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Improved Efficiency of a *Salmonella*-Based Vaccine against Human Papillomavirus Type 16 Virus-Like Particles Achieved by Using a Codon-Optimized Version of L1

David Baud, Françoise Ponci, Martine Bobst, Pierre De Grandi, and Denise Nardelli-Haefliger*

Department of Gynecology, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland

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Cervical cancer results from cervical infection by human papillomaviruses (HPVs), especially HPV16. An effective vaccine against these HPVs is expected to have a dramatic impact on the incidence of this cancer and its precursor lesions. The leading candidate, a subunit prophylactic HPV virus-like particle (VLP) vaccine, can protect women from HPV infection. An alternative improved vaccine that avoids parenteral injection, that is efficient with a single dose, and that induces mucosal immunity might greatly facilitate vaccine implementation in different settings. In this study, we have constructed a new generation of recombinant *Salmonella* organisms that assemble HPV16 VLPs and induce high titers of neutralizing antibodies in mice after a single nasal or oral immunization with live bacteria. This was achieved through the expression of a HPV16 L1 capsid gene whose codon usage was optimized to fit with the most frequently used codons in *Salmonella*. Interestingly, the high immunogenicity of the new recombinant bacteria did not correlate with an increased expression of L1 VLPs but with a greater stability of the L1-expressing plasmid in vitro and in vivo in absence of antibiotic selection. Anti-HPV16 humoral and neutralizing responses were also observed with different *Salmonella enterica* serovar Typhimurium strains whose attenuating deletions have already been shown to be safe after oral vaccination of humans. Thus, our findings are a promising improvement toward a vaccine strain that could be tested in human volunteers.

Cervical cancer is the second leading cause of cancer deaths in women worldwide, and virtually all of these tumors are attributable to infection with a subset of human papillomaviruses (HPVs), of which HPV16 is found most frequently (6, 42). An effective vaccine against these HPVs would, therefore, be expected to have a dramatic impact on the incidence of this cancer and its precursor lesions, as well as on the less common tumors attributable to these viruses. The leading candidate is a prophylactic subunit HPV virus-like particle (VLP) vaccine (reviewed in references 36 and24). A proof of principal efficacy trial showed that women vaccinated with HPV16 VLPs were highly protected against genital mucosal infection by this viral type (19). However, the requirement for multiple injections for a vaccine whose anticipated target population will be older than the population that receives childhood vaccines may represent a substantial hurdle for widespread implementation. This is particularly true in the developing world, which accounts for more than three-quarters of the worldwide cases of cervical cancer (6). 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 mucosal-associated lymphoid tissues. Via this route, both mucosal and systemic immune responses against the carrier and the foreign antigens may be obtained (reviewed in references 11, 22, and 37). We have shown that nasal vaccination of mice with *Salmonella* organisms expressing the HPV16 major capsid protein L1, which self-assembles into VLPs, induces anti-HPV16 conformational and neutralizing antibodies in serum and genital secretions, provided the attenuated *Salmonella enterica* serovar Typhimurium strains exhibit the PhoP^c phenotype (3, 4, 31). However, even with the original PhoP^c strain, a double nasal immunization was required to induce high anti-HPV16 VLP antibody titers, while oral immunization was inefficient (31). The observations of low levels of L1 expression together with a high instability of the L1-encoding plasmids in the absence of antibiotic selection strongly suggested that either the L1 protein or the L1 gene could be toxic to the bacteria. As the viral L1 gene exhibits a highly unfavorable codon usage for expression in *Salmonella*, we designed and tested herein a synthetic nucleotide sequence (referred to as L1S hereafter) encoding the L1 protein and containing the most frequently used codons in *Salmonella*. Our data show that anti-HVP16 VLP humoral and neutralizing responses after either nasal or oral immunization with the new recombinant strains were highly increased. Interestingly, this was not associated with an increased L1 expression but with a remarkable stability of the L1S-expressing plasmid in vitro and in vivo. In addition, immunogenicity was not restricted to PhoP^c, as shown with other *S. enterica* serovar Typhimurium strains whose attenuating deletions are suitable for human use.

MATERIALS AND METHODS

Plasmid constructions and bacterial strains used. The L1S gene was synthesized by Microsynth, Buchs, Switzerland. The open reading frame (ORF) was flanked in 5′ with a NcoI restriction site and in 3′ with a HindIII restriction site. The L1S NcoI-HindIII fragment was inserted in place of the original L1 NcoI-HindIII fragment in the plasmid pFS14nsd HPV16-L1 (31). The resulting plasmid, pFS14nsd HPV16-L1S, was introduced by electroporation (38) into the attenuated *S. enterica* serovar Typhimurium strains PhoP^c (CS022 [27]) and

* Corresponding author. Mailing address: Département de Gynécologie, c/o Institut de Microbiologie, CHUV, Bugnon 48, 1011 Lausanne, Switzerland. Phone: 41 21 314 40 81. Fax: 41 21 314 40 95. E-mail: dnardell@hospvd.ch.

