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DNA Microarray-Based Global Transcriptional Profiling of *Yersinia pestis* in Multicellularity

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***Yersinia pestis*, the causative agent of plague, has a feature of forming multicellular aggregates at liquid-air interface around the wall of glass tube. In this study, we employed the whole-genome DNA microarray of *Y. pestis* to investigate the global transcriptional profile in multicellularity compared with that in its planktonic growth. A total of 177 genes were differentially expressed in *Y. pestis* during early stage of multicellular formation; Seventy genes of them were up-regulated while 107 down-regulated. In addition to a large number of genes encoding unknown functions, most of the induced genes encode cell envelope and transport/binding proteins. The up-regulation of amino acid biosynthesis, the differentially altered genes that are involved in virulence, and the cold shock protein genes were for the first time reported to be associated with the multicellular formation. Our results revealed the global gene expression of *Y. pestis* were changed in the formation of multicellularity, providing insights into the molecular mechanism of multicellular behaviour, which need investigating further.**

***Keywords*:** *Yersinia pestis*, multicellularity, DNA microarray, transcriptional profiling

Many bacteria exhibit two distinct modes of growth, a free-floating planktonic mode and a sessile biofilm mode. Biofilms are surface-associated microbial communities which are thought to be the predominant growth state of bacteria in many natural environments (Davey and O'Toole, 2000). It has been a subject of intense interest in recent years due to its important roles in interactions between nonpathogenic/pathogenic bacteria and host. Biofilms also have a significant impact on medical and industrial settings, due in part to the increased antimicrobial resistance of bacteria in biofilms (Costerton *et al*., 1995; Rose *et al*., 2003). Although there are different vocabularies, autoaggregation (attachment of one species to its clonal decendents), coaggregation (attachment of different species to one another), coadhesion (aggregation) between benthic (sedentary), and planktonic cells (floaters), to describe the various nuances of stickiness relating to biofilm formation etc. (Gilbert and Rickard, 2004), we here refer the 'biofilm', formed around the glass tube at interface between liquid and air (see supplementary Fig. S1), of *Y. pestis* as multicellular aggregates because it is not a typical biofilm.

Yersinia pestis, the causative agent of bubonic and pneumonic plague in humans, persists in populations of wild rodents in many parts of the world, and is transmitted primarily by the bites of infected fleas (Little and Brubaker, 1972). Although cases of human plague can be well controlled by timely administration of antibiotics and the plague is presently not a major health problem, plague is still with us, circulating in various mammalian species in Asia, Africa, and America, where epizootics of plague cause a substantial threat to public health. On the other hand, clinical antibiotics-resistant *Y. pestis* strains have been reported (Galimand *et al*., 1997; Guiyoule *et al*., 2001). Plague has been recognized as a re-emerging disease by the World Health Organization (Titball *et al*., 2003).

To produce a transmissible infection, *Y. pestis* colonizes the flea midgut and forms a biofilm in the proventricular valve, which blocks normal blood feeding (Erickson *et al*., 2006). Furthermore, *Y. pestis* could form biofilms through adhere to the external mouthparts and block feeding of *Caenorhabditis elegans* nematodes (Darby *et al.*, 2005). It has been demonstrated that the biofilm was controlled by the medium composition *in vitro* (Sauer *et al*., 2004; Ngampasutadol *et al*., 2005) and the nutrient is one clue of the biofilm formation. When cultivated in TMH medium, a chemically defined medium, some of *Y. pestis* bacteria formed multicellular aggregates around the wall of glass tube at liquid-air interface, while the other cells remain planktonic growth in the liquid (see Supplementary Fig. S1). The genes and regulatory signals, which determine whether a planktonic cell will switch to an aggregate form, are still poorly understood for *Y. pestis*. Genome-wide transcription profiling has become an important and powerful tool in searching of mechanism for biofilm formation, and has been widely used in investigating biofilm-associated genes for several bacteria, including *Escherichia coli* (Schembri *et al.*, 2003; Beloin *et al.*, 2004), *Pseudomonas aeruginosa* (Bollinger *et al*., 2001), *Bacillus subtilis* (Stanley *et al.*, 2003), and *Staphylococcus*

