Induction of the \textit{phoE} promoter upon invasion of \textit{Salmonella typhimurium} into eukaryotic cells

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Live attenuated \textit{Salmonella typhimurium} strains expressing foreign antigens can be used for vaccination purposes. Due to deleterious effects of constitutive, high-level expression of the heterologous antigens, there is often strong selection pressure against plasmids encoding these antigens, resulting in rapid segregation \textit{in vivo}. \textit{In vivo}-inducible promoters may be a good alternative for constitutive promoters. The outer membrane protein PhoE of \textit{Escherichia coli} is being used as a carrier for foreign antigenic determinants. Here we studied whether its expression from a plasmid is induced in \textit{S. typhimurium} upon invasion of eukaryotic cells. This appeared to be the case. Furthermore, a \textit{S. typhimurium phoE} mutant was constructed and the effects of the mutation on invasion, intracellular survival and virulence were studied. Survival in HEp-2 cells or in the macrophage-like cell line J744 was not, or only slightly, affected. Furthermore, the mutant appeared to be as virulent for mice as the wild-type strain.

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Key words: PhoE; \textit{Salmonella typhimurium}; \textit{in vivo} inducible promoter; intracellular survival; virulence.

Introduction

In order to gain insight in the factors that determine bacterial virulence, a lot of effort has been put into the identification of genes that are expressed after invasion into eukaryotic cells.\textsuperscript{1,2} Furthermore, it has been suggested that \textit{in vivo} inducible genes may contribute to virulence.\textsuperscript{3} Such genes have been identified using \textit{in vivo} expression technology\textsuperscript{1} or transposon mutagenesis.\textsuperscript{2}

It is anticipated that inside eukaryotic cells inorganic phosphate concentrations are very low since phosphate is usually bound in organic compounds. Bacteria like \textit{Escherichia coli} and \textit{Salmonella typhimurium} respond fiercely to phosphate starvation by the induction of the \textit{pho} regulon (reviewed in \textsuperscript{4}). One of these genes is the

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gene encoding the PhoE protein, which is pore-forming outer membrane protein. In the present study, we investigated whether the phoE promoter is induced after invasion, and whether PhoE expression is important for intracellular survival and for the pathogenesis of *S. typhimurium*. These studies are particularly interesting since we are also employing PhoE as a carrier for foreign antigenic determinants (reviewed in 7). The major objective of these studies is the development of live oral vaccines. For this purpose, high level and inducible expression of chimeric proteins by recombinant bacteria upon invasion of eukaryotic cells may be advantageous. When live bacteria that express foreign antigens or antigenic determinants are used for vaccination, features like antigen breakdown by the heterologous host and genetic instability of the recombinant strains, may occur. By using an expression system that is induced upon invasion, such problems may be circumvented.

**Results**

*phoE promoter activity after invasion*

To study whether the *phoE* promoter is induced upon invasion of *S. typhimurium* in eukaryotic cells, the virulent strain SL1344 was transformed either with plasmid pJP29, encoding *E. coli* PhoE, or with the vector pACYC184. Recombinant bacteria were used to infect HEp-2 cells and, after invasion, they were labelled with 35S-methionine. For efficient incorporation of 35S-methionine into bacterial proteins, protein synthesis of the host cells was inhibited with cycloheximide. After labelling, intracellular and extracellular bacteria were isolated and immunoprecipitations were performed using an antibody specific for *E. coli* PhoE trimers. Samples were analysed by SDS-PAGE and autoradiography. Bacteria grown extracellularly did not express PhoE (Fig. 1, lanes 2 and 4). As expected, also intracellular bacteria containing the

![Fig. 1. Autoradiogram of an SDS-PAGE gel on which the immunoprecipitated *E. coli* PhoE was analysed which is expressed after invasion of recombinant *S. typhimurium* in HEp-2 cells. As a control, *E. coli* strain CE1224 containing plasmid pJP29 was grown in low phosphate conditions, labelled for 1 h with 35S-methionine and radiolabelled PhoE was immunoprecipitated (lane 1). *S. typhimurium* strain SL1344 containing pJP29, encoding wild-type PhoE (lanes 2 and 3) or vector pACYC184 (lane 4 and 5) were used to invade HEp-2 cells. Intracellularly (lanes 3 and 5) and extracellularly (lanes 2 and 4) grown bacteria were labelled with 35S-methionine and PhoE was immunoprecipitated as described in Materials and methods.](image-url)
vector plasmid pACYC184 did not express *E. coli* PhoE (Fig. 1, lane 5). In contrast, intracellular bacteria containing plasmid pJP29 did express *E. coli* PhoE after invasion (Fig. 1, lane 3). Similar results were obtained after invasion of Epstein Bar virus-transformed B-cells (data not shown). To exclude a possible effect of cycloheximide on intracellular PhoE expression, similar experiments were performed in the absence of this drug. Also in these experiments, PhoE was expressed after invasion. However, as expected, labelling was less efficient (data not shown). In conclusion, the *E. coli* phoE promoter is active in the bacteria upon invasion of eukaryotic cells and this is consistent with the idea that there is inorganic phosphate limitation inside these cells.

