Identification of *Salmonella enterica* Serovar Typhi DNA Fragments with Transcriptional Activity Under Different Growth Conditions

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Abstract: *Salmonella enterica* serovar Typhi is a human pathogenic microorganism with a very complex infective cycle, involving the transit of bacteria across different microenvironments; to optimize the performance of attenuated *Salmonella* strains suitable as live carriers of heterologous antigens, fine tuning of wild type bacteria gene expression is essential.

Several DNA fragments were obtained from a *Salmonella enterica* serovar Typhi (vi+, fim+) blood isolate and 18 clones were selected according to the dimension of the insert (range <0.2-1.6 kb). These fragments showed a transcriptional activity in a promoterless vector cloned in *Escherichia coli* background, according to homogeneous parameters. The results obtained provide an insight about signals mediating gene activation *in vivo*, particularly in the microenvironments known to exist during the infectious process, even if the fragments are not promoters sequences. Finally, the functional characterization of several fragments showed that they possessed an efficient and homogeneous transcriptional activity, worth to be further investigated.

Keywords: Promoter regions, *Salmonella enterica* serovar Typhi, gene expression, vaccines, live vector.

INTRODUCTION

Salmonella enterica serovar Typhi is the etiological agent of typhoid fever, a human disease. These bacteria infect the human gastrointestinal tract, penetrate the Peyers patches M cells and local lymph nodes and, through the bloodstream and lymph, reach the cells of the reticuloendothelial system. Here the microorganisms multiply within the phagocytes and the persistent bacteremia causes a systemic dissemination. Infected people develop a syndrome characterized by fever, sensorial ailment, splenomegaly, rash and leukopenia [1]. Serovar Typhi is a generalized facultative intracellular parasite and is therefore capable of both extra and intracellular life within the host; *S. enterica* is also able to exist in environmental conditions and this stage allows the completion of the oral-fecal cycle, making infection possible by ingestion of contaminated food products [1].

During the whole infective cycle, inside and outside of the host, enteric pathogens face variations of many parameters, such as pH, osmolarity, temperature and the availability of nutrients and oxygen [2]. In the environmental stage bacteria experience a higher O_2 availability and a temperature that can be sub-optimal with respect to that found in the lumen of the distal ileum, where serovar Typhi begins infection finding a higher temperature and osmolarity, and a lower O_2 tension. When entering the epithelial cells and macrophages, microorganisms encounter a restriction of nutrients, and, in the bloodstream, a low availability of O_2, as most of it is bound by hemoglobin. The bacterial versatility reflects reversible metabolic changes and can only be achieved by a complex regulatory network which warrants not only a tight transcriptional control in spite of a great diversity of conditions [3, 4], but also allows the expression of bacterial virulence (e.g. adherence and invasiveness) [5-9]. Due to its ability to colonize and stimulate important immunological niches, *Salmonella enterica* serovar Typhi has been widely considered as an attractive live vector [10-15]. However, its use is limited by the instability of multiple-copy plasmid systems encoding the heterologous gene, whose expression can place a metabolic burden and favour a negative selection of the vector; furthermore, maintenance of plasmid expression system requests the problematic use of antibiotic selection markers [16]. Moreover, chromosomal integration of the heterologous gene drives an antigen expression that is too low with respect to the ability of generating an effective immune response [17-21]. In previous works, *in vivo*-inducible promoters have been utilized in plasmid vectors to modulate heterologous genes expression in salmonella strains in a manner ideal for bacterial survival and immunological response [22]. More recently, it was demonstrated that, by integrating a single copy of a heterologous gene associated

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with *nirB* promoter into attenuated *Salmonella* chromosome, an inducible expression of foreign antigen was achieved [23]. A similar approach relies on chromosomal insertion of heterologous genes under the control of *in vivo*-inducible promoters that allow a selective expression within human macrophages by bacterial carriers *Salmonella enterica* serovar Typhi [24, 25].

