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Benefits and pitfalls of using microarrays to monitor bacterial gene expression during infection

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The understanding of bacterial pathogenesis is dependent on techniques that elucidate the underlying genetic and biochemical mechanisms. To study the mechanism of bacterial survival and proliferation within host cells we need accurate tools that tell us what is occurring within the infecting organism. It has now become possible to determine the transcriptional status of *in vivo*-derived bacteria at the level of the whole genome. Such expression profiles serve as a monitor of the host cell environment as well as an indicator of the bacterial adaptation to its intracellular niche. Here, we review the methods used to produce microarray data for defining the bacterial intracellular transcriptome, and examine the pitfalls in extracting bacterial RNA from the infected host compartment.

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Introduction

It is almost half a century since it was discovered that bacterial genes were regulated at the transcriptional level [1]. It soon became evident that the process of infection pathogenesis would rely on the accurate regulation of bacterial gene expression. The first evidence was the attenuation of virulence of many regulatory mutants, suggesting that regulatory factors played distinct roles during infection [2]. Initially, bacterial gene expression within mammalian cells was monitored with reporter fusions [3–5]. This reductionist approach necessitated the study of individual bacterial genes, making it difficult to build up a picture of the pattern of expression across the genome. It was clear that a completely new strategy was needed. The first breakthrough came from *in vivo*-expression technology, which was initially developed for plant pathogens [6], and subsequently applied to

mammalian pathogens in the Mekalanos laboratory [7,8]. This approach is based on a genetic selection and has proved applicable to a wide range of bacterial and fungal species for defining *in vivo*-inducible genes [9]. However, since the method can only identify genes that are induced during infection and is relatively labour-intensive, new technologies were needed to monitor the global levels of up- and down-regulation of bacterial gene expression during infection.

The availability of whole genome sequences of bacterial pathogens, and the development of microarray technology in the late 1990s, ushered in the new era of whole genome expression profiling. DNA microarrays are now commercially available for at least seven key bacterial pathogens, and microarray equipment is becoming increasingly accessible to researchers [10]. As well as offering the fastest way to catalogue a bacterial genome [11], microarrays are allowing us to tackle important biological problems at the level of the whole genome, including global mRNA stability [12,13]. Microarray technology has recently been applied to the analysis of bacterial gene expression during infection. Experience has shown that this approach can offer great benefits, but can suffer from pitfalls; these are reviewed below.

The benefits

DNA microarrays can be used to monitor expression of all genes in an organism, simultaneously, at the RNA level. The technology initially allowed us to interrogate RNA preparations that were extracted from *in vitro*-cultivated bacteria [14]. More recently, microarrays have been used to visualise gene expression in more complex multicellular environments. This significant advance relied upon the isolation of sufficient bacterial RNA from infected animal and plant tissue, from infected secretions and from within infected cells (Table 1; Figure 1). An early study used bacterial RNA extracted from infected mammalian cells for qualitative analysis of eight genes by RT-PCR [15]. Until this time all data on bacterial gene expression during infection had been obtained from individual reporter gene fusions [16,17]. More recently, RNA extracted from mouse ear and skin infected with *Borrelia burgdorferi* was used to identify alterations in lipoprotein gene expression during murine infection using a microarray of 137 genes [18••]. Several authors have prepared bacterial RNA from cholera stool samples and intestinal loop contents to study the difference in bacterial gene expression profiles from laboratory- and infection-propagated

