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Immunogenicity against Human Papillomavirus Type 16 Virus-Like Particles Is Strongly Enhanced by the PhoP^c Phenotype in *Salmonella enterica* Serovar Typhimurium

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Recombinant *Salmonella* strains have been widely used to deliver heterologous antigens and induce immune responses in vaccinated animals and humans. It remains to be established, however, how these bacteria mount an immune response; this has prevented the rational design of vaccines. Here we report for the first time that a particular genetic program, PhoP^c, is necessary for recombinant *Salmonella* strains to induce an antibody response to a heterologous antigen, the human papillomaviruses type 16 (HPV16) virus-like particle (VLP). The PhoP^c phenotype results from a point mutation in *phoQ*, the gene encoding the sensor component of a two-component regulatory system (PhoP-PhoQ) that controls the expression of a number of virulence factors in Salmonellae. To demonstrate that immunogenicity of the viral antigen expressed by the bacterial vector was dependent on the PhoP^c phenotype, we have expressed the *phoQ* mutant gene (*phoQ24*) in two differently attenuated *Salmonella enterica* serovar Typhimurium strains. Our data show extrachromosomal *phoQ24* to be dominant over the chromosomal copy of the *phoQ* gene, conferring the PhoP^c phenotype on the recipient strains. In addition, activation of PhoPQ-regulated genes by the plasmid-encoded PhoQ24 did not alter bacterial survival and conferred immunogenicity to the HPV16 VLP expressed in the two *S. enterica* serovar Typhimurium backgrounds, inducing the production of HPV-specific antibodies in mice. This strongly suggests that at least one of the PhoP-regulated genes is necessary for mounting an efficient antibody response to HPV16 VLP. This finding sets the stage for further development of a *Salmonella*-based vaccine against HPV infection and cervical cancer.

The 'high risk' human papillomavirus (HPV) types, most commonly type 16 (HPV16), are etiologically linked to nearly 100% of cervical cancers (49). Cervical cancer is the second most common cause of cancer deaths in women worldwide; this prevalence has encouraged research into the development of a prophylactic vaccine to prevent genital infection by these viruses. Recombinant attenuated *Salmonella* strains that are attenuated yet invasive have been widely used as mucosal vaccine vectors to deliver pathogen-specific protective epitopes into the mucosa-associated lymphoid tissues. Via this route, both mucosal and systemic immune responses to the carrier and the foreign antigens may be obtained (8, 41). We have shown that nasal vaccination of mice with a *Salmonella enterica* serovar Typhimurium PhoP^c strain expressing the HPV16 major capsid protein L1, which self-assembles into virus-like particles (VLPs), induces anti-HPV16 conformational and neutralizing antibodies in serum and genital secretions (33). The PhoP^c strain is attenuated by a single point mutation in *phoQ* (14) (designated *phoQ24*), the gene encoding the sensor component of a two-component system (*phoPQ*) that is involved in the regulation of virulence in *S. enterica* serovar Typhimurium (12, 28, 45). Mutations in the *phoPQ* operon affect the expression of two sets of genes, the PhoP-activated genes (*pag*) and the PhoP-repressed genes (*prg*). In the *phoQ24* background, *pag* genes are permanently activated whereas *prg* genes remain inactive due to constitutive activation of the PhoP regulator protein (PhoP^c). This results in reduced survival of *S. enterica* serovar Typhimurium PhoP^c within macrophages (11, 13, 29), impaired invasion of epithelial cells (4, 36), and altered resistance to antimicrobial compounds and conditions such as defensins, polymyxin B (PMB) and low pH (14, 40, 46). A comparison of differently attenuated but otherwise isogenic recombinant *S. enterica* serovar Typhimurium strains revealed that only the PhoP^c HPV16 strain induced HPV16 VLP-specific antibody responses in mice (5). This suggested that the immunogenicity of recombinant *Salmonella* HPV16 strains was closely related to the PhoP^c phenotype. Unfortunately, the PhoP^c strain cannot be used in humans because of the reported high frequency of reversion of its attenuation (29). We therefore set out to construct recombinants in which both the PhoP^c phenotype and the HPV16 VLP antigen were stably expressed in otherwise safely attenuated *S. enterica* serovar Typhimurium recipient strains (Δ*cya*, Δ*crp*, and Δ*aro*). This would allow us to confirm the implication of *phoQ24* in immunogenicity of the *Salmonella* vector and take the first step toward the construction of a safe *Salmonella* HPV vaccine. We have analyzed the behavior of these new recombinant strains in vitro and in vivo and confirmed the correlation between the expression of the PhoP^c phenotype and immunogenicity against HPV16 VLPs expressed in *S. enterica* serovar Typhimurium.