A further understanding of genetic mechanisms involved in the regulation of serovar Typhi metabolism and virulence could improve development and immunological efficacy of attenuated live *Salmonella* employed as carriers of foreign antigens. This work describes the isolation and functional characterization of DNA fragments of a blood culture *Salmonella enterica* serovar Typhi as putative promoters, activated under different *in vivo*-mimicking conditions, using *Escherichia coli*, genera related to S*almonella*, as the bacterial recipient, which allowed a solution to be found for the instability of constructs in wild type serovar Typhi and preliminary data to be obtained.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The wild type *Salmonella enterica* serovar Typhi strain 30 Ty5 (vi +, fim +), isolated from a blood culture, was used for the extraction of total DNA. The *Escherichia coli* Sure (Stratagene) was used as a recipient for the cloning experiments; this strain was used because most of the constructs were highly unstable in wild type *Salmonella*, even when passed through an intermediate *rec-mod+ Salmonella* strain. The plasmid used in this work was pUJ10 [26], a pCB267 derivative.

Culture Media and Growth Conditions

E. coli strains were grown in Luria-Bertani (LB) broth or LB agar [27], whereas salmonelle were grown in brain heart infusion (BHI) broth or BHI agar (Difco). Selection of bacteria harboring DNA sequences with promoter activity was performed on LB agar supplemented with the chromogenic substrate XP (5-bromo-4-chloro-3-indolyl-phosphate-*p*-toluidene) for alkaline phosphatase [28], or X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) for β-galactosidase [29] at a final concentration of 40 μg/ml.

All the cultures were grown overnight in 250 mL of medium. In order to value the response to different oxygen availability, bacteria were grown at 37 °C in LB broth; the aerobic cultures were achieved in shaking flasks, the microaerophilic cultures were grown with shaking in a 10% CO_2 thermostat and the anaerobic ones were achieved by using a GasPak system (BBL Microbiology Laboratories). To determine the influence of temperature, bacteria were grown under atmospheric O_2 tension in LB broth, at 25°C and 37°C. For the assays under different osmolarities, a low osmolarity medium (73 mmol/l NaCl) [30] was used, the osmolarity was increased, when appropriate, to 0.3 mol/l [30] and the strains were grown at 37°C in presence of atmospheric O_2 tension. Media were supplemented, when indicated, with ampicillin (100 μg/ml).

DNA Manipulations

Total DNA and plasmid isolation, restriction endonuclease digestion, dephosphorylation with fetal calf intestinal phosphatase, ligation with T4 DNA ligase, transformation and DNA analysis by agarose gel electrophoresis were performed following standard protocols [17] or as indicated by the manufacturer. Restriction and modification enzymes were purchased from New England Biolabs and Boehringer Mannheim. 30 specimens of total DNA of the *Salmonella* strain 30 Ty5 (vi+, fim+) were partially digested with serial dilutions of *Sau*3A1 [27], to create small sized fragments. After dephosphorilation with alkaline phosphatase, the DNA fragments were ligated with *Bam*HI-digested pUJ10. The ligation mixture was transformed into the *E. coli* Sure strain. The size of the positive clones inserts were measured by restriction analysis using *Bgl* II and *Sma* I. Primers for sequencing were selected on the two reporter genes and DNA sequencing was performed by the method of Sanger [31] with a *Taq* Dyedeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) and an automated sequencer (model 310; Applied Biosystems). Three of the putative promoters were sequenced (pCG61, pCG88, pCG93) and homologies with known sequences were searched with BLAST 2.2.12 [32].

Enzymatic Assays

To determine alkaline phosphatase and β-galactosidase activity, 1 ml of bacterial culture grown under appropriate conditions were spun down and resuspended in 1 ml of 1 mol/l Tris, pH 8.0 and in 1 mL of Z buffer respectively, to read the optical density at 600 nm (OD_{600}), as described previously [27, 28]; 100 μl of the diluited bacterial suspensions were permeabilized with 50 μl of chloroform and 50 μl of sodium dodecyl sulfate (SDS) 0.1%, and 900 μl of 1 mol/l Tris, pH 8.0 for the alkaline phosphatase and with 900 μl of Z-buffer for the β-galactosidase determination. After equilibrating for 10 min at room temperature, 100 μl of p-nitrophenyl phosphate (PNPP) 0.4% and 200 μl of o-nitrophenyl β-D-galactopiranoside (ONPG) 0.4% were added, and the respective stop solutions were added when a significant yellow colour was observed [27, 28].

