

CODON-OPTIMIZED HPV16 L1 FOR *SALMONELLA* VACCINE STRAINS AGAINST HUMAN PAPILLOMAVIRUS TYPE 16

TECHNICAL FIELD

The present invention relates to a novel nucleic acid sequence (HPV16 L1S) encoding antigenic HPV16 L1 protein as provided in SEQ ID NO: 1, wherein the said sequence has atleast one modified codon for optimum stability of recombinant plasmid vector when transformed in the prokaryotic micro-organism for improved immunogenicity of the resulting prokaryotic micro-organism. The invention also relates to an attenuated strain of a prokaryotic micro-organism transformed with nucleic acid encoding HPV16 (Human Papillomavirus) major capsid protein and expressing the corresponding protein. In addition the invention discloses a process of producing a vaccine based on prokaryotic micro-organism for the treatment of papillomavirus infection and associated risk of cancer.

BACKGROUND AND PRIOR ART REFERENCES

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 sub-set of human papillomaviruses (HPVs), of which HPV16 is found most frequently (6, 41). 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 sub-unit HPV virus-like particle (VLP) vaccine (reviewed by (35) and (24)). A proof of principle 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 those of 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 (11, 22, 36)). We have shown that nasal vaccination of mice with *Salmonella* expressing the HPV 16 major capsid protein L1, that 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 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 as L1S hereafter) encoding the L1 protein with optimized codons for translation in *Salmonella*.

The HPV vaccines based on such VLPs and currently tested in clinical trials have proven to be well tolerated, highly immunogenic, and able to prevent the development of HPV16-induced cervical intraepithelial neoplasia (reviewed by [Schiller, 2004 #1431] and [Lowy, 2003 #1397]). However, these expensive vaccines require multiple intramuscular doses to be efficient and since most of cervical cancers occur in developing countries, such vaccines appear to be inaccessible for people needing them most. It is thus of great importance to develop other strategies that have world wide applicability. Live attenuated *Salmonella* strains may be effective antigen delivery systems, as they are able to express foreign antigens and to elicit mucosal as well as systemic immune responses against homologous and heterologous antigens after oral vaccination (reviewed in [Schödel, 1992 #245; Curtiss, 1994 #466; Levine, 1997 #1416]. In this study, we have further optimized the *Salmonella*-based vaccine against HPV16 so that it could be tested in women. We have first replaced the ampicillin selection marker used for plasmid maintenance by a kanamycine resistance gene that is more acceptable for use in human given its higher biosafety record (FDA approved in 1994 [Administration, 1994 #1522]). Then, we have tested the new plasmid in three *Salmonella enterica* serovar Typhi vaccine strains that have been shown to be safe in human, i.e. Ty21a [Germanier, 1975 #103], the actual licensed typhoid vaccine, as well as Ty800 [Hohmann, 1996 #596] and CVD908htrA [Tacket, 1997 #643, two highly immunogenic typhoid candidate vaccines. Using a model of intranasal immunisation of mice [Galen, 1997 #661], the immune responses elicited by these three strains against the homologous and heterologous antigens were compared. Our data show that anti-HPV16 YLP humoral 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 PhoPc, as shown with other *Salmonella enterica* serovar *Typhimurium* strains whose
5 attenuations are suitable for human use.

BRIEF DESCRIPTION OF ACCOMPANYING DRAWINGS

Figure 1 shows Codon-optimized HPV16 L1S orf. (SEQ ID NO: 1) The nucleotide sequence of L1S is shown with the modified codons underlined and modified nucleotides in
bold.

10 Figure 2 shows HPV16L1 expression in PhoP^c L1 and PhoP^c L1S recombinant strains.

Figure 3 shows L1 and L1S-plasmid stability *in vitro*.

Figure 4 shows Anti-HPV16 VLP systemic (A) and vaginal (B) antibody titers after nasal and oral vaccination with PhoP^c L1S.

15 Figure 5 shows Anti-HPV16 VLP systemic IgG titers after nasal or oral vaccination with %4989 L1S, x4990 L1S, PhoPⁿ L1S and AroA L1S.

Figure 6 is a comparison of serum anti-HPV16 VLP antibody titers after oral vaccination with PhoP^c kan L1S, PhoPⁿ kan L1S and ΔAro A kan L1S.

Figure 7 shows *in vitro* stability of the kan L1S plasmid in different *Salmonella enterica* serovar *Typhi* strains.

20 Figure 8 is comparison of serum anti-HPV16 VLP antibody titers after nasal vaccination with Ty21a kan L1S, Ty800 kan L1S and CVD908htrA kan L1S.