Table 1

Pathogen	Infection model	Details of RNA stabilisation & protocol	Amount of total RNA used per hybridisation	Replicate details	Reference
<i>Borrelia burgdorferi</i>	Mouse skin (ears)	<ul style="list-style-type: none"> No stabilisation Tissue immediately frozen in liquid nitrogen 	NA	<ul style="list-style-type: none"> 3 Technical 	[18**]
<i>Vibrio cholerae</i>	Infected human stools	<ul style="list-style-type: none"> No stabilisation Bacteria harvested from ice-loop fluid by centrifugation within 1h of collection RNA preparation 	NA	<ul style="list-style-type: none"> 2–4 Technical 3 Biological 	[20*]
<i>Vibrio cholerae</i>	Infected human stools	<ul style="list-style-type: none"> No stabilisation Filtration of stools and immediate freezing RNA preparation 	1 µg	<ul style="list-style-type: none"> 4 Technical 	[19**]
<i>Vibrio cholerae</i>	Infected human stools	<ul style="list-style-type: none"> Stabilisation (TRIzol) 	NA	<ul style="list-style-type: none"> 2 Technical 	[21]
<i>Escherichia coli</i> O1:K1:H7	Normal and phagocyte oxidase-deficient human neutrophils (PMN)	<ul style="list-style-type: none"> No stabilisation RNA preparation from PMN containing <i>E. coli</i> Phenol/CHCl₃ extraction in triplicate RNA preparation 	8 to 30 µg	<ul style="list-style-type: none"> 2 Technical 	[22]
<i>Neisseria meningitidis</i>	Cultured HeLa epithelial cells Cultured HBMEC brain microvascular endothelial cells	<ul style="list-style-type: none"> No stabilisation Wash of infected host cells Trypsin/EDTA treatment Centrifugation RNA preparation 	NA	<ul style="list-style-type: none"> 5 Biological 	[39]
<i>Neisseria meningitidis</i> serogroup B	Human 16HBE14 epithelial cells	<ul style="list-style-type: none"> No stabilisation 4 washes Lysis of host cells in 1% Saponin (10 min, 37°C) RNA preparation 	NA	<ul style="list-style-type: none"> 4 Biological 	[40]
<i>Mycobacterium tuberculosis</i>	Bone marrow derived murine macrophages	<ul style="list-style-type: none"> Stabilisation (guanidine thiocyanate) RNA preparation 	5 µg	<ul style="list-style-type: none"> 4–7 Technical 	[25]
<i>Salmonella enterica</i> sv. Typhimurium	Murine macrophage like J774-A.1 cells	<ul style="list-style-type: none"> Stabilisation during lysis of host cells (phenol/ethanol) RNA preparation 	< 5 µg	<ul style="list-style-type: none"> 3–4 Technical 	[26*]
Group A <i>Streptococcus</i>	Mouse dermis and soft tissue	<ul style="list-style-type: none"> Stabilisation (FastPrep) 	NA	<ul style="list-style-type: none"> NA Quantitative RT-PCR only 	[41]
<i>Helicobacter pylori</i>	Human infected stomach biopsies Mouse	<ul style="list-style-type: none"> Stabilisation (RNAlater, TRIzol) RNA preparation 	5–10 µg	<ul style="list-style-type: none"> NA Quantitative RT-PCR only 	[42]

NA = Information not available
 * B = Replicate of infection experiment
 ** T = Replicate of hybridisation experiment

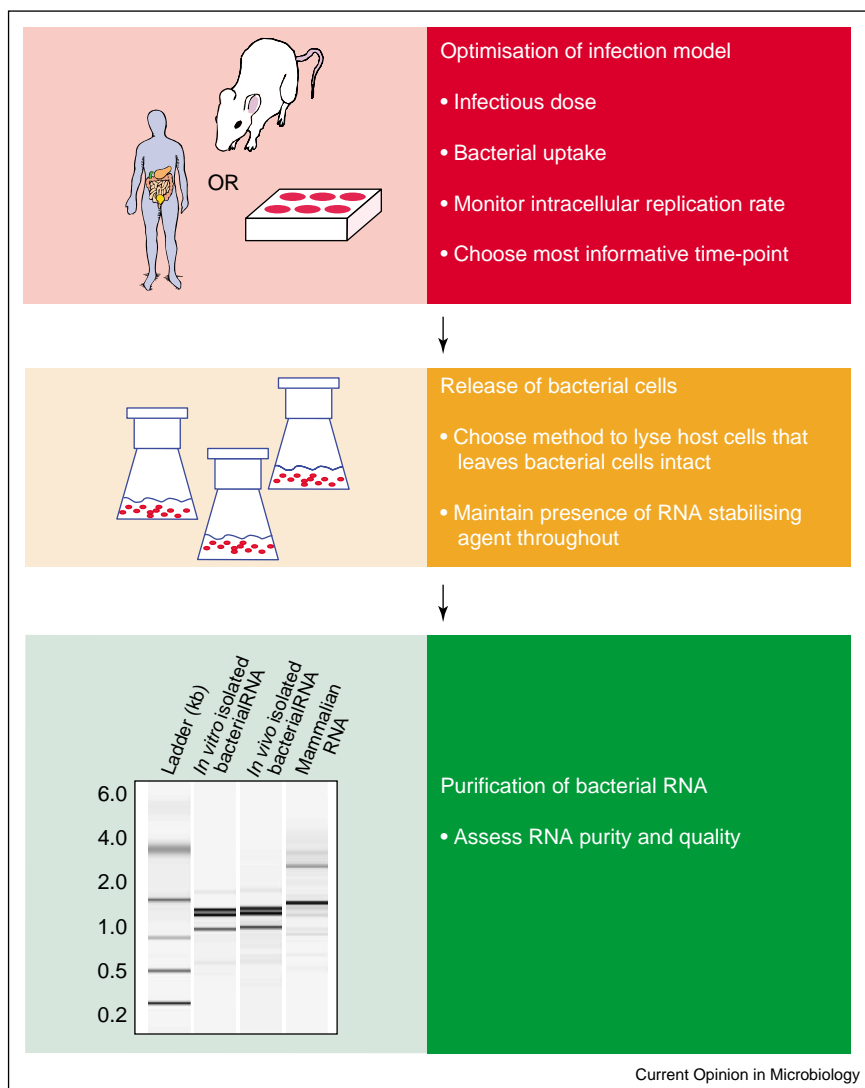
Vibrio cholerae [19**,20*,21]. As well as demonstrating the induction of certain sets of genes, the microarray analyses showed that about 80% of the genes that are differentially expressed in human-shed *Vibrio cholerae* were down-regulated [19**].