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   1  atg gct ctt tgg ctg cct agc gag gcc act gtc tac ctg cct cct gtc ccg
  52  gta tct aag gtt gta agc acg gat gaa tat gtt gca cgc acc aac atc tat
 103  tat cac gca ggc acc tcc cgt ctg ctg gca gtt ggt cac ccg tat ttc ccg
 154  atc aaa aaa ccg aac aac aac aaa atc ctg gtt cct aaa gta tca ggt ctg
 205  caa tac cgt gta ttt cgt atc cac ctg ccg gac ccg aac aag ttc ggt ttc
 256  cct gac acc tca ttc tat aac cca gat act cag cgg ctg gtt tgg gcc tgt
 307  gta ggt gtt gag gta ggt cgt ggt cag cca ctg ggt gtg ggc att agt ggc
 358  cac cct ctg ctg aac aaa ctg gat gac acc gaa aac gct agt gct tat gca
 409  gca aac gca ggt gtg gat aac cgt gaa tgt atc tct atg gat tac aaa caa
 460  act caa ttg tgt ctg att ggt tgc aaa cca cct atc ggt gaa cac tgg ggc
 511  aaa ggt tcc cca tgt acc aac gtt gca gta aac ccg ggt gat tgt ccg ccg
 562  ctg gag ctg atc aac act att att cag gat ggt gat atg gtt gat act ggc
 613  ttc ggt gct atg gac ttc act acc ctg cag gct aac aaa agt gaa gtt cca
 664  ctg gat att tgt act tct att tgc aaa tat cca gat tat att aaa atg gtg
 715  tca gaa cca tat ggc gac agc ctg ttc ttc tat ctg cgt cgt gaa caa atg
 766  ttc gtt cgt cac ctg ttc aac cgt gct ggt gct gtt ggt gaa aac gta cca
 817  gac gat ctg tac att aaa ggc tct ggt tct act gca aac ctg gcc agc tcc
 868  aac tat ttc ccg acc cct agt ggt tct atg gtt acc tct gat gcc caa atc
 919  ttc aac aaa cct tat tgg ctg caa cgt gca cag ggc cac aac aac ggc atc
 970  tgt tgg ggt aac cag ctg ttc gtt act gtt gtt gat act acc cgc agt acc
1021  aac atg tcc ctg tgt gct gcc atc tct act tca gaa act acc tat aaa aac
1072  act aac ttc aag gag tac ctg cga cac ggt gag gaa tat gat ctg cag ttc
1123  atc ttc cag ctg tgc aaa atc acc ctg act gca gac gtt atg acc tac atc
1174  cac tct atg aac tcc act att ttg gag gac tgg aac ttc ggt ctg cag ccg
1225  ccg ccg ggc ggc acc ctg gaa gat act tat cgt ttc gta act tcc cag gca
1276  att gct tgt caa aaa cac acc cct cca gca cct aaa gaa gat cct ctt aaa
1327  aaa tac act ttc tgg gaa gta aac ctg aag gaa aag ttc tct gca gac ctg
1378  gat cag ttc ccg ctg ggc cgt aaa ttc ctg ctg cag gca ggt ttg aag gcc
1429  aaa cca aaa ttc acc ctg ggc aaa cgt aaa gct acc ccg acc acc tca tct
1480  acc tct act act gct aaa cgc aaa aaa cgt aag ctg taa
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FIG. 1. Codon-optimized HPV16 L1S ORF. The nucleotide sequence of L1S is shown with the modified codons underlined; modified nucleotides are in bold.

PhoP$^-$ (CS015 [26]), both a kind gift from John Mekalanos, Boston, Mass., and strains χ4989 (*Δcya Δcrp* [4]), χ4990 (*Δcya Δcrp-cdt* [4]), and *ΔaroA* (SL7207 [16]), a kind gift from Irene Corthésy-Theulaz, Lausanne, Switzerland.

HPV16 L1 and VLP analysis. Expression of L1 in *Salmonella* lysates was analyzed by Western blotting as previously described (31) by using the anti-HPV16 L1 monoclonal antibody, CAMVIR-1 (Anawa). Data were normalized to the content in bacteria as measured by the optical density at 600 nm of the cultures. The HPV16 VLP content was measured by a sandwich enzyme-linked immunosorbent assay (ELISA) as previously described (4) by using two monoclonal antibodies that recognize conformational epitopes on HPV16 VLPs, H16E70, and H16 V5, kindly provided by N. D. Christensen, Hershey, Pa. (9).

Immunization of mice, analysis of anti-HPV16 VLP antibodies, and recovery of *S. enterica* serovar Typhimurium. Six-week-old female BALB/c mice from Iffa Credo, France, were used in all experiments. Twenty microliters of bacterial inoculum was administered orally (10^8 to 10^9 CFU) or intranasally (10^6 to 10^7 CFU) under anesthesia as previously described (17, 31). Sampling of blood and vaginal washes as well as determination of anti-HPV16 VLP antibody titers by ELISA were performed as reported earlier (17, 31). Recovery of *S. enterica* serovar Typhimurium was determined in organs from euthanized mice as previously described (31).

