# EVALUATION BY REAL-TIME PCR OF THE EXPRESSION OF S. FLEXNERI VIRULENCE-ASSOCIATED GENES *ospB* AND *phoN2* UNDER DIFFERENT GENETICAL BACKGROUNDS

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**Under conditions of activated type III secretion *Shigella flexneri* up-regulates the expression of numerous genes, including the virulence plasmid (pINV)-encoded *ospB* and *phoN2* genes. *ospB* and *phoN2* are virulence-associated genes which are part of a bicistronic transcriptional unit encoding OspB, a protein (effector) of unknown function secreted by the type III secretion (TTS) apparatus, and PhoN2 (apyrase or ATP-diphosphohydrolase), a periplasmic protein involved in polar IcsA localization on the surface of *S. flexneri*. In this work we used real-time PCR to measure transcription of *ospB* and *phoN2* of wild-type *S. flexneri* strain M90T as well as of derivative mutants impaired in definite virulence traits. The results obtained confirmed and extended previous reports indicating that the expression of *ospB* and *phoN2* genes is modulated in a *virB*-dependent, *mxiE*-independent manner under conditions of non-activated secretion, while their expression is considerably induced in a *mxiE*-dependent manner under conditions of activated secretion. That the expression of the *ospB-phoN2* operon is up-regulated in condition of activated secretion, indicates that probably the expression of these two genes might be important, especially during the later stages of infection of *S. flexneri*.**

Bacteria belonging to *Shigella* species are human intestinal pathogens which cause the most communicable of bacterial dysenteries, shigellosis. It has been calculated that shigellosis causes approximately 1.1 million deaths and over 164 million cases each year, with the majority of cases occurring in children of developing nations (1). *Shigella* strains contain a virulence plasmid (pINV) of approximately 220 kb which carries genes enabling these microrganisms to invade and disseminate into intestinal epithelial cells, leading to the destruction of the mucosa. Sequence and genetic analysis of pINVs from different *Shigella flexneri* strains indicated that the genes required for entry and diffusion into intestinal epithelial cells, and for the induction of apoptosis of macrophages are clustered in a pINV region of approximately 30 kb designated the entry region (2). The entry region encodes i) a type III secretion (TTS) apparatus (the *mxi-spa* operon), which is used by *S. flexneri*

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to secrete effector molecules in order to induce its own uptake by colonic epithelial cells as well as to exploit and subvert host immune responses (3); ii) translocators (IpaB and IpaC); iii) effectors (IpaD, IpgB1, IpgD,and IcsB); iv) their dedicated chaperones (IpgA, IpgC, IpgE and Spa15); and v) two transcriptional activators (VirB and MxiE) (2). The current model indicates that, upon contact with epithelial cells, the TTS apparatus is activated and secretion of effectors (approximately 25 proteins encoded by genes also located outside the entry region) occurs directly into host cells (2).

The understanding of the molecular mechanisms used by effector molecules in the complex mechanism of pathogenicity of *S. flexneri* is considered crucial for the design of new therapeutical strategies and effective vaccines (4).

*ospB* and *phoN2* are two genes located on the pINV of *Shigella* species and of related enteroinvasive *Escherichia coli* (EIEC) strains (2, 5-6), located outside the entry region. Direct and indirect evidence indicates that *ospB* and *phoN2* contribute to pathogenicity (5-13). Expression of the two genes is transcriptionally linked, being organized in a highly conserved bicistronic operon, thus indicating that the two encoded proteins might be functionally-related. Transcription initiates from an upstream promoter that precedes *ospB* and it is strictly regulated by the same regulatory network (*hns*, *virF*, *virB* and *mxiE*) governing the expression of virulence genes (6-7, 10-11, 14-16).

The regulation of the expression of pINV-encoded virulence genes has been the subject of extensive studies which have provided a wealth of information on their nature and on the mechanisms of their control (12, 14). In this paper, using real-time PCR methodology and selected null-mutants of wild-type *S. flexneri* strain M90T, we investigated transcription of the *ospB* and *phoN2* genes under different genetic backgrounds. The results obtained indicate that the expression of the two genes is up-regulated when its TTS apparatus is activated.

