Comparison of global transcription responses allows identification of *Vibrio cholerae* genes differentially expressed following infection

Soumita Das a, Amit Chakrabortty a,1, Rajat Banerjee a, Susanta Roychoudhury b, Keya Chaudhuri a,.*

* Biophysics Division, Indian Institute of Chemical Biology, 4, Raja S.C. Mullick Road, Jadavpur, Calcutta 700 032, India
* Human Genetics Department, Indian Institute of Chemical Biology, Jadavpur, Calcutta 700 032, India

Received 18 April 2000; received in revised form 18 June 2000; accepted 1 July 2000

Abstract

Comparison of global transcription profiles of *Vibrio cholerae* grown in vitro and in vivo revealed that 20% of the genome was repressed and about 5% was induced under in vivo conditions. Hybridization with the cloned genes revealed that the virulence genes ctx, toxR, toxT and tcpA were induced under in vivo conditions. Dissection of two in vivo induced cosmids identified another set of three genes homologous to *cheY1* involved in motility and chemotaxis, *puc* encoding the major component of the nicotinamide mononucleotide transport system and *icmF* belonging to a cassette involved in multiplication inside host cells. These results demonstrate that the global transcription profile approach might be a powerful method for identification of differentially expressed transcripts under in vivo conditions. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Global transcription profile; In vivo expressed gene; *Vibrio cholerae*

1. Introduction

Cholera still remains a major public health problem in developing countries as well as in some developed countries. The causative organism *Vibrio cholerae*, is a Gram-negative curved rod and the life threatening watery diarrhea is largely due to the action of the secreted cholera toxin (CT) on the epithelium of the small intestine. The so far identified virulence factors of this pathogen include other toxins like Ace, Zot and colonizing factors like TCP [1]. The exact regulatory mechanism of the major virulence genes including *ctx* and *tcp* is yet to be conclusively established.

The current model of the regulation of virulence factor expression in *V. cholerae* postulates that ToxR and TcpP become activated in response to particular environmental signals and act cooperatively to induce expression of ToxT [2]. ToxT in turn directly activates transcription of biosynthetic genes for CT, TCP [3]. In the intestinal lumen, where the pathogen colonizes during infection, conditions are such, that the expression of ToxR controlled virulence factors is repressed [4,5]. Thus to induce ToxR-controlled virulence genes in vivo, *V. cholerae* may recognize other unknown external signals in the host environment [6].

Recently using tnpR operon fusions and signature tagged mutagenesis (STM) to conduct a screen for random insertion mutations, *V. cholerae* genes induced within the host and critical for colonization were isolated [7,8]. Only one gene in the *tcp* operon was isolated suggesting that one third of all the genes responsible could be identified by the STM method. By these methods, involving transposon mutagenesis, only genes which are induced in vivo are identified. However, the genes that are repressed may hold some major information regarding the mechanism of pathogenesis of the organism.

We have used the global transcription response technique to identify regions of the *V. cholerae* genome that are differentially expressed following infection. By this technique, detection, cloning, and mapping of a responding gene or co-regulated gene in response to environmental signals can be achieved. This has been used to identify and map transcriptionally active regions of the *Halofexarx volcanii* genome [9] and to study mRNA levels in an over-
lapping set of clones in λ vectors of the *Escherichia coli* K-12 under various environmental stimuli [10]. In this report we describe the identification of some of the functions that are differentially expressed following infection.

2. Materials and methods

2.1. Bacterial strains and plasmids

The *V. cholerae* 569B and *E. coli* DH5α strains used in this study were maintained at −70°C in Luria–Bertani (LB) medium containing 20% (v/v) glycerol. *E. coli* cells were grown in LB medium and *V. cholerae* cells were grown in nutrient Broth or LB medium. All cloning experiments were done in pBluescript KS+. The plasmids were maintained and amplified in *E. coli* DH5α and XL1 Blue strains. Ampicillin was used at 100 µg ml⁻¹ for *E. coli* and 50 µg ml⁻¹ for *V. cholerae*. The 92 sets of cosmids clones in Lorist M vector [11] were grown in TB medium containing phosphate buffer. Ampicillin (100 µg ml⁻¹) and kanamycin (30 µg ml⁻¹) were used where appropriate.