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TABLE 1. *Salmonella* strains used in this study

Strains (attenuation)	Plasmid electroporated	Abbreviation	Reference
ATTC14028		ATTC14028	28
CS015 (PhoP$^-$, Δ*phoPQ*)		PhoP$^-$	28
CS022 (PhoP^c, *pho-24*)		PhoP^c	29
GL01 (PhoP^c Δ*asd*)		GL01	37
GL01 (PhoP^c Δ*asd*)	pYA3342-HPV16L1	GL01 L1	37
GL01 (PhoP^c Δ*asd*)	pYA3342-HPV16L1-SDPhoQ24	GL01 L1-SDQ24	This work
χ4550 (Δ*cya* Δ*crp* Δ*asd*)		χ4550	43
χ4550 (Δ*cya* Δ*crp* Δ*asd*)	pYA3342-HPV16L1	χ4550 L1	This work
χ4550 (Δ*cya* Δ*crp* Δ*asd*)	pYA3342-HPV16L1-SDPhoQ24	χ4550 L1-SDQ24	This work
χ4550 (Δ*cya* Δ*crp* Δ*asd*)	pYA3342-HPV16L1-PhoPQ24	χ4550 L1-PQ24	This work
SL7207 (Δ*aroA*)			22
GL04 (Δ*aroA* Δ*asd*)		GL04	This work
GL04 (Δ*aroA* Δ*asd*)	pYA3342-HPV16L1	GL04 L1	This work
GL04 (Δ*aroA* Δ*asd*)	pYA3342-HPV16L1-SDPhoQ24	GL04 L1-SDQ24	This work
GL04 (Δ*aroA* Δ*asd*)	pYA3342-HPV16L1-PhoPQ24	GL04 L1-PQ24	This work

MATERIALS AND METHODS

Plasmid constructs and bacterial strains used. We have deleted the *asd* gene from both the PhoP^c mutant, CS022 (29) (a kind gift from John Mekalanos, Boston, Mass.), and the *aroA* mutant, SL7207 (22) (a kind gift from Irene Corthésy-Theulaz, Lausanne, Switzerland), by P22HTint transduction, yielding Δ*asd* derivatives denoted GL01 (37) and GL04, respectively. Diaminopimelic acid-requiring tetracycline-resistant transductants (42) were purified, and tetracycline-sensitive derivatives were obtained from these transductants by fusaric acid selection (27, 42). Stable expression of HPV16 L1 and PhoQ24 was achieved in these backgrounds with the aspartate β-semialdehyde dehydrogenase balanced-lethal vector-host system (9). To this end, the *Nco*I-*Hin*dIII fragment, encoding HPV16 L1, of plasmid pFS14nsdHPV16-L1 (33) was inserted downstream from the trc promoter into the *Nco*I and *Hin*dIII sites of a medium-copy-number *asd*- plasmid (pYA3342). In the resulting plasmid, pYA3342HPV16L1, either an *Xba*I fragment carrying *phoPQ24* or an *Hin*dIII fragment carrying the *phoQ24* open reading frame, including a Shine-Dalgarno (SD) sequence, was inserted. These fragments were generated by PCR performed on the DNA of *S. enterica* serovar Typhimurium strain CS022 as a template. For *phoPQ24*, the following primers were used: a 26-mer located 144 nucleotides upstream from the ATG of *phoP* (45) and containing a *Xba*I site (underlined), 5′-GGGTCTAGACTGGTCGACGAACTTAA-3′, and a 66-mer containing another *Xba*I site (underlined) and a t2 terminator (in italics), 5′-GGGTCTAGA*AAAAGGCCATCCGTCAGGATGGCCTTCT*ATGTTAAGTATCCGCAGGCTGGTATCTG A-3′. For the *Hin*dIII *SDphoQ24* fragment, the primers used were as follows: a 40-mer containing a synthetic SD sequence (in italics), 5′-GGGAAGCTTG*AGGA*AAAGCTA**ATG**AATAAATTTGCTCGCC-3′, and a 25-mer including a *Hin*dIII site (underlined), 5′-GGGAAGCTTGA**AAT**GTTTATTCCTC-3′. The initiation and stop codons are indicated in bold type. The PCR-amplified *Xba*I *phoPQ24* or *Hin*dIII *SDphoQ24* fragments were cloned in the *Xba*I or *Hin*dIII sites of the pYA3342HPV16L1 plasmid, yielding plasmids pYA3342HPV16L1-PhoPQ24 and pYA3342HPV16L1-SDPhoQ24, respectively. The different plasmid DNAs were introduced into the attenuated *S. enterica* serovar Typhimurium strains χ^{4550} (Δ*cya* Δ*crp* Δ*asd* [43]), GL04, and GL01 by electroporation as previously described (44). Strains ATCC14028 and CS015 (PhoP$^-$ [28]) were a kind gift from John Mekalanos. Table 1 summarizes the different strains and abbreviations used in this study.

