elucidated. Meanwhile, heat shock retarded most of the metabolic processes, i.e. RNA transcription, protein translation, aerobic respiration, energy metabolism, small molecule metabolism, peptidoglycan biosynthesis, sulfate uptake and cysteine biosynthesis. In response to cold shock, *Y. pestis* has evolved complex adaptive mechanisms by elevating the transcription of a specific set of genes whose protein products are designed to prevent or eliminate cold-induced DNA or RNA structuring, to remodel cell membrane components for maintenance of normal functions, to elevate the energy generation for ensuring ATP-dependent responses during cold adaptation and to synthesize or transport compatible solutes such as cryoprotectants, and at the same time, by repressing the mRNA level of certain genes whose protein products are not needed for bacterial growth at low temperatures, such as the MHSPs. These results provide a set of new candidate genes for hypothesis-based investigations of their roles in stress response, host adaptation and pathogenicity of this deadly pathogen.

Keywords: *Yersinia pestis*; DNA microarray; Transcriptional profiling; Heat shock; Cold shock

1. Introduction

DNA microarray technology is able to determine changes in mRNA levels simultaneously for all the genes in a cell. In discussing gene expression profiling by microarrays, the concept of stimulons is important. A stimulon is a group of transcription units that are differentially expressed in response to environmental perturbation. DNA microarray technology represents an extremely powerful tool to identify stimulons [1]. For example, DNA microarrays have been used to study the transcriptional response of *Escherichia coli* to heat shock [2], cold shock [3], osmotic stress [4], oxidative stress [5,6], low pH [7,8], low oxygen [9] and sodium dodecyl sulfate (SDS) stress [10]. These studies discovered wide sets of genes whose transcription was enhanced or repressed by specific stress signals, which provides a global perspective allowing one to see that seemingly unrelated activities are modulated together.

Yersinia pestis, a nonmotile Gram-negative bacillus, is the causative agent of plague. The maintenance of plague in nature is dependent upon interactions between flea vectors, mammal reservoirs and the environment. *Y. pestis* in its life cycle or during infection is inevitably exposed to a series of environmental changes that can make its living conditions far from optimal. The molecular basis for stress response in *Y. pestis* is still poorly understood, although the genomic sequences of *Y. pestis* strains CO92 [11], KIM [12] and 91001 [13] have been determined in the past 3 years. In this study, whole-genome DNA microarrays were used to investigate the alter-

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ation of the *Y. pestis* transcriptome induced by heat and cold-shock and furthermore, to define multiple stimulons composed of groups of transcriptional units.

2. Materials and methods

2.1. Strain and growth conditions

Y. pestis 201 was isolated from *Microtus brandti* in Inner Mongolia, China. It has major phenotypes, such as $F1^+$ (able to produce fraction 1 antigen or the capsule), VW^+ (presence of V antigen), Pst^+ (able to produce pesticin) and Pgm^+ (pigmentation on Congo-red media). Strain 201 has an LD_{50} of less than 100 cells for mice by subcutaneous challenge. Strain 201 belongs to a newly established *Y. pestis* biovar, microtus [14]. Biovar microtus strains are supposed to be avirulent to humans, although they are highly lethal to mice. These strains have a unique genomic profile of gene loss and pseudogene distribution, which is thought to be responsible for their human attenuation nature [14].

TMH medium was used for cultivating the bacteria, since TMH is a chemically defined medium containing salts, vitamins, HEPES, potassium gluconate, diverse amino acids, NaS_2O_4, and some variable components such as $CaCl_2$ and $FeSO_4$, as described previously [15]. Overnight culture of strain 201 was used to inoculate the fresh TMH medium, and it was allowed to grow at 26 °C to the exponential growth phase (A_{620} = 0.6). Bacteria were then transferred to grow at 37 °C for 1 h to be ready for stress experiments. For the heat-shock experiments, cells were incubated at 45 °C for 10 min; control culture was allowed to continue growing at 37 °C for the same period of time. For the cold-shock experiments, control samples were taken at 37 °C immediately prior to cold-shock, and test samples were collected 1 h after shock at 10 °C.