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Table 1. The distribution of genes whose transcription was altered in *Y. pestis* multicellularity

Number of genes		Function category
Up-regulated	Down-regulated	
12	16	Cell envelop
12	4	Transport/binding proteins
12	2	Amino acid biosynthesis
9	34	Unknown
7	4	Plasmid borne
3	4	Central intermediary metabolism
3	1	Synthesis and modification of macromolecules
3	1	Adaptions and atypical conditions
2	4	Biosynthesis of cofactors, prosthetic groups and carriers
2	3	Broad regulatory functions
2	0	Others
1	0	Chemotaxis and mobility
1	15	Energy metabolism
1	0	Drug/analogue sensitivity
0	4	Pathogenicity
0	2	Degradation of macromolecules
0	2	Detoxification
0	1	Purines, pyrimidines, nucleosides and nucleotides biosynthesis
0	9	Degradation of small molecule
0	1	Phage-related functions and prophage

aureus (Beenken *et al*., 2004). Biofilm formation apparently requires expression of a distinct set of genes that differentiate sessile cells from planktonic ones.

The aim of this study was to identify genes that are typically expressed in sessile *Y. pestis* populations in contrast to those expressed in their planktonic counterparts. We used transcriptomic profiling by cDNA microarray to identify differentially expressed genes that are associated with formation of multicellular aggregates. A number of genes were found to be differentially expressed between these two groups, including those encoding cell envelop, transport/binding proteins, and virulence factors.

Materials and Methods

Bacterial strain and medium

Y. pestis strain 201 was used in this study. The strain is avirulent for humans, but is highly lethal for mice (Zhou *et al.*, 2004). The detail features of this bacterium was described in our previous paper (Qiu *et al*., 2005). TMH medium was used for cultivating the bacteria, which 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 (Straley *et al.*, 1986).

RNA isolation from multicellular aggregates and planktonic cells

The overnight culture of strain 201 (OD_{620}=0.8) were 1:50 diluted in fresh TMH medium using 20 ml glass tubes and allowed to grow at 26°C with shaking at 180 rpm. The bacterial culture reached the exponential growth phase (A_{620}= 0.8) after 14 h incubation when obvious multicellular aggregates could be observed around the tube wall at liquid-air interface. Planktonic cells in the liquid were aspirated directly into a tube containing of RNA Protect (QIAGEN), vortexed for 5 sec, incubated for 5 min at room temperature, and then pelleted by centrifugation for 10 min at 8,000 rpm. Cell pellets could be stored at -70°C until RNA isolation. The remaining aggregate cells on the tube wall were washed twice with sterile TMH medium and were collected into a tube containing of RNA Protect Bacteria Reagent for RNA isolation. Total RNA was extracted from the suspended aggregate and planktonic cells using the MasterPure^{TM} RNA Purification Kits (Epicenter). RNA quality was monitored by agarose gel electrophoresis and RNA quantity was measured by spectrophotometer.

DNA microarray analysis

Twenty micrograms of RNA from the aggregate or planktonic cultures were used to synthesize cDNA, respectively, in the presence of aminoallyl-dUTP, genome directed primers (GDPs) (Han *et al.*, 2005) and random hexamer primers with the Superscript II System (Invitrogen). The aminoallyl-modified cDNA was then labeled by Cy5 or Cy3 monofunctional dye (Amersham) according to the manufacturer's instruction. Three separated labeled probes were made for each RNA preparation as technical replicates. Pairwise comparisons were made using dye swaps to avoid labeling bias. Glass slides spotted with PCR amplicons representing about 95% of non-redundant annotated genes or ORFs of *Y. pestis* CO92 and 91001 were used for probe hybridization. The DNA microarrays were simultaneously hybridized with the cDNA from aggregate cells or cDNA from planktonic to determine the ratio of gene expression in multicellular aggregates to those in planktonic cells. The detail method has been described in our previous report (Qiu *et al*., 2005).