**Construction of an S. typhimurium phoE mutant strain**

Both in *E. coli* and in *S. typhimurium* PhoE is expressed under phosphate starving conditions and the two promoters are very similar. Since the *E. coli* phoE promoter is active after invasion of pJP29-containing *S. typhimurium* into eukaryotic cells, the *S. typhimurium* phoE promoter is most probably also induced. Therefore, a possible role for PhoE in virulence was considered. To study whether expression of PhoE after invasion is of importance for the intracellular survival of *S. typhimurium*, the chromosomal phoE gene of strain SL1344 was inactivated by gene replacement as described in Materials and methods. Correct gene replacement was verified by Southern blotting (results not shown). A recombinant that showed the correct DNA hybridization pattern on a Southern blot was designated CE1432 and was grown in phosphate-starving conditions. Protein patterns of peptidoglycan-associated proteins were analysed by SDS-PAGE. In contrast to the parental strain (Fig. 2A, lane 3), the phoE mutant did not express PhoE when grown under phosphate limitation (Fig. 2A, lane 5). This result was verified by Western immunoblotting, using a monoclonal antibody that was raised against the denatured form of *E. coli* PhoE and that cross-reacts with *S. typhimurium* PhoE (Fig. 2, panel B).

**Phenotype of the phoE mutant**

To establish possible deleterious effects of the phoE mutation on growth in laboratory conditions, wild-type strain SL1344 and the phoE mutant CE1432 were grown on LB-medium, on phosphate limiting medium and on a synthetic medium, to which 0.25 mM ATP was added as the sole phosphate source. When the growth curves were

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Fig. 2. SDS-PAGE gel (panel A) and Western immunoblot (panel B) of peptidoglycan-associated proteins of *S. typhimurium* strains SL1344 and its phoE mutant derivative CE1432, grown on high phosphate- (lanes 2 and 4) or on low phosphate- (lanes 3 and 5) media. As a control, cell envelopes were isolated of *E. coli* strain CE1224 containing plasmid pJP29, encoding *E. coli* PhoE, which was grown in low phosphate medium (lane 1). The two protein bands that cross-react in the blot with the monoclonal antibody used are the structurally related OmpF and OmpC proteins.
followed by optical density measurements, no differences between the two strains were seen on either of these media (data not shown). Apparently, like in *E. coli*, PhoE expression is not essential for growth in phosphate-starving conditions.

To investigate whether PhoE expression is necessary for intracellular survival, an invasion and replication assay was performed in HEp-2 cells. It appeared that the intracellular survival of the PhoE mutant was not affected (data not shown). *S. typhimurium* is known to invade the gut via M-cells and is subsequently thought to enter and survive inside macrophages. Therefore, survival of the wild-type and the phoE mutant strain was also studied in the mouse macrophage cell line J744. The initial invasion of the mutant appeared to be more efficient than that of the wild-type strain. Three hours after the initiation of invasion, 70 ± 43 and 500 ± 113 colony-forming units of intracellular wild-type or mutant bacteria, respectively, were obtained from 10^5 cells under the experimental conditions employed. However, whereas the amount of intracellular wild-type bacteria increased after the initial invasion due to replication, the amount of intracellular mutant bacteria appeared to decrease (Fig. 3), although approximately 25% were still viable after 48 h. Therefore, it seems that the capability to survive inside this macrophage cell-line is somewhat affected by the phoE mutation.