2.2. DNA microarray analysis

Immediately before harvesting for RNA isolation, bacterial cells were mixed with RNAprotect Bacteria Reagent (Qiagen) to minimize RNA degradation. Total RNA was isolated by using the MasterPure™ RNA Purification kits (Epicenter). RNA quality was monitored by agarose gel electrophoresis, and RNA quantity was measured by spectrophotometer. Fifteen to twenty micrograms of RNA was used to synthesize cDNA in the presence of aminoallyl-dUTP, genome-directed primers (GDPs) and random hexamer primers with the Superscript II system (Invitrogen). The reverse transcription of bacterial RNA by the mixture of GDPs and random hexamers has been proven to be more effective and reliable than with either GDPs only or random hexamers only [16]. The aminoallyl-modified cDNA was then labeled by Cy5 or Cy3 monofunctional dye (Amersham) according to the manufacturer' instructions. Glass slides spotted with PCR amplicons representing about 95% of nonredundant annotated genes or open reading frames (ORFs) of *Y. pestis* CO92 and 91001 were used for probe hybridization [17]. Briefly, based on the genomic sequences of *Y. pestis* CO92 and 91001, a total number of 4015 annotated ORFs were selected after the exclusion of ORFs encoding IS protein, integrase, and transposase. Specific primer pairs were designed to amplify nearly the full length of each gene. 4005 purified, successful ampilcons were spotted on the CSS-1000 silylated glass slides (CEL) by using a SpotArray72 Microarray Printing System (Perkin Elmer Life Sciences) to construct the DNA microarrays. The spotted slides were cross-linked by using a UV Stratalinker (Hoefer). $NaBH_4$ was used to block the free aldehyde groups on the slide surface. The slides were prehybridized in a buffer containing 5 × SSC, 0.1% SDS and 0.1% BSA, and then washed and blown to dry. The two differentially labeled cDNA samples were dried and then resuspended in hybridization solution (50% deionized formamide, 5 × SSC, 0.1% SDS, 5 × Denhardt's solution, and 0.5 μg/μl of sheared salmon sperm DNA). The labeled DNA samples hybridized with the slides at 42 °C for 18–20 h. After hybridization, the slides were washed in 1 × SSC with 0.06% SDS for 2 min, then in 0.06 × SSC for 2 min and finally in ethanol for 2 min. The slides were blown to dry and then were scanned by using a GenePix Personal 4100A Microarray Scanner (Axon Instruments).

The signal intensity and local background measurements were obtained for each spot by using GenePix Pro 4.1 software (Axon Instruments). The data filtering and normalization were processed by using MIDAS program (www.tigr.org/software/tm4/). Briefly, spots with background-corrected signal intensity (median) in both channels lower than twofold of background intensity (median) were rejected from further analysis, and then the remaining data points were normalized by total intensity normalization methods. The normalized $\log_2$ ratio of test/reference signal for each spot was recorded. Finally, the averaged $\log_2$ ratio for each gene with at least three data points were calculated. Significant changes in gene expression were identified with significance analysis of microarrays (SAM) software [18] using one class mode (the measurement is the log (red/green) ratio from two labeled samples hybridized to a cDNA chip, with green denoting before treatment, and red, after treatment). SAM assigns a score to each gene on the basis of change in gene expression relative to the standard deviation of repeated measurements. For genes with scores greater than an adjustable threshold, SAM uses permutations of the repeated measurements to estimate the percentage of genes identified by chance, the false discovery rate.

2.3. Real-time quantitative RT-PCR

Based on genomic location, gene length and transcriptional changes, 11 genes were chosen for real-time PCR analysis. Gene-specific primers are listed in Table 1. cDNA was generated using 5 μg of total RNA and 3 μg of random hexamer primers. Real-time PCR was performed in triplicate for each RNA preparation by using the LightCycler system