The scanning images were processed and the data were

further analyzed by using GenePix Pro 4.1 software (Axon Instruments) in combination with Microsoft Excel software. Spots were analyzed by adaptive quantization, and the local background was subsequently subtracted. Spots with background-corrected signal intensity (median) in both channels less than two fold of background intensity (median) were rejected from further analysis. Data normalization was performed on the remaining spots by total intensity normalization methods. The normalized $\log_2$ ratio of test/reference signal for each spot was recorded. Genes with less than three data points were considered unreliable, and their data points were discarded as well. The averaged $\log_2$ ratio for each remaining gene on the four replicate slides was ultimately collected. Significant changes of gene expression were identified with SAM software (Tusher *et al.*, 2001) using one class mode and the measurement was the $\log_2$ (red/green) ratio from two labelled samples hydridized to a cDNA chip, with green denoting multicellular aggregate and red, plankton.

Real-time quantitative PCR

Real-time quantitative RT-PCR was employed to confirm the microarray data. Eleven genes were chosen, based on genomic location, gene length, and transcriptional changes, to compare data from the two techniques (see Supplementary Table S1). The total RNA preparation and reverse transcription are same as those prepared for microarray analysis. The transcriptional ratio from real-time PCR analysis was logarithm-transformed and then plotted against the average $\log_2$ ratio values obtained by microarray analysis (see Supplementary Table S2) (Han *et al*., 2005; Han *et al*., 2007).

Results and Discussion

Overview of expression profiles of *Y. pestis* biofilm cells

DNA microarrays, comprised of 4,005 open reading frames of the *Y. pestis* genome, were used to monitor the differences in expression profiles between cells grown under multicellular and planktonic conditions. A total of 177 genes were differentially expressed during the biofilm formation. One hundred and seven genes of them were down-regulated and 70 up-regulated (see Supplementary Table S3 for details). As shown in Fig. 1, there was a positive correlation (r=0.9184) between the two techniques, which confirmed the reliability of the microarray data.

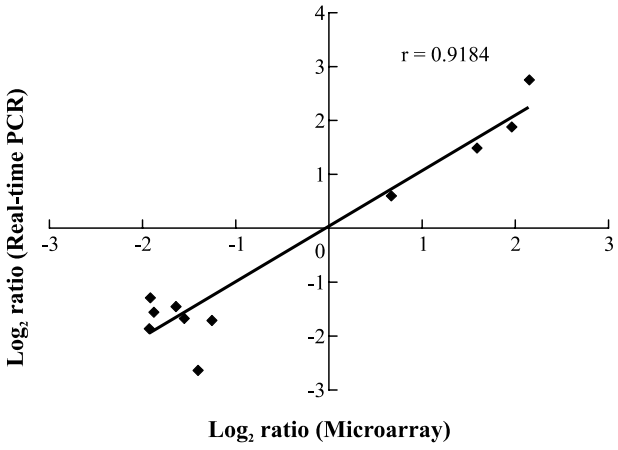

Fig. 1. Comparison of transcription measurements by microarray and real-time PCR assays. The relative transcriptional levels for 11 genes listed in Supplementary Table S1 were determined by microarray and real-time RT-PCR. The real-time RT-PCR $\log_2$ values were plotted against the microarray data $\log_2$ values. The correlation coefficient (r) for comparison of the two datasets is 0.9184.

According to the *Y. pestis* CO92 genome annotation, the differentially regulated genes were classified into different functional groups (Table 1). The results in the present study gave an overall alternated pattern of the gene transcriptional pattern of *Y. pestis* in the state of biofilm. Some of these differentially expressed genes had also been identified during biofilm formation in other bacteria, such as the up-regulation of genes encoding unknown function, cell envelop, transport/binding proteins, ribosome protein biosynthesis, and the down-regulation of cellular metabolism genes, suggesting conserved responses of bacterial cells to biofilm formation. Some of them were first reported to be responsible for the biofilm formation, such as the up-regulation of amino acid biosynthesis, the differentially altered genes involved in virulence, and the cold shock protein genes.