Alkaline phosphatase and β-galactosidase activities of individual clones was measured spectrophotometrically as previously described and compared with the activity of the control, the parental strain containing the promoterless vector. The enzymatic activities were measured in enzyme units (E.U.) that represent the slightest quantity of enzyme capable of releasing 1 μmol of p-nitrophenyl phosphate or o-nitrophenyl β-D-galactopyranoside per minute [27, 28].

The induction of enzymatic activity in recombinants was considered to have taken place when the OD value doubled following a change in the experimental condition relating to a single parameter (*i. e.* temperature, medium osmolarity, O_2 tension**)** and also when a two-fold increase was observed in the recombinant OD value in respect to the value obtained with the control, the promotorless pUJ10.

RESULTS

Cloning of DNA Fragments with Transcriptional Activity

Following partial digestion of *Salmonella* DNA with *Sau*3A1, 25 samples with a prevalent fragment size range from 0.1 kb to 2.8 kb were obtained. After ligation of the inserts with a *Bam*HI-digested pUJ10 vector, harboring the promoterless *lacZ* and *phoA* reporter genes, and transformation of *E. coli* Sure bacteria, the analysis of divergent promoters was made by the screening on substrate agar, and 18 positive recombinant clones were obtained (data not shown). The size of inserts, measured by restriction analysis, ranged from <0.2 to 1.6 kb (Table **1**). Most of the constructs were highly unstable in wild type *Salmonella* strains, even when passed through an intermediate *rec-mod+ Salmonella* strain, suggesting that this instability may be in part due to recombination events. Therefore, transcriptional activation studies were performed using an *Escherichia coli* strain as recipient, which permitted preliminary results to be extrapolated.

Table 1. Dimensions of the Cloned *Salmonella enterica* Serovar Typhi DNA Fragments

Hybrid Plasmid	Size of the Insert
pCG61	<0.200 kb
pCG62	<0.200 kb
pCG71	<0.200 kb
pCG72	0.247 kb
pCG74	0.260 kb
pCG76	0.350 kb
pCG77	0.650 kb
pCG78	0.700 kb
pCG79	0.750 kb
pCG80	0.750 kb
pCG81	0.830 kb
pCG83	0.850 kb
pCG84	0.900 kb
pCG88	0.930 kb
pCG90	0.950 kb
pCG91	1.300 kb
pCG92	1.400 kb
pCG93	1.600 kb

Functional Characterization of DNA Fragments

The effects of incubation temperature on enzymatic activity of the clones were first analyzed: most clones showed a greater alkaline phosphatase activity at 37°C than at 25°C, particularly those harboring plasmids pCG61, pCG78, pCG79 and pCG93; the values of clones harbouring plasmid pCG78 were 3160 and 960 E.U. at 37°C and 25°C, respectively. With regard to β-galactosidase activity, only clones harboring plasmids pCG88 and pCG92 showed a significant increment in the enzymatic activity, more than 100% and 30% respectively, when the temperature was raised from 25°C to 37°C. In contrast, clones containing plasmids pCG81 and pCG91 showed higher enzymatic values at 25°C than at 37°C, with regard to the activity of both enzymes (Table **2**).

Table 2. Alcaline Phosphatase and β-Galactosidase Activities of Clones Harboring *Salmonella enterica* Serovar Typhi DNA Fragments with Reference to Different Incubation Temperature of Bacteria Growth, Measured in Enzymatic Units (E.U.)

Hybrid plasmid	Alkaline Phosphatase		β-Galactosidase	
	37°C	25°C	37°C	25°C
pCG61	1336	522	0	0
pCG62	203	59	0,7	1
pCG71	35	5	7	9
pCG72	262	98	0	0
pCG74	123	16	0	0
pCG76	295	42	0	0
pCG77	260	39	12	0
pCG78	3160	962	3	3
pCG79	620	164	13	11
pCG80	100	18	8	0
pCG81	34	105	70	108
pCG83	95	25	22	8
pCG84	18	5	26	12
pCG88	25	10	551	246
pCG90	23	5	4	7
pCG91	30	73	0,4	298
pCG92	47	10	403	296
pCG93	2517	1403	83	89
pUJ10 (Ctr.)	14	2	7	5

Ctr.: control.