To assess whether the somewhat reduced capability of the phoE mutant strain to survive in macrophages has consequences for the virulence of the strain, groups of three mice were infected with either the wild-type or the phoE mutant strain. Two different doses (5 × 10^6 and 5 × 10^7) of the bacteria were administered intragastrically. As a control, a group of six mice received 10^8 bacteria of strain SL3261, an *aroA* derivative of SL1344. There was no difference in virulence between the wild-type strain and the phoE mutant strain (Table 1). Mice died between 5 and 10 days after infection. In contrast, mice that were infected with the well-defined
Table 1 Infection of mice with SL1344 and its phoE derivative CE1432

<table>
<thead>
<tr>
<th>Strain</th>
<th>Number of bacteria</th>
<th>Death rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL1344</td>
<td>$5 \times 10^6$</td>
<td>2/3</td>
</tr>
<tr>
<td>SL1344</td>
<td>$5 \times 10^7$</td>
<td>3/3</td>
</tr>
<tr>
<td>CE1432 (phoE)</td>
<td>$5 \times 10^6$</td>
<td>3/3</td>
</tr>
<tr>
<td>CE1432 (phoE)</td>
<td>$5 \times 10^7$</td>
<td>3/3</td>
</tr>
<tr>
<td>SL3261 (aroA)</td>
<td>$10^9$</td>
<td>0/6</td>
</tr>
</tbody>
</table>

*Groups of three 8-week-old female Balb/c mice each were immunized orally with a suspension of bacteria in PBS. Mice were followed for 3 weeks. Mice died between 5 and 10 days after immunization.

attenuated strain SL3261 all survived the infection with $10^9$ bacteria. Hence, PhoE expression does not seem to be essential for virulence of SL1344.

Discussion and conclusions

The phoE promoter is normally induced when bacteria are grown in phosphate-starving conditions, and low inorganic phosphate concentration is the actual trigger for its activation. In the present study, we show that the E. coli phoE promoter is induced when S. typhimurium invades eukaryotic cells, indicating that the phoE promoter is an in vivo-inducible promoter. Therefore, it can also be concluded that there is phosphate limitation in the phagocytic compartment in eukaryotic cells, where S. typhimurium resides after invasion. 12

Similar to in E. coli, deletion of the phoE gene in S. typhimurium did not result in less efficient growth in low phosphate conditions, indicating that PhoE expression is not essential for growth. Probably, other pore-forming proteins can compensate for the loss of PhoE function. Of course, it remains very well possible that the phoE mutant strain is less competitive when grown together with the wild-type strain on specific phosphate sources. Also for survival inside eukaryotic cells, PhoE expression is not essential although somewhat less efficient growth inside mouse macrophages (but not in HEp-2 cells) was observed for the mutant. However, deletion of the phoE gene did not result in attenuation of SL1344, indicating that PhoE is not a virulence factor. Rowbury et al. 13 described that in E. coli, phoE is necessary for acid habituation in vitro. This observation suggested that deletion of phoE might result in decreased intracellular survival. However, the phoE mutant was only slightly affected in survival in macrophages. It has been suggested that in vivo inducible genes may contribute to virulence and a lot of effort has been put into the identification of genes that are expressed by Salmonella upon invasion. 1, 2 In this study, we have shown that a gene that is induced upon invasion is not necessarily essential for virulence. The phoE gene is expressed as a part of the pho regulon. 5 Since we observed that the phoE gene is expressed after invasion, it is very likely that the entire pho regulon is expressed under these conditions. Therefore, it remains to be determined whether the products of other pho genes are important for virulence, e.g. by inactivation of the phoB gene, which encodes the transcriptional activator of the regulon.

Attenuated Salmonella strains are good candidates for the delivery of foreign antigens to the immune system. 14–16 High-level expression of foreign or chimeric proteins can easily be accomplished in vitro. However, when recombinant bacteria are used for vaccination studies in vivo, high-level expression of foreign antigens...
can result in rapid plasmid segregation. Different approaches have been used to obtain stable expression of foreign antigens \textit{in vivo}. For instance, Nakayama \textit{et al.}\textsuperscript{17} and Curtiss \textit{et al.}\textsuperscript{18} described a system to prevent plasmid segregation by inclusion of a gene on the plasmid that is essential for survival inside eukaryotic cells. This approach resulted in stable expression of the foreign antigens. However, when such foreign antigens are expressed constitutively, there is always the possibility that, due to deleterious effects of the expressed foreign antigen, mutations will occur that decrease the expression level. On the other hand, when the relevant gene is integrated in the chromosome, expression levels can drop so drastically due to the presence of only one single copy of the gene, that adequate immune responses are not anticipated.\textsuperscript{19,20} Tijhaar \textit{et al.}\textsuperscript{21} described an invertible promoter segment that allows stable expression of foreign antigens in \textit{Salmonella}. Such an invertible promoter segment can either be present in the wrong orientation or it can invert and drive expression of the gene under its control, resulting in expression of the foreign antigen in part of the population. Consequently, there will be no selection against the presence of the plasmid in that part of the population that does not express the foreign antigen. Theoretically, this would imply that even lethal expression levels would not lead to plasmid segregation in the total population. In this system, however, there may be selection against the situation in which the promoter drives expression of the foreign antigen. Therefore, the use of \textit{in vivo}-inducible promoters instead of constitutive promoters, seems to be a good alternative.