Neutralization assays. Neutralizations assays were performed with secreted alkaline phosphatase (SEAP) HPV16 pseudoviruses as described in detail by Pastrana et al. (34). Briefly, OptiPrep-purified SEAP HPV16 pseudoviruses diluted 2,000-fold were incubated on ice for 1 h with twofold serial serum dilutions, and the pseudovirus-antibody mixtures were used to infect 293TT cells for 3 days. The SEAP content in 10 μl of clarified cell supernatant was determined by using a Great EscAPe SEAP chemiluminescence detection kit (BD Biosciences Clontech). Neutralization titers were defined as the reciprocal of the highest serum dilution that caused at least a 50% reduction in SEAP activity (with 100% SEAP activity ranging from 50 to 100 relative light units).

RESULTS

Design of an HPV16 L1 nucleotide sequence with most frequently used codons in *Salmonella*. The codons used for trans-

TABLE 1. Salmonella strains used in this study

Strain (attenuation)	Plasmid electroporated	Abbreviation	Reference
CS022 (PhoP^c, *pho-42*)		PhoP^c	26
	pFS14nsd-HPV16 L1	PhoP^c L1	31
	pFS14nsd-HPV16L1*S*	PhoP^c L1*S*	This work
χ4989 (Δ*cya* Δ*crp*)			4
	pFS14nsd-HPV16 L1	χ4989 L1	4
	pFS14nsd-HPV16L1*S*	χ4989 L1*S*	This work
χ4990 (Δ*cya* Δ*crp-cdf*)			4
	pFS14nsd-HPV16 L1	χ4990 L1	4
	pFS14nsd-HPV16L1*S*	χ4990 L1*S*	This work
CS015 (PhoP$^-$, Δ*phoPQ*)			26
	pFS14nsd-HPV16 L1	PhoP$^-$ L1	4
	pFS14nsd-HPV16L1*S*	PhoP$^-$ L1*S*	This work
SL7207 (Δ*aroA*)			16
	pFS14nsd-HPV16 L1	AroA L1	This work
	pFS14nsd-HPV16L1*S*	AroA L1*S*	This work

lation of major endogenous proteins in *S. enterica* serovar Typhimurium (7, 14) were considered to design an optimized L1 ORF. From the 506 codons of the original HPV16 L1 sequence (HPV16 114/B [18]), 163 were modified in codons most frequently used in *Salmonella* (Fig. 1). This included all the codons of the original L1 sequence which are rarely found in *Salmonella* (136) and some (27 of 72) of the less frequently used codons. The L1 ORF was then replaced in plasmid pFS14nsd-HPV16 L1 (31) by the new L1S ORF, yielding pFS14nsd-HPV16 L1S. The new plasmid was first introduced in the attenuated *S. enterica* serovar Typhimurium strain PhoP^c (27) to generate the recombinant strain, called PhoP^c L1S hereafter. Four others L1S recombinant attenuated *Salmonella* strains were subsequently produced (see below); Table 1 summarizes the different strains and abbreviations used in this study.

HPV16 L1 and VLP expression. The expression of the L1 protein in the lysates of exponential cultures of PhoP^c L1 and PhoP^c L1S were compared by Western blotting (Fig. 2A). Surprisingly, expression of L1 in the bacterial cultures was not improved with the new L1S sequence but rather decreased by twofold (Fig. 2B). This finding was confirmed when the amounts of VLPs produced in the two recombinant strains were compared by sandwich ELISA (Fig. 2C). A striking difference in the growth rate of the two strains was noticed when the time to reach mid-log phase after inoculation of 50 ml of Luria-Bertani (LB) broth with a single colony was compared (ca. 7 h for PhoP^c L1S and ca. 15 h for PhoP^c L1). This may suggest that the optimized codon usage of L1 with respect to the corresponding cognate tRNAs maximized the growth rate without a concomitant increase in L1S translation.

Stability of the L1S-encoding plasmid in vitro and in vivo. We have previously reported that the original L1-encoding plasmid was rapidly lost by plasmid segregation in *Salmonella* in the absence of antibiotic selection in vivo (4, 31). The stability of the L1S- and L1-encoding plasmids was first compared in vitro. For this purpose the percentages of bacteria still harboring the L1- or L1S-encoding plasmids were compared during four successive overnight cultures in the absence of antibiotic selection (Fig. 3). As expected, the L1-encoding plasmid was rapidly lost. In contrast, the L1S-encoding plasmid was recovered in most of the bacteria after ca. 50 generation times in the absence of antibiotic selection. The stability of the L1S-encoding plasmid was further examined in vivo after nasal and oral immunization of mice (Table 2). In contrast to the original L1-encoding plasmid (4), the L1S-encoding plasmid was completely stable for at least 2 weeks in the organs close to the sites of infection or entry. Some instability of the L1S plasmid was, however, observed in more distant organs such as the spleen, where ca. 10% of the bacteria were still harboring the L1S plasmid but no bacteria harboring the L1 plasmid were detected. We should also note that there is no evidence of a higher invasiveness or persistence of the L1S-harboring bacteria, despite the faster growing capacity of these bacteria observed in vitro.