2.2. Ligated rabbit ileal loop model

In vivo growth of *V. cholerae* strains were carried out in the rabbit ileal loop model essentially as described previously [12]. Briefly, 48-h fasted rabbits were anesthetized and the small intestine was tied into consecutive 6-cm segments proximally to the mesoappendix. An inoculum of 0.5 ml of *V. cholerae* strains containing about 5×10⁶ CFU was introduced into each 6-cm segment, while one loop was inoculated with saline as negative control. After 16–18 h the animals were killed and the small intestine removed. Fluid accumulated in each loop was separately collected and measured. *V. cholerae* count in the fluid was measured by plating on thiosulfate–citrate–bile salt-sucrose (TCBS) agar plates. Although no fluid was measured by plating on thiosulfate–citrate–bile salt–sucrose (TCBS) agar plates, usually more than half of the isotope was incorporated. The reaction mixture was incubated at 37°C for 1 h. The reaction was stopped by EDTA to a concentration of 0.5 mM. Labeled DNA was purified from unincorporated [α-³²P]dCTP by passing through a Sephadex G-50 column equilibrated with STE buffer.

2.5. Preparation of α-³²P-labeled total cDNA

The random hexamer priming method [10] was used to prepare labeled cDNA. The reaction mixture contains 15 µg of total RNA, 5 µg of pd(N)₆ (random hexamer from NEB), 0.5 mM dATP, 0.5 mM dGTP, and 0.5 mM dTTP, 5 µM dCTP, 100 µCi of [α-³²P]dCTP (3000 Ci/mmol), 50 mM Tris–HCl (pH 8.3), 6 mM MgCl₂, 40 mM KCl, 0.5 U of RNase block (Stratagene) per µl and 50 U of superscript II RNase H⁻ reverse transcriptase (Life Technologies) in a total volume of 50 µl. The reaction was allowed to continue overnight at room temperature. EDTA and NaOH were then added to final concentrations of 50 mM and 0.25 M, respectively, and the mixture was incubated at 65°C for 30 min to degrade the RNA templates. The cDNA was then ready to use after neutralization by adding HCl and Tris buffer. Free nucleotides were not removed. Usually more than half of the isotope was incorporated.

2.6. Dot blot hybridization

A set of 92 cosmids DNAs were denatured by boiling at 100°C for 5 min and with subsequent chilling at 4°C. Individual samples were spotted on a membrane filter and the membrane was dried and baked in a vacuum desiccator at 70°C for 2–3 h. For hybridization with appropriate probes, the blots were prehybridized for 6 h at 60°C in the prehybridization solution and hybridized overnight with α-³²P-labeled DNA fragment at 60°C in 6×SSC and 0.5% SDS. The blots were washed successively (15 min each at room temperature) in 2×SSC, 0.5% SDS and in 2×SSC, 0.1% SDS with a final stringency wash in 1×SSC, 0.5% SDS at 60°C for 2 h. The blots were dried and exposed to Kodak XR-5 film using an intensifying
screen or to a phosphorimager (Bio-Rad, USA) for analysis.

2.7. Subcloning and DNA sequencing

Selected cosmids were digested with BamHI and HindIII and cloned into BamHI–HindIII-digested pBluescript. The nucleotide sequence was determined with double stranded plasmid DNA as the template by cycle sequencing in an ABI prism 377 DNA sequencer (Applied Biosystems, Perkin-Elmer) using company supplied kit reagents and protocols.

2.8. Nucleotide sequence and accession number

The nucleotide and deduced amino acid sequences of the gene fragments appear in EMBL, GenBank, DDBJ databases under the accession numbers AF171076, AF239736 and AF239738.

3. Results and discussion

High molecular mass RNA was isolated from V. cholerae 569B cells grown overnight in rabbit ileal loop (in vivo) and in rich media (in vitro) conditions. α32P-Labeled cDNA was prepared by reverse transcription primed with mixed hexanucleotides and hybridized with dot blots of a minimal set of 92 cosmid clones representing about 90% of the V. cholerae genome. The minimal set of cosmid clones were taken from the cosmid library constructed in the Lorist M vector by Chatterjee et al. [11] in our laboratory. The control and experimental hybridizations were performed at least thrice by reusing the same blot. The resulting data were analyzed in a phosphorimager (Bio-Rad, USA) instrument. The percentage of total counts for each spot was obtained directly from the machine after subtracting the background level, which was taken from the blank area of each blot (Fig. 1). Each cosmid clone contained an insert of V. cholerae genomic DNA between 20 and 40 kb. The restriction map of each clone was verified with the original data [11], to check for deletions. The clones containing rrn operons were excluded as they gave saturated values and caused errors during calculations. From the hybridization patterns (Fig. 1), ratios of in vivo to in vitro counts were calculated and plotted in Microsoft Excel (Fig. 1). We regarded induction or repression ratios of >2 and <0.5 as significant. The same blot was used with stripping to reduce errors. The experiments were repeated with at least five different batches of RNA.

In comparison to baseline expression, some clones showed differential expression when the bacteria were grown in the animal host. About 20% of the clones showed repressed expression under in vivo conditions, and about 5% were induced. It is not surprising that most of the clones showed a repression in vivo, as in the stressful environment within the host it is possible that very few proteins were allowed to be synthesized at a high rate. This helps the pathogen to make proper utilization of the limited resources in vivo and synthesize proteins essential for virulence and survival within the host. However, the cosmid clones containing 20–40 kb of V. cholerae DNA need to be further dissected for fine tuning of the expression profile.