The effects of variations in osmolarity and O_2 availability on enzymatic activation were also monitored. Under conditions of hyperosmolarity greater *phoA* expression was observed for clones harbouring pCG61, pCG72, pCG76 and pCG93, and greater *lacZ* expression was observed for clones harboring pCG84, pCG88, pCG91 and pCG92 (the alkaline phosphatase values of pCG93 were 620 and 2209 U.E., and the β-galactosidase values of pCG88 were 792 and 4766, in low and high osmolarity medium, respectively). The alkaline phosphatase values increased when measured in low osmolarity in clones harboring plasmids pCG77, PCG88 and pCG91 (Table **3**).

When clones containing pCG61, pCG78, pCG79 and pCG93 were tested in anaerobiosis the alkaline phosphatase activity increased (the values expressed by clone pCG61 were 1220, 5333 and 3184 U.E. in atmospheric O_2, anaerobiosis and microaerophilia conditions respectively), and

clones containing pCG71, pCG79 and pCG91 showed minimal β-galactosidase activity which was otherwise undetectable in aerobic conditions. In contrast, clones harbouring pCG81 and pCG88 showed a minor increase in β-galactosidase expression in the presence of atmospheric O_2 and a disappearance in anaerobiosis; a reduction in microaerophilia was only observed in the clone harbouring pCG88 (Table **4**).

Table 3. Alcaline Phosphatase and β-Galactosidase Activities of Clones Harboring *Salmonella enterica* Serovar Typhi DNA Fragments with Reference to Different Medium Osmolarities, Measured in Enzymatic Units (E.U.)

Hybrid Plasmid	Alkaline Phosphatase		β-Galactosidase	
	H.o.m.	L.o.m	H.o.m.	L.o.m
pCG61	122	62	4	6
pCG62	85	0	0	0
pCG71	37	29	17	11
pCG72	409	121	2,5	4
pCG74	28	57	6	8
pCG76	302	122	1	0
pCG77	140	762	0	2
pCG78	105	113	6	3
pCG79	46	44	5	0
pCG80	14	58	3	0
pCG81	51	67	104	71
pCG83	125	152	67	37
pCG84	23	60	416	29
pCG88	64	188	4766	792
pCG90	40	91	23	9
pCG91	245	670	1238	501
pCG92	179	161	587	56
pCG93	2209	620	111	68
pUJ10 (ctr.)	59	40	2,5	0

Ctr.: control; H.o.m.: high osmolarity medium; L.o.m.: low osmolarity medium.

DNA Sequence Analysis

The 89-bp fragment isolated from clone pCG61 showed close homology with known DNA-directed RNA polymerase, beta'-subunit sequence of *Salmonella enterica* serovar Typhi and other sources: *Salmonella enterica* serovar Typhi Ty2 and CT18, 100%; *Salmonella enterica* serovar Paratyphi A, 100%; *Salmonella enterica* serovar Typhimurium LT2, 98%.

The 258-bp fragment isolated from clone pCG88 shared a close homology with known *Salmonella enterica* serovar Typhi Ty2 and CT18 (100%) and *Salmonella enterica* serovar Paratyphi A (98%) sequences, both coding for a penicillin-binding protein and a rRNA guanine-N1-methyltransferase.

Table 4. Alcaline Phosphatase and β-Galactosidase Activities of Clones Harboring *Salmonella enterica* Serovar Typhi DNA Fragments with Reference to Different Oxygen Availability, Measured in Enzymatic Units (E.U.)