The \textit{nirB} promoter, which is induced in anaerobic conditions, has been shown to be suitable to obtain stable expression of foreign antigens in \textit{Salmonella}.\textsuperscript{22} From the data presented here, it is feasible that stable expression of foreign antigens can be obtained \textit{in vivo}, using the \textit{phoE} promoter. Moreover, by genetic manipulation short amino acid sequences can be inserted in outer membrane protein PhoE,\textsuperscript{7} which can be expressed at high levels in \textit{S. typhimurium}.\textsuperscript{23} This approach circumvents the problem of proteolytic antigen degradation, which often occurs when complete foreign antigens are expressed in bacteria. All these observations indicate that attenuated \textit{Salmonella} strains expressing chimeric PhoE proteins are ideal candidates for the delivery of foreign antigenic determinants to the immune system.

\section*{Materials and methods}

\textbf{Bacterial strains, plasmids and growth conditions.} The virulent \textit{S. typhimurium} strain SL1344\textsuperscript{24} and its \textit{aroA} derivative SL3261\textsuperscript{24} were kindly provided by B. A. D. Stocker (Department of Medical Microbiology, Stanford University, CA, U.S.A.). \textit{E. coli} strain CE1224\textsuperscript{25} does not produce pore proteins due to mutations in the \textit{phoE} and \textit{ompR} genes. Plasmids pJP2926 and pST3\textsuperscript{26}, encode the \textit{E. coli} and \textit{S. typhimurium} PhoE, respectively. pACYC184\textsuperscript{27} is a cloning vector and was also used for the isolation of the DNA fragment encoding a tetracycline resistance gene. Bacteria were grown at 37°C under aeration in Luria-Bertani medium, in phosphate-limiting medium\textsuperscript{5} or in a synthetic medium in which the phosphate concentration can be varied.\textsuperscript{5} When necessary tetracycline (20 \(\mu\)g/ml) or chloramphenicol (25 \(\mu\)g/ml) was added.

\textbf{Cell cultures.} All tissue culture reagents were obtained from GIBCO laboratories (Grand Island, NY, U.S.A.). Cells were grown in RPMI-1640 or in DMEM, supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 IU/ml) and streptomycin (100 \(\mu\)g/ml) at 37°C in a CO\textsubscript{2}-incubator. The human larynx epithelial cell line HEP-2 (ATCC CCL 23) was obtained from the American Type Culture collection (Rockville, MD, U.S.A.). The Epstein Bar virus-transformed B-cell line JY has been described.\textsuperscript{28} J774 cells, a continuous macrophage-like cell line derived from a reticulum cell sarcoma, has been described by Ralph \textit{et al.}\textsuperscript{30} For invasion assays, \(2 \times 10^5\) HEP-2 cells were seeded in 6-well dishes and incubated to semiconfluent
monolayers (approximately $5 \times 10^5$ cells/well). Prior to the bacterial invasion assay, the cell lines were incubated overnight in culture medium without antibiotics.

**Invasion and intracellular replication assay.** An invasion assay was performed as described previously. Briefly, cells were infected with suspensions of logarithmically growing bacteria, at a nominal multiplicity of infection (MOI) of 10. Adhesion and subsequent invasion of bacteria was allowed to take place for 30 min at 37°C. Extracellular bacteria were killed by incubation in RPMI-1640 supplemented with 10% fetal calf serum and 200 µg/ml gentamycin for 1 h at 37°C in a CO₂ incubator. Cells were maintained in RPMI-1640 supplemented with 10% fetal calf serum and 20 µg/ml gentamycin to prevent reinfection. At various time points after invasion, cells were lysed by incubation with PBS containing 1% (w/v) saponin (1 ml/sample) for 5 min at room temperature to release intracellular bacteria. To determine the number of intracellular bacteria, suspensions were plated on LB-plates. To monitor intracellular bacterial protein synthesis, cells were incubated 1.5 h after invasion for 30 min in cycloheximide (10 µg/ml) to inhibit protein synthesis of the host cells. Subsequently, bacterial proteins were labelled for 2 h with $^{35}$S-methionine. Extracellular bacteria grown in the absence of cells in RPMI supplemented with 10% fetal calf serum, were also labelled for 2 h with $^{35}$S-methionine.