Differential alteration of virulence-related genes

Typical *Y. pestis* strains contain three plasmids (pPCP1, pCD1, and pMT1), which have been reported to play significant roles in different stages of *Y. pestis* pathogenesis (Cornelis *et al.*, 1998). Plasmid pCD1 is a common virulent plasmid shared by the three pathogenic *Yersinia* species, i.e., *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*, and it could secrete virulent factors via a type III secretory system (T3SS) into host cells when they contact each other. In the present study, five genes encoding T3SS, including type III secretion proteins (*yscB*, *yscC*, and *yscD*), Yops secretion ATP synthase (*yscN*), and putative virulence determinant protein (yopK/yopQ), were up-regulated in biofilm cells, indicating that the T3SS system may play a role in the biofilm formation. It has heen confirmed by that the *Erwinia chrysanthemi* T3SS is required for multicellular behavior (Yapici *et al*., 2005).

Plasmid pPCP1 encodes a virulence-specific adhesion/invasion, plasminogen activator (Pla). Pla has been proven to be essential for effectively invading human epithelial and endothelial cells, which plays a vital role in establishing subcutaneous infection. Plasmid pMT1 is a *Y. pestis*-specific plasmid that encodes two major virulence-related factors: F1 capsular protein, which can help *Y. pestis* escape from phagocytosis of the host immune system, and *Yersinia* murine toxin (*Ymt*), which is essential for transmission of *Y. pestis* by flea vectors (Zavialov *et al*., 2003). These three genes were down-regulated in the biofilm cells, indicating the biofilm formation *in vitro* does not need the help of these virulence genes.

Our results also showed that some putative virulence-associated genes, such as urease-associated genes, were found to be down-regulated, indicating their piddling role in the biofilm formation. The higher level of urease activity in *Streptococcus salivarius* growing in biofilms was reported (Li *et al.*, 2000). The ability of *S. salivarius* biofilm cells to up-

regulate urease expression in response to pH gradients and to accumulate greater quantities of urease enzyme as growing in biofilms may have a significant impact on oral biofilm pH homeostasis and microbial ecology *in vivo* (Li *et al.*, 2000). As mentioned below, the up-regulation of the gene encoding the putative acid shock protein Asr accounts for the fact that up-regulation of urease-associated genes is an automatic response of *Y. pestis* to the micro-environment within biofilm although these genes are inactivated in the bacterium.

Cell envelope and transport/binding protein

It was reported that a number of surface-associated proteins are important for biofilm formation (Costerton *et al.*, 1995; Foster and Hook, 1998). Consistent with these reports, in the present study, the genes encoding cell envelop were among the most number of the altered gene categories. Totally 28 genes encoding cell envelop were changed differentially, among them 12 were up-regulated, 16 down-regulated. Apart from one up-regulated gene *ompC2* and five down-regulated genes (*ompC*, *perm*, *ureG*, *ybaV*, and *yrbD*) in the multicellular cells, the other altered cell envelop genes belonged to different kinds of putative cell envelop proteins. The results indicate that during the multicellular formation the bacteria adapt themselves to the metabolism in multicellularity by the cell envelop remodeling. The dramatically altered genes encoding cell envelop could explain, at least partly, why biofilm cells are so resistant to shear forces *in vivo* and why they cannot be easily accessed by the host immune system. The specific roles played by these altered genes in biofilm need to be investigated further.