Anti-HPV16 VLP antibody and HPV16 neutralization titers induced by PhoP^c L1S or PhoP^c L1. Our final aim was to test whether expression of the HPV16 L1S gene would improve the immunogenicity of the HPV16 VLP antigen in *S. enterica* serovar Typhimurium. Direct comparisons of the serum immune responses induced after nasal immunization of female BALB/c mice with PhoP^c harboring either the original L1 sequence or the codon-optimized L1S sequence are shown in Fig. 4. In addition to anti-HPV16 VLP conformational antibody titers (Fig. 4A), HPV16 neutralization titers are shown in Fig. 4B. We used the SEAP HPV16 pseudovirus assay (34) to determine the endpoint neutralization titers. These neutralization titers are only slightly lower than the VLP ELISA titers and confirm the prophylactic potential of a *Salmonella*-based vaccine. Comparison of single nasal immunizations shows that a major improvement with the L1S strain is that HPV16 ELISA and neutralization titers are two orders of magnitude higher than those achieved with the original PhoP^c L1 strain (Fig. 4 and references 4 and 31). The anti-VLP antibody titers measured in serum and vaginal secretions of the mice at 4 to 6, 8, and 24 weeks after a single immunization with PhoP^c L1S are shown in Fig. 5. A single nasal vaccination induced high and long-lasting anti-HPV16 VLP immunoglobulin G (IgG) titers

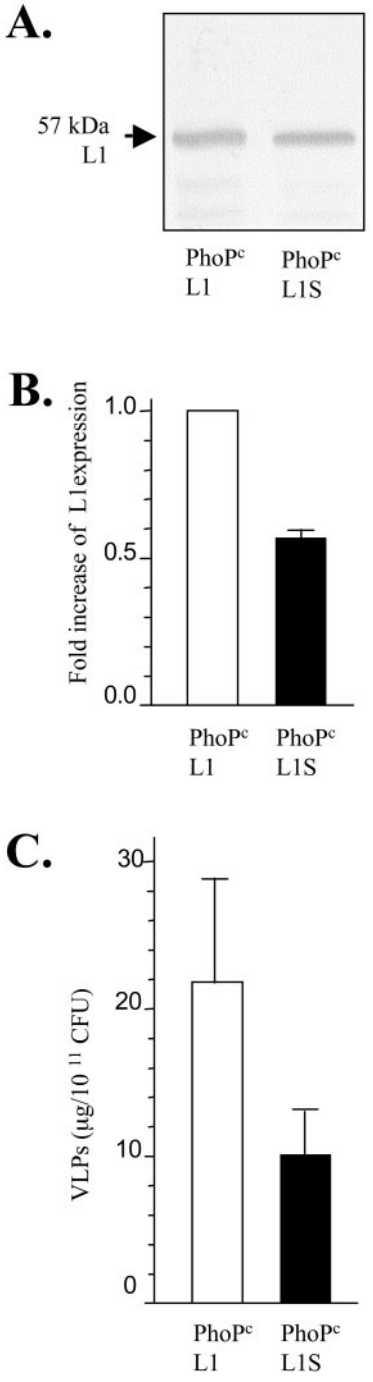

FIG. 2. HPV16L1 expression in PhoP^c L1 and PhoP^c L1S recombinant strains. An immunoblot of bacterial lysates with anti-HVP16 L1 monoclonal antibody is shown, and the 57-kDa protein band identified as L1 is indicated by an arrow (A). Scanning of the L1 protein bands obtained after immunoblotting of the bacterial lysates from the two recombinant strains (three independent experiments) was performed by using National Institutes of Health Image software. The results are shown as the means of pixel densities of the L1 protein bands normalized to the content in bacteria and are expressed as increases in expression (*n*-fold) in comparison to PhoP^c L1 (B). The amounts of VLPs determined by sandwich ELISA are shown in micrograms per 10^{11} CFU (C).

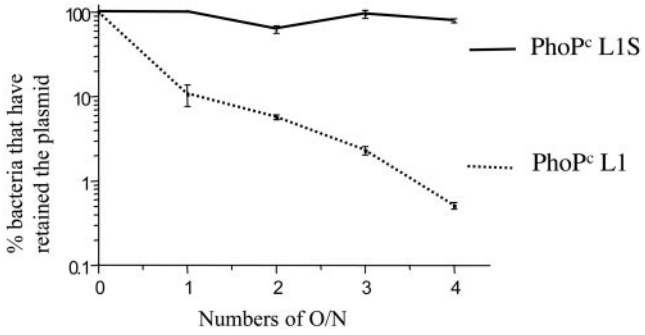

FIG. 3. L1 and L1S plasmid stability in vitro. The number of successive overnight cultures at 1/1000 dilution performed in LB broth without antibiotic is indicated on the horizontal axis. Each morning, bacteria were plated on LB agar in the presence or absence of antibiotic. The vertical axis represents the percentage of bacteria that have retained the plasmid. Error bars indicate the standard errors of the means. O/N, overnight cultures.

in serum, as well as specific IgG and IgA titers in vaginal washes. These antibody titers are similar to those induced after a double nasal vaccination with the original PhoP^c L1 strain (4, 31). Interestingly, oral vaccination with PhoP^c L1S was also highly immunogenic, although less than nasal vaccination, while even a double oral vaccination with the original PhoP^c strain was inefficient (31). Administration of two nasal (Fig. 4) or three oral doses of PhoP^c L1S (data not shown) did not increase the immune responses.

