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

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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, mucosal vaccine vectors to deliver pathogen-specific protective epitopes into the mucosal-associated lymphoid tissues. Via this route, both mucosal and systemic immune responses are obtainable 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 \textit{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 \textit{Salmonella}. Our data show that anti-HPV16 VLP humoral and neutralizing responses after either nasal or oral immunization with the new recombinant strains were highly increased. 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 \textit{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.

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Pho<sup>+</sup> (CS015 [26]), both a kind gift from John Mekalanos, Boston, Mass., and strains y4989 (Δcya Δcrp [4]), y4990 (Δcya Δcrp-cdt [4]), and y9004 (Δcya Δcrp-cdtΔaroA [16]), a kind gift from Irene Corthézy-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<sup>8</sup> to 10<sup>9</sup> CFU) or intranasally (10<sup>6</sup> to 10<sup>7</sup> 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 nucleotide sequence of L1 is shown with the modified codons underlined; modified nucleotides are in bold.

FIG. 1. Codon-optimized HPV16 L1S ORF. The nucleotide sequence of L1S is shown with the modified codons underlined; modified nucleotides are in bold.
lution 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 L1 ORF. From the 506 codons of the original HPV16 L1 sequence which are rarely found most frequently used in sequence (HPV16 114/B [18]), 163 were modified in codons L1 ORF. From the 506 codons of the original HPV16 L1 sequence (27) to generate the recombinant strain, called PhoP c L1S were compared by Western blotting (Fig. 2A). 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 used codons. The L1 ORF was then replaced in plasmid pFS14nsd-HPV16 L1S by the new L1S ORF, yielding in codons of the original L1 sequence which are rarely found most frequently used in sequence (HPV16 114/B [18]), 163 were modified in codons L1 ORF. From the 506 codons of the original HPV16 L1 Typhimurium (7, 14) were considered to design an optimized PhoP c L1S were compared by Western blotting (Fig. 2A).

**TABLE 1. Salmonella strains used in this study**

| Strain (attenuation) | Plasmid electroporated | Abbreviation | Reference |
|----------------------|------------------------|--------------|-----------|
| CS022 (PhoP\(^\text{c}\), \text{pho}-42) | pFS14nsd-HPV16 L1 | PhoP\(^\text{c}\) L1 | This work |
| | pFS14nsd-HPV16 L1S | PhoP\(^\text{c}\) L1S | This work |
| \(\chi4989 (\Delta\text{gya} \Delta\text{crp})\) | pFS14nsd-HPV16 L1 | \(\chi4989\) L1 | This work |
| | pFS14nsd-HPV16 L1S | \(\chi4989\) L1S | This work |
| \(\chi4990 (\Delta\text{gya} \Delta\text{crp-cdf})\) | pFS14nsd-HPV16 L1 | \(\chi4990\) L1 | This work |
| | pFS14nsd-HPV16 L1S | \(\chi4990\) L1S | This work |
| CS015 (PhoP\(^\text{c}\), \Delta\text{phoPQ}) | pFS14nsd-HPV16 L1 | PhoP\(^\text{c}\) L1 | This work |
| | pFS14nsd-HPV16 L1S | PhoP\(^\text{c}\) L1S | This work |
| SL7207 (\text{aroA}) | pFS14nsd-HPV16 L1 | AroA L1 | This work |
| | pFS14nsd-HPV16 L1S | AroA L1S | This work |

**HPV16 L1 and VLP expression.** The expression of the L1 protein in the lysates of exponential cultures of PhoP\(^\text{c}\) L1 and PhoP\(^\text{c}\) 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\(^\text{c}\) L1S and ca. 15 h for PhoP\(^\text{c}\) 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 LIS-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\(^\text{c}\) L1S or PhoP\(^\text{c}\) 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\(^\text{c}\) 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\(^\text{c}\) 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\(^\text{c}\) L1S are shown in Fig. 5. A single nasal vaccination induced high and long-lasting anti-HPV16 VLP immunoglobulin G (IgG) titers.

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The table above lists the Salmonella strains used in this study.
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 PhoPc strain expressing the L1S encoding plasmid, we further introduced this plasmid in different strains including \(^4\)989, \(^4\)990, PhoP\(^-\), and AroA (Table 1 gives precise attenuations, abbreviations, and references). The serum anti-HPV16 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 PhoPc 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-
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 (PhoPc [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 x 10^6 to 2 x 10^7 CFU for the AroA L1S strain to 2 x 10^6 to 2 x 10^7 CFU for the AroA L1 strain) compared to the expression of the original L1 sequence (VLP amounts between 20 and 60 x 10^6 CFU). 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-

### TABLE 2. Recovery of *Salmonella* PhoPc carrying L1- or L1S-encoding plasmids 2 weeks after nasal or oral immunization

| Route of immunization | Organ(s) analyzed | Means of total *Salmonella* recovered<sup>a</sup> | % of *Salmonella* bearing the plasmids: |
|-----------------------|------------------|-----------------------------------------------|-----------------------------------|
|                       |                  | PhoPc L1<sup>b</sup> | PhoPc L1S | PhoPc L1 | PhoPc 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<sup>c</sup> | 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      |

<sup>a</sup> Means (log<sub>10</sub>) of CFU/organ ± standard error of the means.

<sup>b</sup> Data after nasal immunization with PhoPc L1 are taken from Benyacoub et al. (4).

<sup>c</sup> Not detectable.

![FIG. 4. Comparison of serum anti-HPV16 VLP antibody and HPV16 neutralization titers after nasal vaccination with PhoPc L1S or PhoPc L1. Groups of five BALB/c mice were intranasally vaccinated with 10<sup>7</sup> to 10<sup>9</sup> CFU of PhoPc L1S or PhoPc 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<sub>10</sub>) 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.](http://jvi.asm.org/article-pdf/v78/p2004/2004_2007c_9947_08314422.pdf)
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 PhoPc 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...
nasal immunization with PhoPc 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 PhoPc 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 PhoPc 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 (χ4989, PhoPc, 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

![Figure 6](http://jvi.asm.org/Downloaded from http://jvi.asm.org)
PhoP\(^{\text{a}}\) 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 encouraging as the PhoPc strain, which is in agreement with previous findings (3, 6908 BAUD ET AL. J. VIROL.

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