Analysis of *pagN* promoter activity. A *pagN*::*lac*Z indicator plasmid was first generated. The promoter region of the *pagN* gene was cloned and identified by cleavage of a PCR fragment containing the *S. enterica* serovar Typhimurium ATCC 14028 *pagN* coding sequence (15) and upstream sequences with the enzyme *Ssp*I. The resulting 310-bp fragment was inserted into the *Sma*I site of the transposon Tn *3*-based vector pGMK1337 (25), enabling activation of β-galactosidase expression. The plasmid pGMK1338, bearing a 310-bp *Ssp*I *pagN* promoter fragment, and a control plasmid, pGMK1339, bearing an unregulated weak promoter sequence located upstream of the *pagN* gene, were constructed. To facilitate the analyses, the transposable sequences were integrated into stringently replicating plasmid R751, yielding R751::TnP *pagN*::*fol*A::*lac*Z or R751::TnP::*fol*A::*lac*Z. These plasmids were introduced into the recombinant *Salmonella* strains by conjugation. The *Salmonella* exconjugants were selected for resistance to ampicillin (conferred by the conjugative plasmid) and growth on minimal medium. Specific positive agglutination with *Salmonella* O antiserum group B (Difco) was used to confirm the identity of the exconjugants. β-Galactosidase activity assays were performed as described by Miller (30). Portions (100 μl) of the bacterial cultures were mixed with 900 μl of Z-buffer (0.06 M $Na_2HPO_4 \cdot 7H_2O$, 0.04 M $NaH_2PO_4 \cdot H_2O$, 0.01 M KCl, 0.001 M $MgSO_4 \cdot 7H_2O$, 0.05 M β-mercaptoethanol [pH 7]) containing 0.1% sodium dodecyl sulfate (SDS) and 1% chloroform. A 200-μl volum of *o*-nitrophenyl-β-D-galactoside (ONPG, 4 mg/ml in Z-buffer [Sigma]) was then added as substrate, and the optical density (OD) was recorded at both 420 and 550 nm. The light-scattering correction factor ($CF = OD_{420}/OD_{550}$) was first determined for each *Salmonella* strain in Luria-Bertani (LB) broth and then the β-galactosidase activity was calculated using Miller's formula: Ui β-Galactosidase = $1{,}000OD_{420} - (CF \times OD_{550})/t \times v \times OD_{600}$, where t is time of the reaction in minutes and v is the volume of the culture used in the assay in milliliters.

PMB resistance assay. PMB resistance was assessed by using a method modified from those of Gunn et al. (14) and Roland et al (40). Briefly, bacterial strains were grown to mid-log phase and diluted to a concentration of approximately 10^4 CFU/ml in tryptone saline. Cells (100 μl/well) were mixed in a microtiter plate with various concentrations of PMB (Sigma) and incubated at 37°C for 1 h. Then 100 μl of PMB-treated cells was plated on LB agar medium and the relative strain resistances (compared with untreated cells) were determined by CFU counting. The PMB resistance best-fitting sigmoid curves were drawn, and 50% inhibitory concentration were calculated with GraphPad Prism.

Nonspecific acid phosphatase (PhoN) activity assay. PhoN activity was assessed using a method modified from that of Kier et al. (24). Briefly, *Salmonella* strains were grown overnight, diluted in LB broth at 1:100, and grown to an OD_{600} of 0.6. The bacteria were pelleted and resuspended in 1 ml of 1 M Tris (pH 8.0). The cells were mixed with 200 μl of 0.4% *p*-nitrophenyl phosphate (Sigma) in 1 M Tris (pH 8) and incubated at 37°C until a yellow color appeared; the reaction was then stopped by adding 200 μl of 1 M K_2HPO_4. The PhoN activity was measured in arbitrary units at $OD_{420/550}$ by using the same equation used for the β-galactosidase activity.

Protein analysis. Recombinant *Salmonella* cells from an exponential-phase culture were lysed in 2.5% SDS. After 30 min at room temperature, the bacteria were boiled for 10 min in a final solution of 3% SDS–2 mM EDTA–50 mM Tris-HCl (pH 7.0)–8% glycerol–1% β-mercaptoethanol–0.1% bromophenol blue. Bacterial lysates were separated on SDS–10% polyacrylamide gels. Expression of L1 in the *Salmonella* lysates, normalized to the OD_{600} of the cultures, was analyzed by Western blotting as previously described (33), using the anti-HPV16 L1 monoclonal antibody, CAMVIR-1 (Anawa).

Immunization of mice, analysis of the immune response, and recovery of *S. enterica* serovar Typhimurium. Six-week-old female BALB/c mice were used in all experiments. A 20-μl volume of bacterial inoculum was administered intranasally under anesthesia as described previously (23, 33). For the inoculum, bacteria were grown to mid-log phase and diluted to ca. 10^7 CFU/20 μl. Sampling of blood, as well as determination of anti-lipopolysaccharide (LPS) and anti-HPV16 VLP titers by enzyme-linked immunosorbent assay were performed as previously described (23, 33). A good correlation was shown between HPV16 VLP-specific antibody titers and HPV16 neutralization (33, 34, 39). Recovery of *S. enterica* serovar Typhimurium was determined in organs from euthanized mice as previously described (33).