Serum anti-HPV16 VLP antibody and HPV16 neutralization titers following nasal or oral vaccination with differently attenuated *S. enterica* serovar Typhimurium strains expressing the codon-optimized or the original L1 gene. We have previously shown that nasal vaccination of mice with differently attenuated *S. enterica* serovar Typhimurium strains expressing the original L1-encoding plasmid induced only low levels of or no anti-HPV16 VLPs antibodies (4). Given the high immunogenicity observed with the PhoP^c strain expressing the L1S encoding plasmid, we further introduced this plasmid in different strains including χ4989, χ4990, PhoP$^-$, and AroA (Table 1 gives precise attenuations, abbreviations, and references). The serum anti-HVP16 VLP IgG and neutralizing titers measured in mice 6 to 7 weeks after a single nasal or oral vaccination with these new recombinant strains are shown in Fig. 6. In contrast to the strains expressing the original L1 gene, all the new recombinant strains induced consistent anti-HPV16 VLP humoral and HPV16 neutralizing responses after a single nasal vaccination, although the titers are about one order of magnitude lower than those achieved with the PhoP^c L1S strain (Fig. 4 and 5). As expected, oral vaccination was less immunogenic, with the exception of the AroA L1S strain, which induced similar anti-HPV16 VLP IgG and HPV16 neutralizing titers after both routes of vaccination.

DISCUSSION

The development of a *Salmonella*-based vaccine against HPV infection and associated lesions would be of great value for worldwide implementation with the theoretical advantage of inducing long-lasting systemic and mucosal immunity with a single oral vaccination. However, although we showed the fea-

TABLE 2. Recovery of *Salmonella* PhoP^c carrying L1- or L1S-encoding plasmids 2 weeks after nasal or oral immunization

Route of immunization	Organ(s) analyzed	Means of total *Salmonella* recovered[a]		% of *Salmonella* bearing the plasmid:	
		PhoP^c L1[b]	PhoP^c L1S	PhoP^c L1	PhoP^c L1S
Nasal	Lung	5.30 ± 0.02	4.14 ± 0.21	3.2	100
	Cervical lymph nodes	3.39 ± 0.09	2.95 ± 0.09	10	100
	Peyer's patches	2.51 ± 0.34	2.17 ± 0.42	ND[c]	100
	Spleen	3.76 ± 0.13	2.88 ± 0.14	ND	8
Oral	Peyer's patches	2.02 ± 0.29	2.38 ± 0.29	ND	100
	Mesenteric lymph nodes	1.70 ± 0.75	2.67 ± 0.15	ND	27
	Spleen	2.07 ± 0.87	2.46 ± 0.12	ND	16

[a] Means ($\log_{10}$) of CFU/organ ± standard error of the means.
[b] Data after nasal immunization with PhoP^c L1 are taken from Benyacoub et al. (4).
[c] Not detectable.

sibility of such a strategy in mice (31), several drawbacks had to be addressed before a *Salmonella*-based vaccine could be safely tested in women. The drawbacks included the requirement of a particular *Salmonella* phenotype (PhoP^c [3, 4]) and the use of the nasal route of immunization to efficiently induce neutralizing antibody responses, as well as the observation that the L1-encoding plasmid was unstable without antibiotic selection (31, 4) or poorly expressed when stabilized with a semilethal complementation system (3). Here we report that most of these problems are solved by using a codon optimization strategy for the expression of the HPV16 L1 capsid gene (HPV16 L1S). Indeed, expression of the synthetic L1S gene is stable in *Salmonella* and results in higher immunogenicity when differently attenuated bacteria are delivered by either the nasal or oral route. Immunogenicity strongly correlated with HPV16 neutralization as assayed with the SEAP HPV16 pseudovirus assay. This further demonstrates the great potential of *Salmonella*-based vaccines to prevent HPV16 infections.

Expression of native papillomavirus capsid genes is limited in mammalian cells, but the resulting lack of immunogenicity of HPV DNA vaccines could be relieved by codon optimization (20, 23, 43). The influence of codon usage on immunogenicity has been recognized for other DNA vaccines (1, 12, 32, 39), where higher expression of the heterologous genes resulted in higher immunogenicity. As the codon usage of the original HPV16 capsid gene is also suboptimal for translation in *Salmonella*, we anticipated that expression of a codon-optimized L1S gene would result in higher VLP expression and, consequently, higher immunogenicity of the recombinant *Salmonella*. To our surprise, the higher immunogenicity of the differently attenuated L1S recombinant *Salmonella* does not correlate with higher amounts of L1 or VLPs produced in these bacteria. In fact, the opposite is true, and lower amounts of HPV16 VLPs were produced when the L1S gene was expressed (ranging from ca. 3 $\mu g/10^{11}$ CFU for the AroA L1S strain to 23 $\mu g/10^{11}$ CFU for χ4989 L1S) compared to the expression of the original L1 sequence (VLP amounts between 20 and 60 $\mu g/10^{11}$ CFU[4]). This is in contrast to the $>10^4$ increase in L1 expression obtained in mammalian cells with a human-optimized HPV16 L1 gene (20). We should note, how-