Hybrid Plasmid	Alkaline Phosphatase			β-Galactosidase		
	Anaer.	Micro.	Atm O_2	Anaer.	Micro.	Atm O_2
pCG61	5333	3184	1220	0,3	0	0
pCG62	458	238	145	9	8	0
pCG71	121	19	81	17	4	0
pCG72	430	148	410	1	0,7	0
pCG74	52	9	60	0,3	0,5	0
pCG76	311	94	322	0,3	0,5	0
pCG77	294	82	256	0,7	0,4	0
pCG78	2940	924	2005	8	7	0
pCG79	1703	305	1035	30	7	0
pCG80	282	125	163	0	1,5	0
pCG81	125	91	94	0	79	84
pCG83	77	38	117	29	17	29
pCG84	8	3	12	7	6	0
pCG88	13	8	24	1	68	295
pCG90	46	24	35	9	9	0
pCG91	23	7	42	75	36	5
pCG92	118	101	85	221	232	187
pCG93	9070	1476	4390	169	8	180
pUJ10 (Ctr.)	43	22	28	0	1,6	0,5

Ctr.: control; Anaer.: anaerobiosis; Micro.: microaerophilia; Atm O_2: atmospheric O_2 tension.

The 285 nucleotide from clone pCG93 also showed close identity to known *Salmonella enterica* serovar Typhi Ty2 and CT18 (99%) and *Salmonella enterica* serovar Typhimurium LT2 (98%) sequences, both coding for a conserved hypothetical protein.

DISCUSSION

Salmonella enterica serovar Typhi, like other facultative intracellular parasites (*e.g. Yersinia, Brucella, Legionella, Listeria monocytogenes* and *Mycobacterium*), encounters multiple microenvironments *in vivo*, growing and replicating extracellularly or within leukocytes and macrophages. During parasitic life serovar Typhi not only adapts itself to different environments, but is also able to escape from non-specific host clearance mechanisms, such as complement and phagocytic lysis, through virulence factors. *Salmonella* has not lost the capacity for a saprophytic life (*e.g.* in a milk, freshwater or saltwater environment) where habitats are not hostile but nutritional limitations, as well as sub-optimal temperatures, may represent stringent challenges for the survival

[1]. In a microorganism as versatile as *Salmonella enterica* serovar Typhi some functions, which are essential (*e.g.* metabolic), although modulated under different conditions, must be always present; in contrast, other functions are activated only when it is possible and/or necessary, in response to specific signals [2]. A coordinate regulation of gene expression allows bacteria to survive and replicate both inside the host and in the environment through sequential signals that, affecting RNA polymerase sigma factors [33-36] and/or the efficiency of different promoters that regulate a single operon [3, 4], permit changes in transcription. It is therefore important to understand and to imitate the natural bacterial versatility to avoid compromising the competitiveness and efficacy of *Salmonella* when used as vaccine delivery system; this could be developed by selecting wild type promoters up-regulated at different time points or niches during infection, choosing the promoters whose transcriptional activity is the greatest in conditions similar to that which bacteria find in the organism and, particularly, in the antigen presenting cells, and the lowest in the environmental ones, such as during fermentation of vaccine manufacture [37, 38].

In this work, we have isolated 18 DNA fragments from *Salmonella enterica* serovar Typhi whose transcriptional efficacy was valued by inserting them into a promoterless plasmide, encoding *lacZ* and *phoA* as reporter genes, and cloning into *Escherichia coli* background. After growing all the recombinants in seven different conditions, we obtained 23 activations (four low activation, with E. U. value under 100) and six significant increases, relating to 12 clones (Tables **5** and **6**). Only four enzymatic activations (related to recombinants pCG72, pCG76, pCG77 and pCG84) were obtained following a single change in the growth parameters, while other activations or increases were obtained for at least two homogeneous conditions, i.e. 37°C, high osmolarity medium and anaerobiosis or 25°C, low osmolarity medium and atmospheric O_2 tension. Four recombinants exhibited an increase, relating to the same enzyme, in two experimental conditions similar to that of ileum mucosa and, in particular, two recombinants (pCG78 and pCG79) showed the greatest alkaline phosphatase value at 37°C and in anaerobiosis while two recombinants (pCG88 and pCG92) attained major β-galactosidase activity at 37°C and in high osmolarity medium; finally, it is also interesting to note that clones containing pCG61 and pCG93 showed enzymatic activation or a significant increase at 37°C, in hyperosmolarity and in anaerobiosis (Table **5**). Recombinants pCG61, pCG78, pCG79 and pCG93 showed a high baseline activation for a reporter gene, *phoA*, that has been probably enhanced by a culture condition change.