## MATERIALS AND METHODS

*Bacterial strains, plasmids and culture conditions*

The bacterial strains and plasmids used in this study are listed in Table I. Growth media used were Trypticase soy broth (BBL Microbiology Systems) and Luria–Bertani (LB) broth medium (17). The solid media contained 1.5% agar. Congo red (Cr) (Sigma) was added at 0.01% to Trypticase soy agar to determine Cr binding. To induce *in vitro* activation of secretion of the TTS machinery, exponentially-growing bacteria were resuspended in phosphate-buffered saline (PBS) and incubated at 37°C for 30 min in the presence of 7 μg/ml of Cr, as described (18). When appropriate, antibiotics (Sigma) were added at the following concentrations: 100 μg/ml ampicillin (Ap); 30 μg/ml chloramphenicol (Cm); 30 μg/ml kanamycin (Km).

*DNA isolation, manipulation and PCR amplification*

DNA extraction, isolation of plasmids, restriction digestion, electrophoresis, purification of DNA fragments, construction of recombinant plasmids, transformation and electroporation were performed by standard methods (19).

Primers used throughout this study and their relevant characteristics are listed in Table II. Thermal cycling conditions were as described previously (7). DNA sequence data were compared to known nucleotide sequences using the BLAST Server (National Center for Biotechnology Information Bethesda, Md).

*Construction of mutant strains and plasmids*

All *S. flexneri* strains were derived from serotype 5a wild-type strain M90T (Table I). Oligonucleotide primers were designed based on the *S. flexneri* pINV sequence (2). M90T non-polar deletion mutants of *virB*, *mxiA* and *mxiE* were constructed by the PCR-based gene-disruption method reported by Datsenko and Wanner, as previously described (7, 20). In this way, M90T derivative strains HND54 (Δ*virB*), HND43 (Δ*mxiA*) and HND63 (Δ*mxiE*) were generated. SF1076 (*ipaB4*) was obtained from C. Parsot (21).

*RNA extraction and cDNA synthesis*

Total RNAs from wild-type strain *S. flexneri* M90T and from its relative mutants (Table I) were isolated as follows. Overnight cultures of bacteria grown at 30°C in LB medium was diluted 1:100 in fresh medium and incubated at the same temperature until bacteria reached $OD_{600}$ = 0.1. At this time, bacteria were incubated either at 30°C or shifted to 37°C until bacteria reached their mid-logarithmic phase of growth ($OD_{600}$ = 1.2). Aliquots of broth-cultures grown at 37°C were taken at different time points (time-course experiment). Total RNAs were prepared with the RiboPure Bacteria Kit (Ambion), as recommended by the manufacturer. Contaminating DNA was removed by DNase treatments as recommended by the manufacturer. The quality and yield of the RNA preparations were assessed by measuring the $A_{260}/A_{280}$

ratios and by visualization of 16S and 23S rRNAs by 2% agarose gel electrophoresis.

First-strand cDNA synthesis was performed on 1 μg of DNase-treated total RNA with QuantiScripts Reverse Transcriptase (Qiagen) using a blend of poly-T and random primer oligonucleotides (Qiagen). cDNA samples were diluted 10-fold with RNAse free water and 2 μl aliquots were used as templates in each real time PCR reaction.

*Real-time PCR studies*

Real-time PCR were performed with a LightCycler thermocycler (Roche) by use of a QuantiTect SYBR Green PCR kit (Qiagen). For each reaction, 2 μl of diluted reverse-transcribed cDNA was subjected to PCR amplification in a 20 μl final volume containing 500 nM of each primer and 10 μl of 2X SYBR green master mix. The following conditions were used for amplification: 1 cycle at 95°C for 15 min and 40 cycles at 95°C for 15 s, 53°C for 20 s and 72°C for 15 s. To ensure the specificity of the PCR products, melting curve analysis was performed by heating products to 95°C for 15 s, followed by cooling to 60°C and slowly heating to 95°C while monitoring fluorescence (data not shown). In each PCR experiment appropriate negative controls were also included.