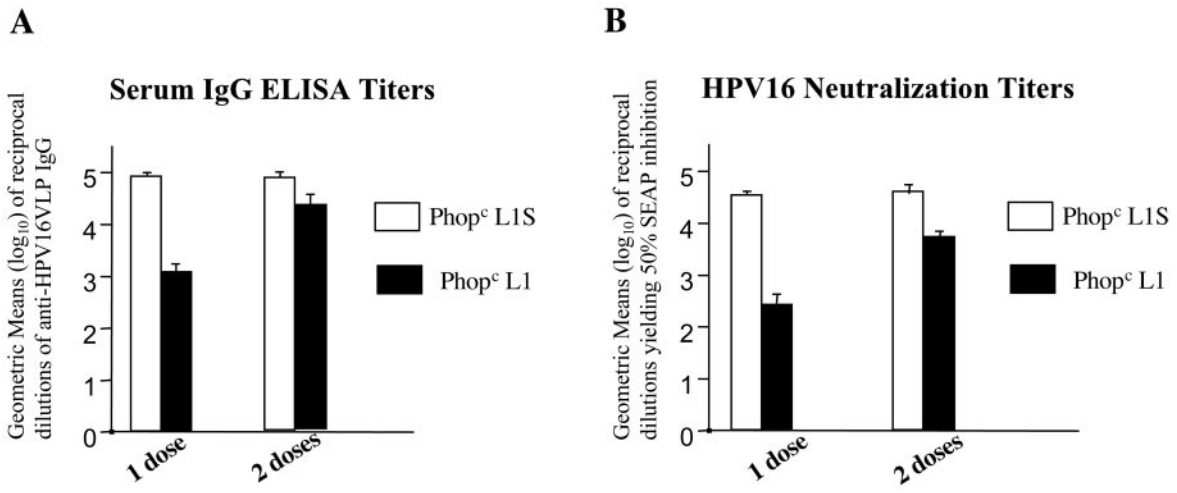

FIG. 4. Comparison of serum anti-HPV16 VLP antibody and HPV16 neutralization titers after nasal vaccination with PhoPc L1S or PhoP^c L1. Groups of five BALB/c mice were intranasally vaccinated with 10^6 to 10^7 CFU of PhoP^c L1S or PhoP^c L1 as a single dose or as two doses at week 0 and week 2. Serum was sampled 4 weeks after the last immunization, and HPV16 VLP-specific IgG (A) and HPV16 neutralization (B) titers are indicated. Data are expressed as the geometric means ($\log_{10}$) of the reciprocal serum dilutions of specific IgG (A) or reciprocal serum dilutions yielding 50% SEAP inhibition (B) from individual mice. Error bars indicate the standard errors of the means.