TABLE 2. PhoN activity in *Salmonella* strains

Strain	PhoN activity (arbitrary units)[a]
PhoP^c	552±57
PhoP$^-$	9±1
ATCC14028	33±2
χ4550 L1	31±1
χ4550 L1-SDQ24	22±2
χ4550 L1-PQ24	146±15
GL04 L1	27±1
GL04 L1-SDQ24	25±2
GL04 L1-PQ24	214±14

[a] Results are given as mean ± standard error of the mean.

Statistics. Comparisons of the different data were made by one-way analysis of variance and a Bonferoni post-test using GraphPad Prism.

RESULTS

Extrachromosomal expression of *phoPQ24* activates the *pag* gene *phoN* in both χ4550 and GL04. The mutant *phoQ* gene, *phoQ24*, was inserted into plasmid pYA3342-HPV16L1, already carrying the HPV16 L1 gene and the *asd* gene but no antibiotic resistance marker. The two plasmids constructed encode either the complete *phoPQm* operon, including the promoter-operator, or only the *phoQ24* coding sequence, including an SD sequence. Both constructs were introduced into the attenuated *S. enterica* serovar Typhimurium strains, χ4550 (Δ*cya* Δ*crp* Δ*asd* [43]) and GL04 (Δ*aroA* Δ*asd* [Table 1]). In the resulting *S. enterica* serovar Typhimurium recombinants, plasmid-located *phoQ24* is expected to be dominant over the chromosomal *phoQ* and thus to induce the PhoP^c phenotype. The phenotypes of strains bearing plasmid-located *phoQ24* and those in which the full *phoPQ24* operon was present were anticipated to be different. In the former, the global regulatory protein PhoP will be mostly in the activated state due to unregulated phosphorylation by PhoQ24, whereas in the latter, overphosphorylation of PhoP is expected to be accompanied by higher expression of the *phoPQ24* operon as a result of a positive autoregulatory loop (45). However, in both strains, the expression of the *pag* and *prg* genes is expected to be deregulated in comparison to that in strains harboring the wild-type *phoQ*.

One of the aspects of the complex PhoP^c phenotype is its high phosphatase activity, which helps the identification of this strain on solid media as dark blue colonies when 5-bromo-4-chloro-3-indolylphosphate (BCIP) is added to the medium (29). Indeed, both strains χ4550 and GL04 carrying the plasmid containing *phoPQ24* generated dark blue colonies on BCIP-containing plates. To our surprise, however, we could not distinguish χ4550 and GLO4 recombinants, harboring the plasmid containing *phoQ24* from those bearing pYA3342-HPV16L1 as controls, since they all generated light blue colonies on BCIP-containing plates. Determination of the non-acid phosphatase PhoN activities (24) in all these strains further confirmed these observations (Table 2). PhoN activities were similar in the three strains harboring a wild-type *phoPQ* operon (33U, 31U, and 27U in ATCC14028, χ4550 L1, and GL04 L1, respectively) and plasmid-located *phoQ24* (22U and 25U in χ4550 L1-SDQm and GL04 L1-SDQm, respectively).

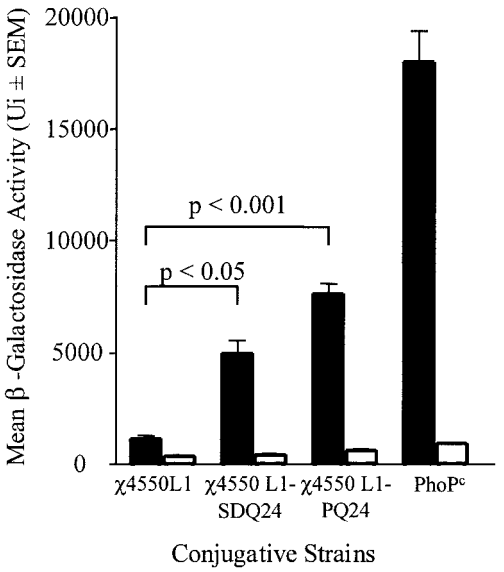

FIG. 1. Extrachromosomal expression of PhoQ24 is able to activate the *pagN* promoter. β-Galactosidase activity was measured in the different conjugative strains, as indicated in Materials and Methods. Mean β-galactosidase activities in three independent experiment are shown for each conjugative strain. Black and white bars indicate strains containing plasmids encoding β-galactosidase driven by the *pagN* promoter and the control promoter, respectively. SEM, standard error of the mean.

In contrast, PhoN activity was 5- and 8-fold higher in χ4550L1-PQm and GL04L1-PQm ($P < 0.05$ for 146U and $P < 0.001$ for 214U), while even higher activation of PhoN (17-fold [$p < 0.001$]) was observed in the PhoP^c strain compared to the wild-type strain. These data show that extrachromosomal expression of *phoPQ24*, but not *phoQ24* activates *phoN*.