*Detection, quantitation and statistical analysis*

Data collection was carried out by use of LightCycler ver 4.0 software (Roche). Data were normalized to levels of *rrsA* (16S rRNA) and analyzed by use of the comparative critical threshold (CT) method as described (22). The relative comparison method was used to compare the level of expression of target genes in the different experimental conditions. No contamination by genomic DNA was detected by PCR for *ospB*, *phoN2* or *rrsA* after DNase treatment of RNA (data not shown). For real-time data presentation, error bars represent standard deviations of the mean for three independent experiments. Statistical significance was calculated with Student's *t* test. A $P$ value $<0.05$ was considered significant.

## RESULTS

*Real-time PCR analysis of ospB-phoN2 expression*

In this paper we sought to determine, by real-time PCR methodology, the transcriptional profile of *ospB* and *phoN2*, two virulence-associated genes located on the pINV of wild-type *S. flexneri* strain M90T, as well as of selected M90T derivative mutants impaired in important virulence traits [namely HND43 (*virB*), SF1076 (*ipaB4*), HND43 (*mxiA*) and HND63 (*mxiE*)] (Table I). Firstly, to better define the relative levels of the two transcripts as well as the influence of temperature on transcription, we evaluated the expression of the two genes in a classical time course experiment. 16S rRNA (*rrsA*) levels were used to normalize real-time data and to adjust for various bacterial numbers present at each time point. Fig. 1 shows that the expression of *phoN2* is strongly induced when bacteria reach their mid-exponential phase of growth. At 180 min, corresponding to an $OD_{600}$ of 1.2, we measured the peak of expression whose level was about three-fold higher compared to that at 120 min ($OD_{600} = 0.8$). On the other hand, *ospB* reaches its peak expression at 120 min ($OD_{600} = 0.8$) (we measured an approximately four-fold increase, if compared to 60 min, and about a four-fold decrease compared to 180 min time points). As expected, we found basal level of expression of the two genes when bacteria were grown at the non-permissive temperature of 30°C ($OD_{600} = 1.2$).

To better understand the regulation of *ospB-phoN2* expression, we evaluated the relative expression of the two genes under genetic conditions known to influence the expression of virulence genes in *S. flexneri*. Based on the results shown in Fig. 1, we decided to compare the expression using bacteria grown solely at $OD_{600} = 0.8$, because at this optical density we obtained a relatively high peak of expression of both genes.

*ospB and phoN2 expression is up-regulated in conditions of activated TTS secretion*

Congo red (Cr) has been shown to induce activation of the TTS secretion machinery in *S. flexneri* and, in turn, TTS activation has been shown to induce the expression of the *ospB-phoN2* transcriptional activator MxiE (11, 18). As shown in Fig. 2, the activation of the TTS secretion machinery by Cr, significantly up-regulates the relative expression of *ospB* and *phoN2* genes by about 6.9 and 6.5 fold, respectively, compared to bacteria grown in conditions of non-activated TTS secretion. This result is in agreement with previous reports indicating that *mxiE* is involved in the induction of the expression of the *ospB-phoN2* operon (14). To confirm these results we evaluated the relative expression of the two genes using HND63 (*mxiE*), HND43 (*mxiA*) and SF1076 (*ipaB4*) M90T mutants grown in the absence and in the presence (when