As expected the clones containing the virulence cassettes ctxAB, zot, ace, orfU, cep showed an induction in the level of expression in vivo. So did the toxR and tcpAB, toxT.
containing clones. Since toxR tcpA, ctxAB, and toxT constitute the virulence cassette of the V. cholerae genome, we decided to investigate this further. Northern blot experiments with the cloned genes (laboratory collection) revealed that under in vivo conditions ctx, toxR, toxT and tcpA were induced, the expression of tcpA was found to be always higher compared to the other three (Fig. 2). Very recently using RIVET technology it has been suggested that tcpA induction is biphasic and is required both at the initial and later stages of infection [13] which might be the cause of higher tcpA expression in our experiments.

The clones showing an overall induced level of expression were digested with BamHI and HindIII and then were further cloned in pBSK+ to identify the genes responsible for the increased signal in vivo. Northern blot experiments were used to confirm the induction of these genes. Three in vivo induced segments identified using this strategy were sequenced and the database was searched for homology using the BLASTX [14] programme (Table 1).

Interestingly, one of the in vivo induced segments identified in this study is homologous to cheY1 that relates to motility and chemotaxis in several Gram-negative enteric bacteria. Induction of such a gene is quite expected as it has been found that V. cholerae isolated from rabbit intestine is 350% more motile than those grown under normal laboratory conditions [12]. Some evidence has also suggested that motility and chemotaxis are prerequisites for full virulence of V. cholerae [15–17]. Since V. cholerae adheres to the mucus coat in the small intestine, these motility and chemotaxis genes may play an important role in penetration of the mucous coat.

The presence of the icmF gene in V. cholerae and its induction within the host is a new mystery in the pathogenesis of this organism. In Legionella pneumophila, icmF belongs to the icm locus shown to be essential for virulence in guinea pigs and for intracellular multiplication of Legionella within human macrophages [18]. Although intracellular multiplication has not been documented in non invasive V. cholerae 569B, it is still exciting as we have recently shown that V. cholerae 569B grown under in vivo conditions becomes resistant to human serum [12] in which icmF might play an important role.

The increased expression of pncC, the major component of the nicotinamide mononucleotide (NMN) transport system under in vivo conditions in V. cholerae might be required for survival of V. cholerae in the anaerobic environment prevailing inside rabbit intestine. Under anaerobic conditions the NAD pool goes down [19] and increased NMN transport will help the organism to synthesize more NAD.

Including this report, in the last 5 years there has been at least five different approaches to identify differential expression and regulation of V. cholerae genes during growth in vivo [7,8,12,13]. Our results suggest that with the sequencing of V. cholerae genome nearing completion, a genomic approach towards identification of in vivo expressed genes in this organism using DNA microarrays would possibly yield more detailed information necessary to understand its complex mechanism of pathogenesis.

Acknowledgements

This work was supported by the research Grant SP/SO/D-56/96 from the Department of Science and Technology, Government of India. A.C is grateful to the Council of Scientific and Industrial Research for a research fellowship. We thank S.N. Dey and I. Guhathakurta for excellent technical support.

References


Table 1

Clones from the V. cholerae genome induced in vivo

<table>
<thead>
<tr>
<th>Clone#/gene(s)</th>
<th>Homologous to</th>
<th>Tentative function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sh1</td>
<td>PnuC protein of E. coli</td>
<td>NMN transport</td>
<td>Present study</td>
</tr>
<tr>
<td>Sh2</td>
<td>IcmF protein of L. pneumophila</td>
<td>Involved in intracellular multiplication</td>
<td>Present study</td>
</tr>
<tr>
<td>Sh3</td>
<td>CheY1 protein of E. coli</td>
<td>Chemotaxis</td>
<td>Present study</td>
</tr>
<tr>
<td>toxR</td>
<td>ToxR of V. cholerae</td>
<td>Virulence regulator</td>
<td>[20]</td>
</tr>
<tr>
<td>toxT</td>
<td>ToxT of V. cholerae</td>
<td>Virulence regulator</td>
<td>[21]</td>
</tr>
<tr>
<td>ctxAB</td>
<td>CtxAB of V. cholerae</td>
<td>Enterotoxin</td>
<td>[22]</td>
</tr>
<tr>
<td>tcpA</td>
<td>TcpA of V. cholerae</td>
<td>Pilus required for colonization</td>
<td>[21]</td>
</tr>
</tbody>
</table>
fusions to identify *Vibrio cholerae* genes induced during infection. Mol. Microbiol. 18, 671–683.