Bacterial pathogens that replicate intracellularly offer a particular challenge to the medical establishment, and have become a fertile area of cellular microbiology. The availability of gene expression profiles obtained from bacteria replicating in an intracellular environment has shed light on the mechanisms of bacterial proliferation within mammalian cells. Some examples of the biological

information gained through microarray-based experiments are discussed below.

Staudinger *et al.* demonstrated the biological importance of the bacterial OxyR response to oxidative stress by comparing the *E. coli* gene expression profiles in normal and phagocyte oxidase-deficient human neutrophils [22]. In these experiments the *E. coli* RNA was extracted from a mixture of host and bacterial cells. Similarly, the adaptation of yeast to antimicrobial responses of neutrophils and macrophages has also been analysed [23,24]. In this case the microorganisms were separated from host cells before RNA extraction to enrich the proportion of

Figure 1



Optimisation procedure for isolation of high quality bacterial RNA from host environment. The main steps to be optimised for the extraction of reliable, good quality bacterial RNA as shown on the chromatographic separation in the lower green panel of the figure are shown here. This analysis of *Salmonella* RNA extracted from infected macrophages (reproduced from Eriksson *et al.* 2003 with permission from Blackwell Publishing) shows that the differential lysis approach allows isolation of *in vivo* bacterial RNA of comparable quality to *in vitro* RNA, with no contamination by mammalian RNA.

microbial RNA. This approach has now been refined by including a differential lysis step, which led to identification of the intracellular transcriptome of *Mycobacterium tuberculosis* [25] and *Salmonella enterica* [26^{*}]. This work has been proceeding rapidly, and we have used the *Salmonella* protocol [26^{*}] to determine gene expression profiles for *S. enterica* in human epithelial cells (I Hautefort, unpublished) and bone marrow-derived primary murine macrophages (S Töttemeyer, S Ugrinovic, C Bryant, P Mastroeni and D Maskell, personal communication). The same protocol has also been successfully applied to *Shigella flexneri* during infection of human macrophages and epithelial cells (J Yu, H Liu and S Lucchini, personal

communication). When considered together, the transcriptomes from infecting bacteria tend to reveal a larger proportion of down-regulated than up-regulated genes, as the bacteria transit from the external environment to that of the host.

Isolation of bacterial RNA from infected mammalian cells

Crucial technical details need to be considered when using microarrays to study pathogens during infection. Firstly, the number of bacteria that can be isolated from the infection model must be maximised. As indicated in Figure 1, the infection protocol must be optimised for

each pathogen to allow sufficient RNA to be obtained. The procedure for extracting bacterial RNA from infected cells must also be modified for each infection system to achieve maximal purity and quality of the bacterial RNA. We recommend a differential lysis approach that involves the physical separation of bacteria from host cells before RNA preparation. The aim is to leave the bacteria intact while generating efficient lysis of the host cells or tissue; detergents such as sodium dodecyl sulphate, [26[•]], sodium deoxycholate or Triton should be tested with this in mind. In the case of Gram-positive pathogens such as *Mycobacterium*, harsher lysis reagents such as guanidine thiocyanate can be used [25,27[•]]. Differential centrifugation is an efficient way to separate the released bacteria from host debris [26[•]]. The higher the purity of bacterial RNA, the more efficient the subsequent fluorescent labelling becomes; this in turn generates good signal-to-noise ratios following microarray hybridisation. When high quality RNA is analysed on microarrays with appropriate biological and technical replicates of each experiment (Table 1), the resulting data are robust.

And the pitfalls

The need for bacteria to respond extremely rapidly to changes in the environment has led to the development of tight control of transcription and of rapid RNA degradation systems for the removal of transcripts. This means that genes can be switched on in a matter of a few minutes [28]. If the extraction of bacterial RNA involves any environmental change, such as chilling on ice, the expression of sub-sets of genes will be induced (e.g. the cold-shock regulon) [29]. Conversely, the efficiency of RNA degradation is illustrated by the short average half-life of bacterial mRNA, which is around five minutes [12,13]. Therefore, any delay in the extraction of the bacterial RNA will have dramatic effects on microarray data, as the most labile transcripts will be degraded, and absent from such RNA preparations. It is therefore crucial for the lysis procedure to be performed under conditions that ensure the immediate cessation of transcription and simultaneous prevention of RNA degradation. The use of efficient stabilising agents such as phenol/ethanol for Gram-negative bacteria [30] or guanidinium for Gram-positive bacteria [25] guarantees the purification of a comprehensive population of transcripts that accurately represents the bacterial response to a given environment. Researchers must remember that microarray data are only as good as the structural integrity of the RNA used in the experiment, and the quality of the experimental design. A poorly executed gene expression profile will not provide the biological insights we are searching for.