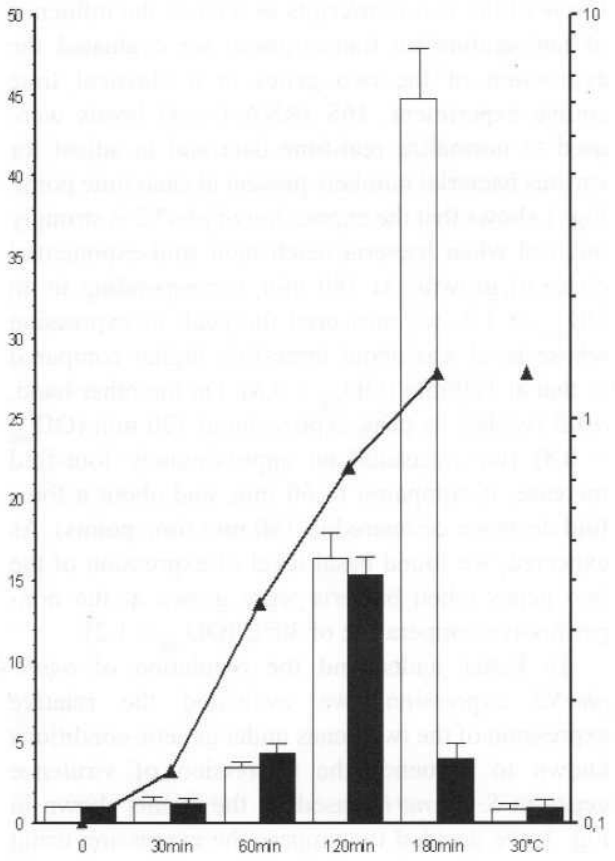


**Fig. 1.** *Transcriptional expression of the ospB and phoN2 genes was measured during 180 min time course experiment by real time PCR as described in Materials and Methods. Wild-type S. flexneri strain M90T was grown at 37 and 30°C as described in Materials and Methods. At the indicated time points (▲, corresponding to the different $OD_{600}$) total RNA was extracted. The growth curve of S. flexneri M90T at 37°C is indicated as a continuous black line while the $OD_{600}$ of bacteria grown at 30°C is indicated as a single black arrowhead. The histogram shows the increases of ospB (black bars) and phoN2 (open bars) expression relative to that of the two genes measured at 0 min time point. CT values were normalized to levels of rrsA (16S rRNA) to correct for variations in bacterial numbers. Results shown are means and standard deviations from triplicate experiments (P<0.05).*

appropriate) of Cr. Fig. 2 shows that the Cr-induced activation of the TTS apparatus in the absence of a functional *mxiE* gene (HND63 Cr) or the lack of a functional TTS apparatus (HND43, a mutant of M90T defective for TTS secretion) does not influence *ospB* and *phoN2* expression, whose levels are comparable to those measured for the wild type strain, grown in conditions of non-activated secretion. On the other hand, the expression of the two genes in SF1076 (a constitutive secretion deregulated mutant) is up-regulated at approximately the same level measured in M90T grown in the presence of Cr, indicating that MxiE is necessary to induce up-regulated expression of the *ospB* and *phoN2* only in condition of activated secretion. These results are in agreement with those reported by Le Gall et al. that used microarrays analysis to evaluate the expression of the two genes (14).

## DISCUSSION

*ospB* and *phoN2* are two virulence-associated genes located on the virulence plasmid (pINV) of *S. flexneri*, outside the entry region (2, 5-6). *ospB* encodes for an effector of unknown function (OspB) secreted by the TTS apparatus of *S. flexneri* (2, 7, 14). *phoN2* encodes for apyrase, a periplasmic enzyme which belongs to the family of ATP-hydrolyzing enzymes (5, 9, 23), able to sequentially hydrolyze nucleoside triphosphates to diphosphates and then to monophosphates. Recently we have shown that *phoN2* is a virulence gene, involved in actin-based motility of *S. flexneri* since *phoN2* null non-polar mutants of *S. flexneri* strain M90T presented an altered surface distribution of IcsA, an outer membrane protein responsible for nucleation of host cell actin which allows *S. flexneri* to move intra- and inter-cellularly (7).

The two genes are organized in a single highly conserved bicistronic operon and are co-transcribed as a 2 kb bicistronic temperature-regulated mRNA (expression of the bicistronic operon as well as of virulence genes is repressed at the non-permissive temperature of 30°C), initiating from an upstream promoter that precedes *ospB* (7). The 2 kb mRNA is post-transcriptionally processed in the intercistronic *ospB–phoN2* region, leading to a considerable accumulation of a more stable 1 kb *phoN2*-specific mRNA (6).