Activation of the *pagN* promoter in different *phoQ24* backgrounds. An indirect functional analysis of *phoQ24* was performed by introduction of a plasmid (*PpagN-lacZ*) in which *lacZ* is expressed under the control of the *phoPQ*-regulated *pagN* promoter (15). A PhoP^c strain bearing the *PpagN-folA*::*lacZ* plasmid exhibited 20-fold-higher β-galactosidase activity than did the same strain bearing a plasmid constitutively expressing low levels of LacZ (control-*lacZ*) (Fig. 1); β-galactosidase activities in PhoP$^-$ recombinants invariably remained low (data not shown). When the same plasmid was introduced into χ4550L1-SDQ24 or χ4550L1-PQ24, β-galactosidase activities were 12- and 13-fold higher, respectively, than in the control strains that expressed *lacZ* from a *phoPQ*-independent promoter. Most importantly, a significant increase (five- to sevenfold [fig. 1]) in the β-galactosidase activity could be clearly attributed to *phoQ24* expression. Interestingly, and in contrast to what we had observed in the regulation of *phoN*, activation of *pagN* was similar in χ4550 L1-SDQ24 and χ4550 L1-PQ24, suggesting that different threshold levels of the activated PhoP are necessary to turn on the expression of different *pag* genes.

Expression of *phoQ24* or *phoPQ24* in *S. enterica* serovar Typhimurium χ4550 and GL04 confers resistance to PMB. We next examined whether extrachromosomal expression of *phoQ24* or *phoPQ24* could influence the expression of another

TABLE 3. PMB resistance of *Salmonella* strains

Strain	PMB IC_{50} (μg/ml) (95% CI)[a]
PhoP^c	7.39(6.29–8.68)
PhoP$^-$	0.17(0.09–0.32)
ATCC 14028	1.03(0.98–1.08)
χ4550 L1	0.58(0.48–0.70)
χ4550 L1-SDQ24	4.42(3.51–5.57)
χ4550 L1-PQ24	10.16(8.08–12.77)
GL04 L1	0.52(0.48–0.56)
GL04 L1-SDQ24	2.27(1.77–2.92)
GL04 L1-PQ24	3.23(2.85–3.66)

[a] IC_{50}, 50% inhibitory concentration; CI, confidence interval.

endogenous PhoPQ-regulated gene. The *pmrAB* locus, a *pag* gene responsible for the PMB resistance (14), was chosen for this assay. The 50% inhibitory concentrations of PMB in different *S. enterica* serovar Typhimurium strains were determined (Table 3). As expected, the PhoP^c strain exhibited a significant increase of PMB resistance (sevenfold) compared to the wild-type ATCC 14028 ($P < 0.001$) whereas the PhoP$^-$ strain was more sensitive to PMB than was the wild-type strain ($P < 0.001$), which is consistent with the inactivation of the PhoPQ regulatory system in this strain (11, 31). Interestingly, a significant increase in PMB resistance was conferred on χ4550 and GL04 harboring either the L1-SDQ24 or L1-PQ24 plasmid (7.5- and 17.5-fold, respectively, for χ4550 and 4.4- and 6.2-fold, respectively, for GL04 [$P < 0.001$]). In addition, similar to *phoN*, a higher activation of *pmrAB* was induced when *phoPQ24* was expressed in both strains χ4550 and GL04 ($P < 0.01$).

Extrachromosomal expression of *phoPQ24*, but not *phoQ24*, altered the ability of χ4550 and GL04 to invade and persist in the mouse. Combinations of Δ*cya* Δ*crp* mutations (χ4550) with mutations in the *phoPQ* locus have not yet been evaluated in animal models, while a double PhoP^c Δ*aroA* mutant was shown to be 100-fold more attenuated than the single Δ*aroA* mutant (32). It was therefore important to determine whether expression of PhoQ24 in a strain that already harbored attenuating mutations would not cut short the infectious pathway in the mouse. This might result in poor antigen presentation due to cumulative effect of attenuating mutations on mouse virulence. For this purpose, we intranasally immunized groups of mice with the different recombinant *Salmonella* strains. The mice were euthanized 2 weeks after immunization, and recombinant *Salmonella* organisms were recovered from representative sites of bacterial invasion and persistence, i.e., lungs, cervical lymph nodes, Peyer's patches, and spleen (33). While the two strains harboring the *phoPQ24*-containing plasmid showed a dramatic decrease in survival and/or replication in all organs examined (Fig. 2), only a slight decrease was observed in the lungs when these strains were harboring the *phoQ24*-containing plasmid, suggesting that *phoPQ24*, but not *phoQ24*, had deleterious effects on invasiveness and persistence of the Δ*cya* Δ*crp* and Δ*aroA Salmonella* strains in vivo.