Labelling sub-microgram levels of bacterial RNA

Until recently, another limitation for *in vivo* experiments was the amount of RNA available for successful labelling

and hybridisation reactions. Despite optimisation of infection protocols, *in vivo* derived bacteria tend to be scarce and usually yield sub-microgram quantities of RNA. All of the common RNA labelling techniques require several micrograms of total RNA as starting material; the fluorescent probe is generated by 'direct labelling' which involves reverse transcription of the RNA in the presence of Cy3- or Cy5-dCTP [31]. Alternatively, 'indirect labelling' involves the reverse transcription of similar amounts of RNA in the presence of an amino-allyl linked dNTP, followed by crosslinking to the appropriate Cy-dye ester.

A modified method for labelling sub-microgram quantities of RNA is referred to as 'cold labelling', and takes advantage of the greater efficiency of incorporation of labelled nucleotides by Klenow polymerase compared to reverse transcriptase. Briefly, it involves reverse transcription of RNA with unlabelled nucleotides followed by random priming of the cDNA to incorporate Cy-dyes [32]. We have demonstrated that the cold labelling method yields comparable microarray data to the well-established approaches described above [26[•]; for details, see <http://www.ifr.ac.uk/safety/microarrays/>].

Interpretation and standardisation of microarray data

Gene expression profiles from infection models generally need to be compared to an appropriate control bacterial culture grown *in vitro*. This concept of a universal control culture is particularly important if comparisons are to be made between different bacterial mutants or between the infection of different cell lines. Therefore, bacterial researchers are moving away from the traditional microarray experiment that only allowed the comparison between two RNA samples [19^{••}]. We now favour the hybridisation of fluorescently labelled genomic DNA as the reference channel for each two-colour microarray experiment involving RNA [31,33[•]]. As well as allowing the comparison of many distinct experiments, this approach offers a quality control for spots on the array due to hybridisation of genomic DNA.

To ensure that the microarray data are valid, one should perform data centering and statistical procedures. Skewing of the raw intensity data due to unequal incorporation of Cy-dyes and uneven hybridisation across the array can be compensated for by a block-by-block data centering method, where a block is defined as the group of spots printed by the same pin [34]. For statistical analysis one can, for example, use a parametric filter based on a two-sample *t* test for two groups or ANOVA for multiple groups; the Benjamini and Hochberg multiple testing correction is ideal for the adjustment of individual p-values [35]. These tests are features of the GeneSpringTM (Silicon Genetics) microarray analysis software

package that can be used for data visualisation and mining purposes.

Conclusions: the way ahead for bacterial transcriptional profiling during infection

Just two years ago, the microbiological community was sceptical that bacterial microarray data would ever be obtained from infected mammalian cells [36]; Table 1 shows that this has now been realised. We now face the real challenge of isolating sufficient amounts of stabilised bacterial RNA from infected animal organs or tissue to allow visualisation of bacterial gene expression during authentic infection. To successfully release bacteria from organs such as lung or liver, it may be necessary to borrow from protocols that were initially developed for mammalian *in situ* hybridisation, and involve initial perfusion with fixatives such as paraformaldehyde to stabilise mammalian RNA [37]. The small levels of bacterial RNA that can be isolated will pose problems for subsequent microarray analyses, and it would be ideal if a procedure could be devised to amplify bacterial RNA in a linear fashion. Such an amplification procedure is being actively developed, but may never prove to completely reflect the original pool of mRNA transcripts [27]. For microarray-based strategies, it may be more productive to focus on a new strategy to improve the fluorescent labelling of cDNA, and so reduce the requirement for RNA from micrograms to the level of nanograms.

As real-time PCR can already generate robust expression data for particular genes from as little as 100 pg of total bacterial RNA, it already allows us to accurately determine the levels of expression of selected bacterial genes of interest, in the context of a genuine animal infection [38].

The availability of gene expression profiles for several bacterial pathogens during infection is beginning to represent a completely new type of resource for the investigation of the microbiology of infection. We can now use these bacteria as 'sensors' to report the environment they experience at selected phases during the infection pathogenesis. For example, *S. flexneri* can be used to probe the cytoplasmic compartment of macrophages, whereas *S. enterica* and *M. tuberculosis* describe different modified phagosomal vacuoles. As more expression profiles are obtained for pathogens adapted to the intracellular milieu, we look forward to the construction of more complete pictures of intracellular parasitism.

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