To evaluate the relative levels of the two transcripts as well as the influence of temperature on transcription, we measured by real-time PCR the relative expression of the two genes in time course

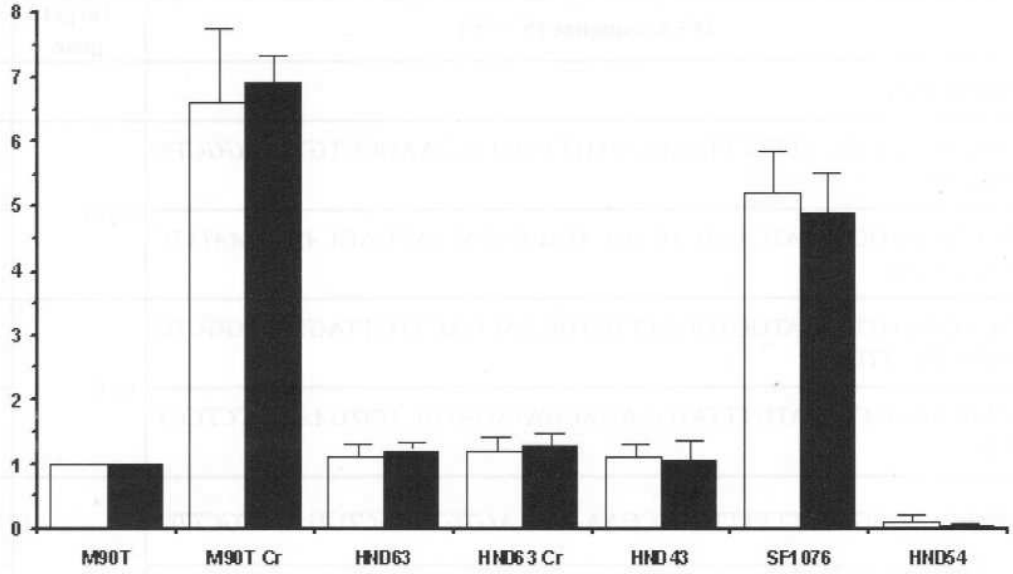


**Fig. 2.** *Relative expression of the ospB and phoN2 genes in wild-type S. flexneri strain M90T and its relative mutants. Total RNA was extracted from bacteria grown at 37°C ($OD_{600}$ = 0.8, corresponding to 120 min of growth). The histogram shows the increases of ospB (black bars) and phoN2 (open bars) expression relative to that of the two genes of wild-type M90T. CT values were normalized to levels of rrsA (16S rRNA) to correct for variations in bacterial numbers. The strains analysed were M90T, M90T Cr (with the addition of Congo red to activate TTS secretion), HND63 (mxiE), HND63 (mxiE) Cr, HND43 (mxiA), SF1076 (ipaB4) and HND54 (virB) (see the Results and Discussion section for details). Results shown are means and standard deviations from triplicate experiments (P<0.05).*

**Table I.** *Bacterial strains and plasmids used in this study.*

| Strains or plasmid | Description | Reference or source |
|---|---|---|
| **Bacterial strains** | | |
| M90T | Wild-type *S. flexneri* serotype 5a | 24 |
| HND43 | M90T Δ*mxiA*; susceptible | 7 |
| HND54 | M90T Δ*virB*; susceptible | This study |
| HND63 | M90T Δ*mxiE*; susceptible | This study |
| SF1076 | *ipaB4* | 21 |
| **Plasmids for mutant construction** | | |
| pKD46 | λ Red helper plasmid: *oriR101 repA101*(Ts) *P-araB-gam-bet-exo* $Ap^r$ | 20 |
| pKD4 | Template plasmid carrying a $Km^r$ gene with FRT sequence. | 20 |
| pCP20 | FLP helper plasmid; pSC101 replicon (Ts) *bla cat* Flp (λR*p*) *cI*857 $Ap^r$ $Cm^r$ | 20 |

*$Ap^r$, ampicillin-resistant; $Km^r$, kanamycin-resistant; $Cm^r$ cloramphenicol-resitant*