Expression of *phoQ24* in both χ4550 and GL04 L1-expressing strains confers immunogenicity against HPV16 L1 VLPs. The aim of this study was to test whether the expression of the *phoQ24* gene in *S. enterica* serovar Typhimurium backgrounds that were previously shown to be poorly immunogenic would

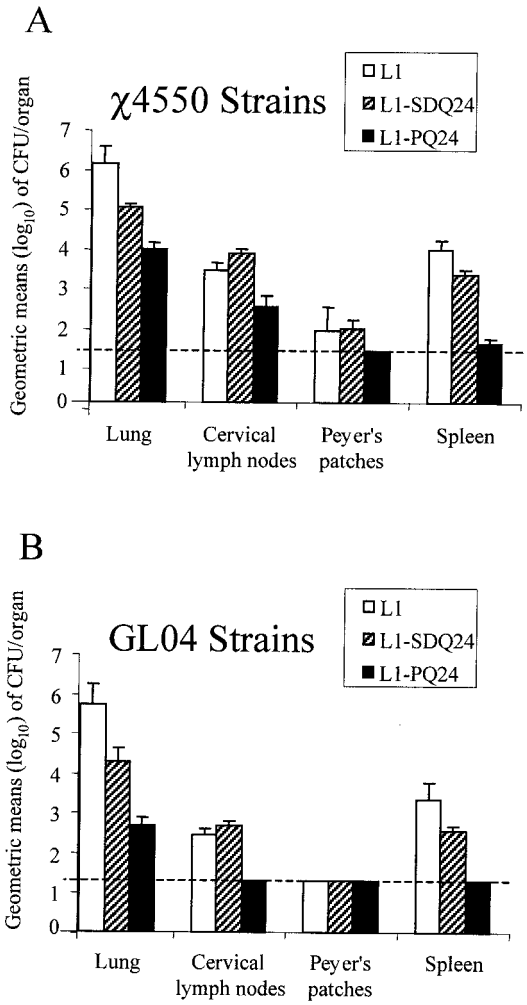

FIG. 2. Recovery of *S. typhimurium* χ4550 (A) and GL04 (B) 2 weeks after intranasal vaccination. Groups of three to five 6-week-old BALB/c mice were immunized with ca. 10^7 CFU of χ4550 or GL04 recombinant strains. The different organs were prepared and plated on agar as previously described (33). Data are expressed as the geometric mean (log_{10}) CFU per organ. The level of detection (20 CFU/organ, or log_{10} 1.3) is indicated by a horizontal dashed line. Error bars indicate the standard error of the means.

improve the immunogenicity of the HPV16VLP antigens. Although successfully used to induce protective immunities against a number of other heterologous antigens, *cya crp*, and *aroA* mutants yielded only barely detectable antibody responses to the HPV16 L1 protein (5; J. Benyacoub, unpublished results). Strains χ4550 and GL04 harbor a chromosomal deletion that eliminates the *asd* gene. This gene is essential for the growth of bacteria on unsupplemented media, but its defect can be complemented in *trans* by using a plasmid. This creates a balanced lethal state that can be exploited to ensure stable plasmid inheritance. Since we had previously encountered serious problems in the maintenance of L1-encoding plasmids in χ4989 and other isogenic strains (5), we decided to use this system to evaluate the role of *phoQ24* in the immunogenicity of HPV16 VLPs delivered by live *Salmonella* strains in the mouse. The levels of expression of HPV16L1 were

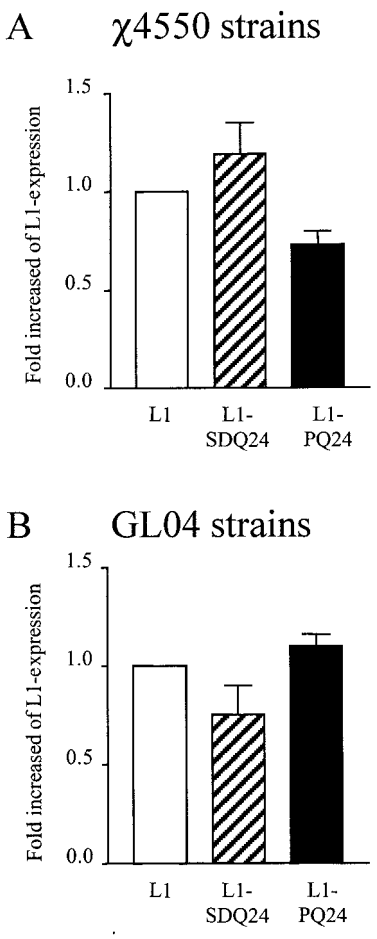

FIG. 3. HPV16 L1 expression in the χ4550 (A) and GL04 (B) recombinant strains. Scanning of the L1 protein bands obtained after Western blotting (four independent experiments) of the bacterial lysates from the different recombinant strains was performed by using NIH Image software. The results are shown as the means of the pixel densities of the L1 proteins bands normalized to the content in bacteria (OD_{600} of the culture) and are expressed as fold increase in comparison to the strains harboring the plasmid-encoded L1.