**Immunoprecipitations.** For immunoprecipitations, bacteria were lysed by rapid freezing in liquid nitrogen and incubated for 30 min in 0.5 M NaCl, 10 mM EDTA. Subsequently, Triton-buffer was added (final concentration 2% Triton X-100, 50 mM Tris/HCl pH 8.0 and 0.1% BSA). Suspensions were centrifuged for 10 min and a monoclonal antibody, 5B7 (kindly provided by M. Kleerebezem), that recognizes *E. coli* PhoE trimers was added to the supernatants. After overnight incubation at 4°C, 2.5 mg of protein A-sepharose CL-4B (Pharmacia, Uppsala, Sweden) was added, and the mixture was incubated at room temperature under gentle shaking. Sepharose pellets were washed three times in Triton-buffer. Samples were analysed by SDS-PAGE and autoradiography.

**DNA manipulations and gene replacement.** Standard DNA manipulations were performed according to Maniatis *et al.* Restriction enzymes and other DNA modifying enzymes were purchased from Pharmacia (Uppsala, Sweden). The *S. typhimurium* phoE gene was excised from pST360 with *ClaI* and *BglII* and subcloned into pACYC184, digested with *ClaI* and *BamHI*. The resulting plasmid, pRM44 (Fig. 4), contains a unique *EcoRV* site in the *phoE* gene. This *EcoRV* site was used for the insertion of a tetracycline resistance box. This box was isolated from pACYC184 by digestion with *XbaI* and *AvaI*. Protruding ends were filled-in with the

![Fig. 4. Restriction map of plasmid pRM44. Fat lines represent DNA from the vector pACYC184 and the thin line chromosome-derived *S. typhimurium* DNA. In pRM45, a 1500 bp DNA fragment from pACYC184, containing a tetracycline-resistance marker is inserted in the *EcoRV* site of pRM44. The positions of the *phoE* gene, the chloramphenicol-resistance marker (Cm) and the tetracycline resistance marker (Tc) are indicated by arrows.](image)
Klenow fragment of DNA polymerase and the tetracycline resistance box was ligated into EcoRV-digested pRM44. The resulting plasmid, pRM45, was first modified by passage through SL3261 in order to increase the electroporation efficiency. The BstEII fragment (Fig. 4), containing the inactivated phoE gene and the flanking DNA sequence was isolated from a low melting point agarose gel using β-agarase (New England Biolabs, Schwalbach, Germany). The linear fragment, which does not contain the origin of replication, was ligated in order to obtain circular DNA and concatemeres to improve the efficiency of homologous recombination. The ligated DNA was used to transform SL1344 via standard electroporation using a Biorad gene pulser, and recombinants were selected on tetracycline. Tetracycline-resistant recombinants were tested for chloramphenicol-sensitivity to identify and discard clones that contained the intact plasmid. Among the chloramphenicol-sensitive clones, correct homologous recombination was verified by Southern blotting. Chromosomal DNA was isolated and digested with PstI and Clal. DNA fragments were separated on a 0.6% agarose gel and transferred to nylon membranes using a vacuum blotter (Bio-Rad, Hercules, U.S.A.) according to the manufacturers' instructions. As a probe, the EcoRV-PstI fragment of pRM44 (Fig. 4) was used. Labelling of the probe, hybridization and detection were performed with digoxigenin labelling and detection kits (Boehringer-Mannheim, Germany) according to the manufacturers' instructions.

**Characterization of cell fractions.** Protein patterns of peptidoglycan-associated proteins of recombinants were analysed on SDS-PAGE and Western immunoblotting. Peptidoglycan-associated proteins were isolated after ultrasonic disintegration of cells. Cell envelopes were pelleted and incubated for 30 min at 50°C in a buffer containing 2% SDS. Peptidoglycan-associated proteins were pelleted by ultracentrifugation. A monoclonal antibody, mE138 raised against E. coli PhoE monomers, that cross-reacts with S. typhimurium PhoE, was used for detection.

**Infection of mice.** Female Balb/c mice of 8 weeks were immunized orally with $5 \times 10^8$, $5 \times 10^7$ or $10^9$ bacteria in 0.5 ml of PBS, and death rates were determined.

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