**Table II.** *Primers used in this study.*

| Primers | DNA sequence (5'→3') | Target gene | Reference or source |
|---|---|---|---|
| **Nonpolar Mutagenesis** | | | |
| **M 4 3 Fw**[a] | GTGATCCAGTCTTTTCTTAAGCAAGTAAGTACTAAGCCT*GTGTAGGCTG GAGCTGCTTC* | *mxiA* | 7 |
| **M 4 3 Rv**[a] | TGGTATATGCTTCATCAATCTCAGCATACGATATAACGAG*CATATGAATATC CTCCTTAG* | | |
| **V 3 3 Fw**[a] | ACAGGGTGTGATATGGTGGATTTGTGCAACGACTTGTTA*GTGTAGGCTG GAGCTGCTTC* | *virB* | 7 |
| **V33 Rv**[a] | CATCAGTGTTCGATGTTTATGAAGACGATAGATG*CATATGAATATCCTCCT TAG* | | |
| **E23 Fw**[a] | ATGGAAGGGTTTTTTTTTGTCCGAAATCAA*GTGTAGGCTGGAGCTGCTTC* | *mxiE* | This study |
| **E23Rv**[a] | AATATAAAATGTGTAGGCTGGAGCTGCTTC*CATATGAATATCCTCCTTAG* | | |
| **Real time PCR** | | | |
| **rrsa Fw** | CACGATTACTAGCGATTCCGACTT | *rrsA* | 25 |
| **rrsa Rv** | CGTCGTAGTCCGGATTGGA | | |
| **o s p B Fw** | ATTTAGATGGTGTTAGACCATACTGT | *ospB* | This study |
| **o s p B Rv** | GATAGAACATCATTGCTATCACAA | | |
| **p h o N 2 Fw** | GCAAATGCTCTGAAGGCAGAA | *phoN2* | This study |
| **p h o N 2 Rv** | GCGGCCGAATTCATAATGAGC | | |

[a] *Primers used to construct M90T non-polar deletion mutants. Primers were designed to anneal to template plasmid pKD4 (Table 1) in order to amplify the Km*$^r$ *resistance cassette and flanking FRT sites (in italics). Primers contain extensions that are homologous to regions adjacent to pWR100-encoded target genes (GenBank accession no AL391753).*

experiments, using 16S rRNA (*rrsA*) as an internal calibrator. Under these experimental conditions, expression of *phoN2* was shown to be strongly induced when bacteria reach their mid-exponential phase of growth ($OD_{600}$ = 1.2) while *ospB* reached its peak expression when bacteria were at the exponential phase of growth ($OD_{600}$ = 0.8) (Fig. 1). As expected, basal level of expression of the two genes were detected when bacteria were grown at the non-permissive temperature of 30°C.

Furthermore, to study the regulation of *ospB* and *phoN2* expression, the relative expression of the two genes was evaluated under different genetic backgrounds known to influence the expression of virulence genes in *S. flexneri*. Expression of gene encoding proteins (effectors) secreted by the TTS apparatus can be classified into three categories: i) those that are controlled by *virB*; ii) those that are controlled by *mxiE*; and iii) those that are controlled by both *virB* and *mxiE* (14). To understand whether the expression of the *ospB* and *phoN2* genes are under the control of the two genes, we evaluated

their expression in wild-type strain M90T, in HND54 (*virB*), HND63 (*mxiE*) and SF1076 (*ipaB4*) under conditions of activated and non-activated secretion. The results reported in Fig. 2 clearly show that the expression of the *ospB* and *phoN2* is strongly repressed in HND54 (*virB*), indicating that these genes are regulated in a *virB*-dependent, *mxiE*-independent manner under conditions of non-activated secretion (Fig. 2, M90T, HND63, HND63 Cr and HND54), while their expression increases, in a *mxiE*-dependent manner, under conditions of activated secretion (Fig. 2; M90T Cr and SF1076).

In conclusion, our study confirms and extends previous reports indicating that the expression of the *ospB-phoN2* operon, being up-regulated in condition of activated secretion, might be important, especially during late phases of invasion of host cells by *S. flexneri*.

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