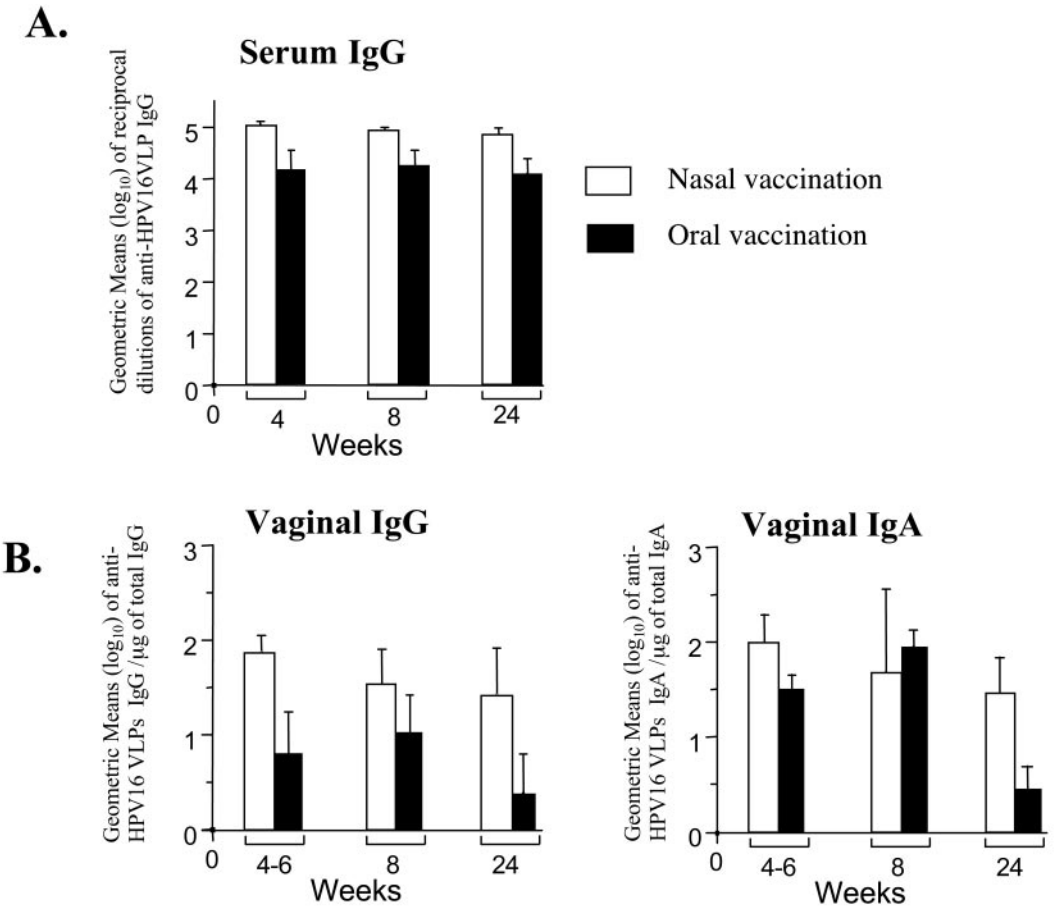

FIG. 5. Anti-HPV16 VLP systemic (A) and vaginal (B) antibody titers after nasal and oral vaccination with PhoP^c L1S. Groups of five to eight BALB/c mice were immunized with 10^6 to 10^7 CFU by the nasal route or 10^8 to 10^9 CFU by the oral route. Serum and vaginal washes were sampled at the indicated weeks after immunization. Data are expressed as the geometric means (log_{10}) of the reciprocal dilutions of specific IgG from individual mice in serum (A) and specific IgG and IgA per microgram of total IgG and IgA, respectively, in secretions (B). Error bars indicate the standard errors of the means.

ever, that we cannot exclude the possibility that the amounts of VLPs expressed in the bacteria may vary when the *Salmonella* are invading the mouse tissues, where the metabolic constraints are different. Unfortunately, we are unable to measure VLP expression in vivo, given the relatively low number of bacteria recovered (10^3 to 10^4 CFU/organ) and the low VLP expression achieved (<1 fg/bacteria).

Another notable feature associated with the expression of the codon-optimized L1S sequence is the improved stability of the L1S-expressing plasmid in vitro and in vivo in the absence of antibiotic selection. This may contribute to the higher immunogenicity of the recombinant *Salmonella*, as it results in a longer persistence of the VLP antigen carried by the bacteria. Such an explanation is in agreement with the idea that a longer persistence of antigens in the mucosa-associated lymphoid tissues is a key mechanism that underlies the immune responses elicited by *Salmonella* vaccine strains (35) and contrasts with the other suggestion that the initial amount of antigen that primes the mucosal lymphoid tissue is the critical point for inducing efficient immune responses (8, 10). Different approaches have been used to improve plasmid stability in bacterial carriers (reviewed in references 13 and 25). These approaches include the use of in vivo inducible promoters or balanced lethal plasmid stabilization systems, but to our knowledge codon optimization of heterologous antigens was not previously reported to induce plasmid stabilization.

Interestingly, plasmid stability and lower VLP expression were associated with a faster growth rate of the L1S-expressing bacteria in vitro. It is assumed that the investment in the translation system is optimized to provide a maximal growth rate of bacteria, and this is achieved by an adequate balance between the different tRNAs and their cognate codons (5). Our observations suggest that optimizing the codon usage of the heterologous L1 gene released the tRNA pool, allowing translation of endogenous bacterial protein and thereby increasing the growth rate to the detriment of L1 or VLP expression. This increased growth rate in vitro did not correlate with an increased invasion and/or persistence of the bacteria in vivo, and, therefore, we do not anticipate that L1S expression may affect the safety of a *Salmonella* vaccine strain.

The immunogenicity of PhoP^c L1S in mice is really improved and compares well with that induced with purified HPV16 VLPs, the leading prototype prophylactic subunit vaccine now in phase III clinical trials (reviewed in reference 24). A single