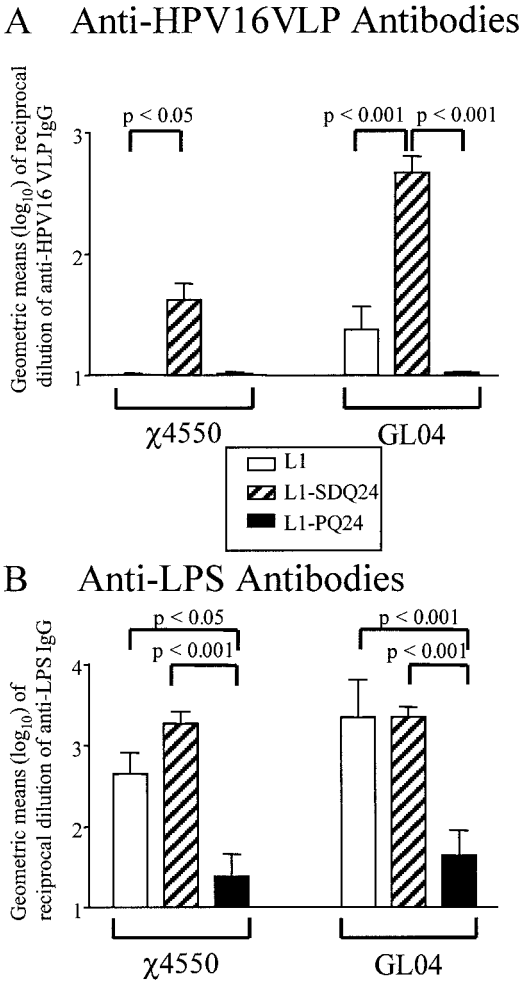

FIG. 4. Anti-HPV16 VLP (A) and anti-LPS (B) IgG in the serum of immunized mice. BALB/c mice were intranasally immunized twice with ca. 10^7 CFU of the different recombinant strains, and serum samples were obtained 4 weeks later. Data are expressed as the geometric means (log_{10}) of the reciprocal dilutions of specific IgG from individual mice. Error bars indicate the standard errors of the means.

examined in all the strains to determine whether addition of *phoQ24* to the L1-encoding plasmid would alter L1 expression. This did not turn out to be the case, as illustrated in Fig. 3. To test the immunogenicity of the VPLs produced by *S. enterica* serovar Typhimurium recombinants, mice were intranasally vaccinated twice with the L1-expressing χ4550 and GL04 recombinants and specific antibody responses were measured in serum 4 weeks after the second immunization. As expected, χ4550 L1 and GL04 L1 induced low or barely detectable HPV16 VLP antibodies. In contrast, expression of *phoQ24* in these backgrounds induced a significant increase of HPV16 VLP immunoglobulin G (IgG) titers in both χ4550 L1-SDQ24 and GL04 L1-SDQ24 ($P < 0.05$ and $P < 0.001$, respectively [Fig. 4]). In contrast to this, and in agreement with the observed penetration and survival of *Salmonella* recombinants in vivo, bacteria expressing *phoPQ24* induced only very low antibody titers against HPV16 VLP, the heterologous antigen, and against bacterial LPS. This supported our hypothesis that these recombinants were overattenuated (Fig. 4). There was no statistical difference in the anti-LPS responses induced by the other four strains (Fig. 4B), suggesting similar infection of the mice. We confirmed by a sandwich ELISA (14) that in agreement with the Western blot analysis, no difference in VLP assembly occurred whether PhoQ24 was expressed or not (7.0 ± 1.3 and 5.1 ± 0.4 μg of VLP/10^{11} CFU for χ4550 L1 and χ4550 L1-SDQ24, respectively; 5.6 ± 0.3 and 7.3 ± 1.5 μg of VLP/10^{11} CFU for GL04 L1 and GL04 L1-SDQ24, respectively). In addition, we did not find any statistical difference in the anti-HPV16 VLP IgG titers induced by GL01, a $PhoP^c$ Δ*asd* strain (37), harboring either the L1 gene or the L1-*phoQ24*-carrying plasmid (anti-HPV16 VLP IgG log_{10} titers of 2.42 ± 0.34 and 2.84 ± 0.22 [mean ± standard error of the mean] respectively). Taken together, our data demonstrate that a relatively low expression level of PhoQ24 can be combined with attenuations in Δ*aro*, as well as in Δ*cya* and Δ*crp*,

although to a lesser extent, to induce immunogenicity against the HPV16 VLP antigen.