Different clones showed the opposite behaviour, having the greatest transcriptional activity in the environmental conditions. Growing at 25°C clone pCG81 reached a double value for alkaline phosphatase and a minor increase in β-galactosidase, in respect to 37°C, and, in the presence of atmospheric O_2 tension, showed a low activation of β-galactosidase in respect to the anaerobiosis condition. Recombinant pCG91 attained major alkaline phosphatase at 25°C and in low osmolarity medium, whereas it attained major β-galactosidase activity at 25°C but also in two conditions similar to that of ileum mucosa, i.e. high osmolarity medium and anaerobiosis. Recombinant pCG88 also showed un-homogeneous behaviour, as β-galactosidase was activated by growth at 37°C and in high osmolarity medium as well as in aerobiosis, while alkaline phosphatase was only activated in low osmolarity medium (Table **6**).

Table 5. Alcaline Phosphatase and β-Galactosidase Activities of Clones Harboring *Salmonella enterica* Serovar Typhi DNA Fragments which Exhibited Preferential Activation in Experimental Conditions Alike to that of Ileum Mucosa

Hybrid Plasmid	Alkaline Phosphatase			β-Galactosidase		
	37°C	H.o.m	Anaer.	37°C	H.o.m	Anaer.
pCG61	x	(x)	x			
pCG93	(x)	x	x			
pCG78	x		(x)			
pCG79	x		(x)			
pCG88				x	x	
pCG91					x	x-
pCG92	x-			(x)	x	
pCG72		x				
pCG76		x				
pCG84					x	

H.o.m.: high osmolarity medium; Anaer.: anaerobiosis.
x : activation (enzymatic value higher than 100 E. U. and double in respect to the relating parameter and to the control value).
x-: low activation (enzymatic value under 100 E. U. but double in respect to the relating parameter and to the control value).
(x): increase of activity (increasing in enzymatic value but not double in respect to the relating parameter and to the control value).

Table 6. Alcaline Phosphatase and β-Galactosidase Activities of Clones Harboring *Salmonella enterica* Serovar Typhi DNA Fragments which Exhibited Preferential Activation in the Environmental Conditions

Hybrid Plasmid	Alkaline Phosphatase			β-Galactosidase		
	25°C	L.o.m	Atm O_2	25°C	L.o.m	Atm O_2
pCG91	x-	x		x		
pCG77		x				
pCG81	X			(x)		x-
pCG88		x				x

L.o.m.: low osmolarity medium; Atm O_2: atmospheric O_2 tension
x: activation (enzymatic value higher than 100 E. U. and double in respect to the relating parameter and to the control value).
x-: low activation (enzymatic value under 100 E. U. but double in respect to the relating parameter and to the control value).
(x): increase of activity (increasing in enzymatic value but not double in respect to the relating parameter and to the control value).

Although functional characterization of several fragments showed that they possessed an efficient transcriptional activity, and some of these demonstrated homogeneous behaviour in respect to growth parameters, when further investigated by sequence analysis, it emerges that three clones contained sequences which did not correspond to known serovar Typhi promoter sequences; yet, it is clear that they were bacterial DNA fragments, encoding for the beta'-subunit of DNA-

directed RNA polymerase and other proteins, which are highly conserved in the genus salmonella. Although they are not promoters, the expression took place as if a suitable promoter was located upstream of the corresponding reporter gene. We intend to perform additional studies in order to fully understand the significance of our findings.

The versatility of *Salmonella enterica* serovar Typhi and moreover its ability to live in host cells are factors which are difficult to maintain in the genetically-engineered strains. It is necessary to understand and exploit these characteristics, so that the attenuated microorganism does not loose its capacity to penetrate the enteric cells and macrophage, in order to obtaine an important advantage for the oral administration of live attenuated bacterial vaccines.

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