A number of genes encoding transport/binding proteins of *E. coli* and *Pseudomonas fluorescens* were found to be up-regulated in the formation of biofilms (Hinsa *et al.*, 2003; Schembri *et al.*, 2003). Consistent with these reports, in our present study, sixteen genes encoding transport/binding protein were also changed differently. The 12 up-regulated genes of transport/binding proteins consist of genes related to the transport of molecules such as anions, carbohydrate organic acids and alcohols, amino acids, cations, and these transport/binding proteins are involved in diverse cellular processes such as nutrient uptake, maintenance of osmotic homeostasis etc., indicating that the genes encoding transport/binding proteins were another important change to fit for metabolism of the biofilm in the biofilm formation.

Ribosome protein and amino acid biosynthesis

It was reported that the expression of ribosomal genes were up-regulated in biofilms (Schembri *et al.*, 2003). In the study on *E. coli* biofilm, ribosomal genes were found to be activated compared to stationary phase cells (Schembri *et al.*, 2003). It was also reported that the gene expression pattern of the biofilm cells resembles stationary phase cells in many other respects. One would expect the planktonic cells to have the greatest exposure to nutrients and the fastest growth rates. What could trigger the increased expression of ribosomal protein genes in biofilms? Could some cells achieve a faster growth rate by catabolism of the biofilm matrix or of dead cells? In the present study, we found only two genes encoding ribosomal protein synthesis were up-regulated and one gene down-regulated in the multicellularity, indicating that the ribosomal protein synthesis is not actively regulated *in vitro* in *Y. pestis* multicellular formation.

It was a surprising that 12 genes involving amino acid biosynthesis were up-regulated in the multicellular cells. They belong to glutamate family biosythesis (7 genes), asparatate family biosynthesis (3 genes), and pyruvate family biosynthesis (2 genes). It needs to be further study to verify their roles in multicellular formation of *Y. pestis*.

Down-regulation of cellular metabolism in biofilm cells

A number of genes involved in cellular metabolism, such as energy metabolism, central intermediary metabolism, and degradation of small molecules, were down-regulated in the biofilm cells compared to the cells in the planktonic conditions, indicating the retardance of most of the metabolic processes in the biofilm. It is increasingly clear that bacteria in biofilm display special phenotypes controlled by the gene regulation. Among the genes that appear to be differentially regulated in biofilms are those involved in metabolism or starvation responses (Inglesby *et al.*, 2000; Jackson *et al*., 2002; Sauer *et al*., 2002). This regulation could easily be resulted from incomplete penetration of nutrients or electron acceptors into the biofilm.

It is logical to hypothesize that any slowing of the growth rate in biofilm cells might be reflected in a down-turn of the level of metabolic enzymes, even though care should be taken in interpreting such a reduction as implying a corresponding down-turn of metabolites' flux. The planktonic growth rates of the bacterium in the various media indicated that the specific growth rate varied depending on the medium composition (Sauer *et al.*, 2004; Ngampasutadol *et al.*, 2005).

Stress proteins

Stress proteins are usually designated on the basis of their inducibility by the major stresses, although a stress protein could be induced by a number of different stresses. The major cold shock protein CspA acts as a chaperone to bind nascent mRNA transcripts for preventing the formation of mRNA secondary structure induced by cold shock. The cold shock genes can be induced not only by the temperature but also by other stresses (Han *et al.*, 2005), our previous result showed that the cold shock genes were induced to a certain extent following the addition of group of antibiotics such as chloramphenicol and tetracycline (Qiu *et al.*, 2006). In this paper, we demonstrated the genes encoding cold shock protein (*cspa2* and *cspa1*) were up-regulated in the biofilm cells, which indicated that the relationship between cold shock protein and biofilm formation.

The *asr* gene encoding putative acid shock protein can be induced in the acid environment (Foster, 1993). We could also see its up-regulation in the multicellular cells, suggesting that a low pH environment might be formed in the biofilm (Li *et al.*, 2000), so that the acid shock protein was up-regulated.