DISCUSSION

Attenuated *Salmonella* vaccine strains were initially developed to produce live typhoid vaccines. Ideally, these strains had to be safe, stably attenuated, and protective. For these purposes, several means of attenuation have been used, including introduction of auxotrophic mutations and mutations in regulatory genes which render the bacteria avirulent while preserving different degrees of invasiveness (26). When these strains were used to carry heterologous antigens, the specific immune responses induced were highly variable depending on the antigen expressed, the location of the antigen in the bacteria, the type of attenuation, and the route of immunization (5, 10, 20, 21, 23, 38). However, to our knowledge this is the first time that a specific genetic program of *Salmonella* has been shown to be required to obtain an immune response to an heterologous antigen. We have demonstrated that the PhoP^c phenotype is essential for the induction of antibody responses to HPV16 VLPs. This genetic program is normally activated when the *Salmonella* organisms enter the intracellular environment, and it is thought to be triggered by reduced magnesium levels in the phagosome (12). Constitutive activation of PhoP, resulting in expression of the *pag* and *prg* genes, has important consequences since it is sufficient to attenuate the strain for mouse virulence (28). Only some of the functions of *pag* and *prg* genes have been demonstrated; they include genes involved in modification of LPS, which can affect cytokine and chemokine secretion (18), in antimicrobial peptide resistance (PMB [16, 17]), in the invasion and secretion of proteins by a type III mechanism (36), in the formation of spacious phagosomes (1), and in antigen presentation (48, 50). One or several of these genes could influence the immune responses in the mouse. Antigen processing and presentation by dendritic cells might be critical in this process (6). Interestingly, antigen presentation by major histocompatibility complex class II molecules on dendritic cells infected with a PhoP^c strain expressing the model antigen Crl-OVA was reported to be less efficient than (48) or similarly efficient to (35) that obtained with the nonattenuated wild-type strain. In contrast, our preliminary data, obtained using bone marrow-derived dendritic cells and an HPV16 L1-specific CD4 T-cell hybridoma (2), revealed that antigen presentation was more efficient after infection with the PhoP^c HPV16 strain (data not shown). We cannot exclude the possibility that the effect of the PhoP^c phenotype is limited to the HPV16 VLP antigen. Indeed, these VLPs are able to induce efficient immune responses in the absence of adjuvant in both mice and humans (3, 19), probably due to their particulate nature and regular surface structure. However, this contrasts with the difficulty in obtaining antibody responses when HPV16 VLPs are expressed by *Salmonella.* Even the strain that most efficiently induced antibody responses to the HPV16 VLPs, the PhoP^c strain, could do so only after a double nasal immunization, while oral immunization was inefficient (33). This might be linked to the low level of HPV16 VLP that can be expressed in *Salmonella* (ca. 50 $\mu g/10^{11}$ CFU [33] and only ca. 5 $\mu g/10^{11}$ CFU when stable expression is achieved with the *asd* system), most probably because of an unfavorable codon usage. Higher levels of expression (about 1% of the total protein content, the equivalent of approximately 150 μg antigen per 10^{11} bacteria) have previously been shown to be necessary to induce specific antibody responses with recombinant *Salmonella* strains (7, 47). It is possible that our finding that the PhoP^c phenotype is required to obtain significant immunogenicity of HPV16 VLPs expressed in *Salmonella* will eventually reveal some of the mechanisms behind the presentation of antigens expressed by these bacteria.

The main objective of these studies is to develop a live recombinant *Salmonella* vaccine against HPV16 infection and cervical cancer. The PhoP^c strain cannot be used as a vector in humans due to the high frequency of reversion of its attenuation (29). However, we had previously found that this was the only useful background for this particular antigen. In the present study, we have shown that the mutant *phoQ24* gene can be introduced into safely attenuated recipient strains by using a plasmid encoding the antigen L1 and carrying the *phoQ24* gene. In the resulting recombinants, the extrachromosomal *phoQ24* genes induce activation of *pag* and repression of *prg* via the endogenous phosphorylated PhoP protein (14, 45), and reversion of all *phoQ24* copies is highly unlikely. In these constructs, the extrachromosomal expression of *phoQ24* was driven either by its own promoter or by a plasmid promoter. This resulted in strains exhibiting at least some of the features of the PhoP^c phenotype, as shown by activation of three different *pag* genes. Interestingly, the more complete PhoP^c phenotype, as judged by the expression levels of a number of PhoP-associated markers, was observed when *phoPQ24* was expressed. However, these recombinants suffered from strongly reduced invasiveness and poor immunogenicity, which may be due to overattenuation. In contrast, expression of *phoQ24* did not alter the ability to colonize and survive in mice and the resulting strains were immunogenic, i.e, induction of anti-HPV16 VLP antibodies was obtained. Although the titers were slightly lower than those obtained with the PhoP^c HPV16 strain (ca. 10^4 for IgG [33]), they are similar to those obtained with the Δ*asd* derivative, GL01, probably because of the lower HPV16 VLP expression observed in these strains. Provided that the anti-HPV16 VLP titers can be induced at higher level, this strategy is a promising step toward a vaccine strain that could be tested in human volunteers.

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