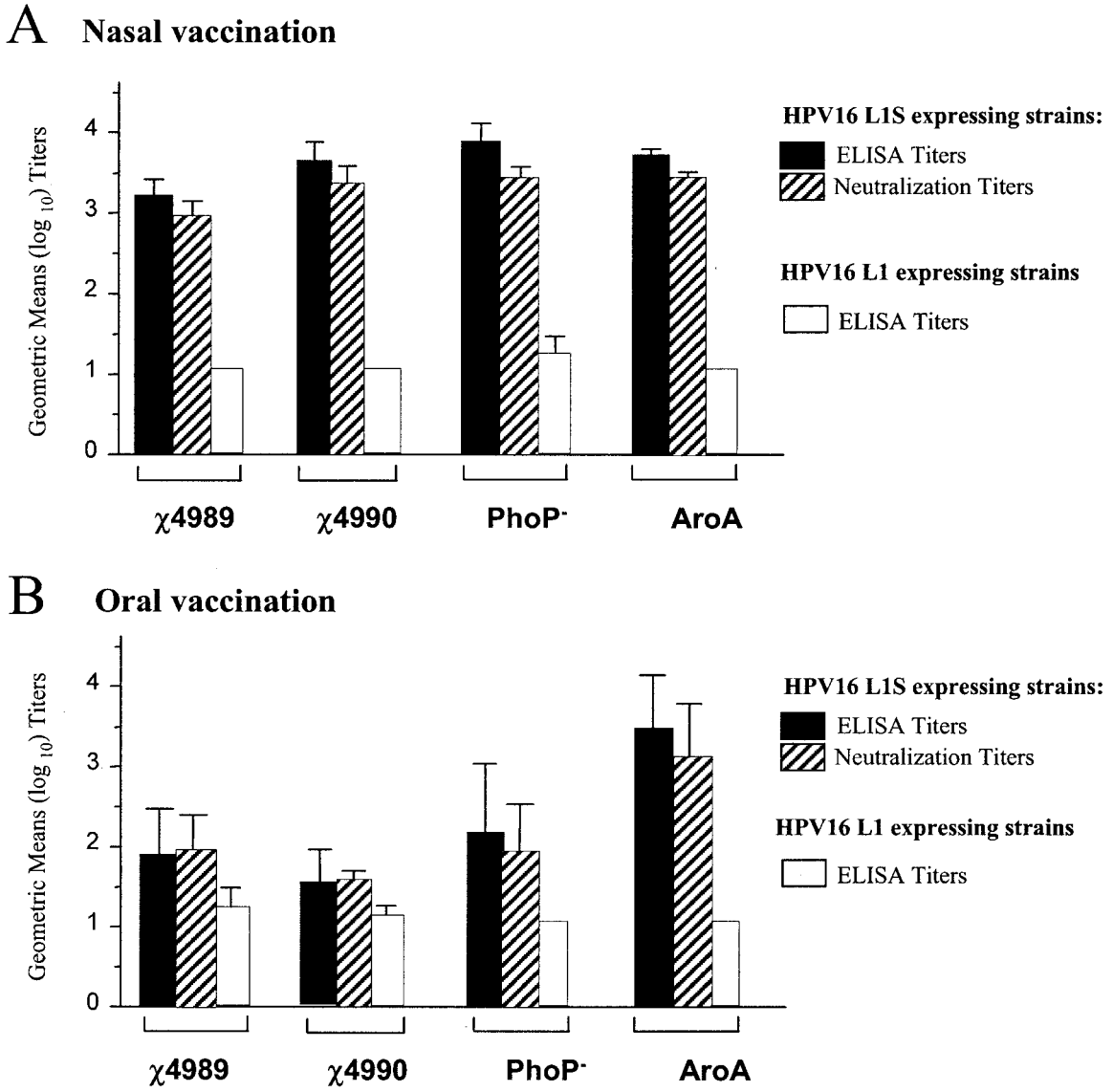

FIG. 6. Serum anti-HPV16 VLP IgG and HPV16-neutralization titers after nasal or oral vaccination with χ4989, χ4990, PhoP$^-$, and AroA expressing L1S or L1. Groups of four to seven BALB/c mice were immunized with ca. 10^6 to 10^7 CFU by the nasal route (A) or ca. 10^8 to 10^9 CFU by the oral route (B) with the indicated recombinant strains expressing L1S or L1. Serum was sampled 6 to 7 weeks after immunization, and HPV16 VLP-specific IgG (plain bars) or HPV16 neutralization (striped bars) titers are shown. Data are expressed as the geometric means ($\log_{10}$) of the reciprocal serum dilutions of specific IgG (A) or reciprocal serum dilutions yielding 50% SEAP inhibition (B) from individual mice. Error bars indicate the standard errors of the means.

nasal immunization with PhoP^c L1S induced serum and vaginal anti-HVP16 VLPs IgG titers that were similar to results with three subcutaneous injections with 1 μg of purified HPV16 VLPs or three nasal/aerosol immunizations with 5 μg of VLP doses together with the mucosal adjuvant cholera toxin, including induction of specific IgA in vaginal washes for the mucosal protocols (2, 29). Although we have shown that nasal vaccination with recombinant *Salmonella* can be highly efficient at low doses and without concomitant lung inflammation (28), there are still safety concerns for using such a route of immunization in humans. Here we report that the safer oral route can be used since a single oral vaccination with PhoP^c L1S was immunogenic, and though the VLP-specific titers are lower than following nasal immunization, they are similar to those induced after three nasal or aerosol doses of 5 μg of VLP without adjuvant (2).

One of the major limitations for testing an HPV16 *Salmonella*-based vaccine in humans was the reported reversibility of the PhoP^c strain, which harbors a single attenuating mutation (PhoQ24 [27]), and the necessity of this phenotype for inducing efficient anti-VLP responses in mice (3, 4). Here we show that other *S. enterica* serovar Typhimurium strains (χ4990, PhoP$^-$, and *aroA*) whose attenuating mutations have been tested in *S. enterica* serovar Typhi and have been shown to be safe in humans (χ4632 [30, 40], Ty800 [15], and CVD908-htrA [41]) can induce anti-VLP and HPV16-neutralizing responses in mice after nasal vaccination. The titers are, however, one or two orders of magnitude lower than those induced by the

PhoP^c strain, which is in agreement with previous findings (3, 4). Whether expression of PhoQ24 (3) may enhance the immunogenicity of the new L1S recombinant strains remains to be tested. Although oral immunization was less efficient than nasal immunization, the immunogenicity of AroA L1S was less affected by immunization by the oral route. This result is highly encouraging as the *S. enterica* serovar Typhi vaccine strains that harbor Aro deletions (CVD908 *htrA*) has the best record of safety and immunogenicity in humans (reviewed in reference 13, 21, and 33). Thus, a recombinant CVD908 *htrA* L1S strain may represent the best candidate oral live vaccine to test in human volunteers for the prophylaxis of HPV16 infections and associated lesions.

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