Regulatory function

A number of global regulatory genes such as *rpoS*, *spoOA*, and *ccpA* have been implicated in biofilm formation

(Lazazzera, 2005). In the present study, two genes (*phoB* and *phnF*) and three genes (*rhaR*, *phoP*, and *pyrI*) involving global regulatory functions were up-regulated and down-regulated, respectively. Interestingly, *phoB* and *phoP* were regulated in a reverse manner. It has been confirmed that expression of the *phoB* negatively regulates biofilm formation by *Pseudomonas aureofaciens* in low-Pi environments (Monds *et al*., 2001), but in *Y. pestis* multicellularity this gene is up-regulated, which may indicates that i) the homologue gene in different bacteria plays diverse roles, and even the reverse role; and ii) the *Y. pestis* biofilm collected for microarray analysis is in its later stage and *phoB* is up-regulated in order to activate its downstream genes to constraint the biofilm formation. These postulates need to be verified. *Y. pestis* has the PhoP/PhoQ two-component system consisting of the Mg^{2+} sensor PhoQ and the responsive regulator PhoP. The PhoP/PhoQ two-component system has been shown to be required for the bacterial growth in macrophages (Oyston *et al.*, 2000). PhoP/PhoQ appears to govern a complex of cellular pathways in *Y. pestis* (Zhou *et al.*, 2005). The role of *phoP* up-regulation in biofilm formation is worth investigating further.

Chemotaxis and mobility

It was reported that one of the major classes of genes differentially expressed in biofilm cells was those involved in motility. Such as a number of flagellar and chemotaxis genes were expressed at lower levels in *Vibrio cholerae* biofilm cells (Butterton *et al.*, 2000), and the same result also seen in the *P. aeruginosa* and *E. coli* (O'Toole and Kolter, 1998; Pratt and Kolter, 1998), revealing that inhibition of flagellar-gene transcription may be a general phenomenon for bacteria in biofilms. It has been reported that chemotaxis plays a stage-specific role in formation of the *Vibrio cholerae* monolayer (Moorthy and Watnick, 2005). In our results, only one gene (*cheD*) was up-regulated in biofilm, suggesting the role of chemotaxis in the biofilm formation of *Y. pestis*.

Prophage

The induction of prophage genes may represent another general phenotype of bacteria in biofilms. A number of genes in the *B. subtilis* genome involved in prophage production were highly expressed in the biofilm. It has been proposed that prophage production may have a role in generating genetic diversity in the biofilm (Stanley *et al.*, 2003). The higher expression levels of prophage genes in biofilm cells were also demonstrated in the *P. aeruginosa* study (Webb *et al*., 2004). A bacteriophage capable of superinfecting and lysing the *P. aeruginosa* parent strain was detected in the fluid effluent from the biofilm during the onset of biofilm killing and during biofilm development thereafter (Webb *et al.*, 2003), and therefore the authors propose that prophage-mediated cell death is an important mechanism of differentiation inside microcolonies that facilitates dispersal of a subpopulation of surviving cells. In our result, only one gene (YPO1096) related to the prophage was down-regulated in the biofilm cells. Its role in biofilm formation should be investigated.

Quorum sensing system

Quorum sensing has been shown to play a role in the control of biofilm architecture in some bacteria, such as *P. aeruginosa*, *Burkholderia cepacia*, and *Aeromonas hydrophila* (Bollinger *et al.*, 2001; Labbate *et al.*, 2004; Balestrino *et al.*, 2005; Ngampasutadol *et al.*, 2005). In the genome of *Y. pestis*, three quorum sensing system was predicted, quorum sensing (QS) regulates a large set of genes involved in both basic cellular functions and virulence, also affected the biofilm formation (Bobrov *et al.*, 2007). In our microarray data, the QS genes did not changed differently, to assess these genes in the biofilm formation, we use the quorum sensing mutant (Chen *et al.*, 2006) of *Y. pestis* to define the impact of QS on biofilm formation. The result showed that the double mutant of two *luxI* QS genes did not affect the multicellularity formation when cultivated in TMH medium at 26°C (unpublished data).

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