Figure 9 is comparison of HPV16 VLP and Flagellin -specific CD4⁺ T cell proliferations.

Figure 10 shows HPV16-neutralizing and anti-HPV16 VLP antibodies in serum and vaginal secretions of mice nasally vaccinated with Ty21a kan L1S.

25 Figure 11 shows HPV16 neutralizing and anti-HPV16 VLP antibodies in serum and vaginal secretions of mice nasally vaccinated with Ty21a kan L1S alone or primed with purified VLPs.

DETAILS OF TABLES REFERRED IN THE SPECIFICATION

TABLE 1 & 1A refers to *Salmonella* strains used in this study

30 TABLE 2 refers to recovery of *Salmonella* PhoP^c carrying L1- or L1S-encoding plasmids two weeks after nasal or oral immunization

TABLE 2A refers to recovery of *Salmonella* PhoP^c L1S-encoding plasmids carrying the

ampiciline or the kanamycine resistant genes two weeks after oral immunization

TABLE 3 refers to recovery of different *Salmonella enterica* serovar *Typhi* carrying kan L1S plasmids one week after nasal immunization.

5 OBJECTS OF THE INVENTION

The main object of the present invention is to provide a novel nucleic acid sequence (HPV16 L1S) encoding antigenic HPV16 L1 protein as provided in SEQ ID NO: 1, wherein said sequence has at least one modified codon for optimum stability of recombinant plasmid vector when transformed in the prokaryotic micro-organism for improved immunogenicity of the resulting prokaryotic micro-organism.

Another object of the present invention is to construct recombinant vectors pFS14nsdHPV16L1, pFS14nsdHPV16 kan L1S and pFSnsd-kan HinS-HPV16L1S harboring SEQ ID NO: 1, wherein the former carries Ampicillin and the latter, Kanamycin as a selection marker.

15 Yet another object of the invention provides an attenuated strain of a prokaryotic micro-organism transformed with nucleic acid encoding HPV16 (Human Papillomavirus) major capsid protein and expressing the corresponding protein.

One more object of the invention is to provide a process of producing a vaccine based on prokaryotic micro-organism for the treatment of papillomavirus infection and associated risk of cancer.

20 SUMMARY OF INVENTION

Accordingly, the present invention provides a novel nucleic acid sequence (HPV16 L1S) as provided in SEQ ID NO: 1, based on Human Papilloma Virus Type 16 (HPV 16) encoding antigenic HPV16 L1 protein, wherein the said sequence has at least one modified codon for optimum stability of recombinant plasmid vector when transformed in the prokaryotic micro-organism for improved immunogenicity of the resulting prokaryotic micro-organism, preferably the strains belonging to *Salmonella* species. The invention further relates to constructing recombinant vectors pFS14nsdHPV16L1, pFS14nsdHPV16 kan L1S and pFSnsd-kan HinS-HPV16L1S harboring SEQ ID NO: 1, wherein the former carries Ampicillin and the latter, Kanamycin as a selection marker. The invention further provides an attenuated strain belonging to *Salmonella* species transformed with pFS14nsdHPV16L1, pFS14nsdHPV16 kan L1S or pFSnsd-kan HinS-HPV16L1S harboring SEQ ID NO: 1. In addition the invention discloses a process for producing a

Salmonella based vaccine for the treatment of papillomavirus infection and associated risk of cancer.

DETAILED DESCRIPTION OF THE INVENTION

The development of a *Salmonella*-based vaccine against HPV infection and associated lesions would be of great value for a worldwide implementation with the theoretical advantage to induce long-lasting systemic and mucosal immunity with a single oral vaccination. However, despite we showed the feasibility of such a strategy in mice (31), several drawbacks had to be addressed before a *Salmonella*-based vaccine could be safely tested in women. Those drawbacks included the requirement of a particular *Salmonella* phenotype (PhoP^c, (3, 4)) and 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 semi-lethal 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 (HPV16L1S). 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.

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 and 42). The influence of codon usage on the immunogenicity has been recognized for other DNA vaccines (1, 12, 32 and 38), where higher expression of the heterologous genes resulted in higher immunogenicity. As the codon usage of the original HPV 16 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/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. 3µg/10¹¹ CFU for the AroA LIS strain to 23µg/10¹¹ CFU for χ 4989 LIS) as compared to the expression of the original L1 sequence (VLP amounts between 20 and 60µg/10¹¹ CFU, (4)). This is in contrast to the >10⁴ increase in L1 expression obtained in mammalian cells with a human-optimized HPV16 L1 gene (20). We should note however, that we cannot exclude 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 - 10^4 CFU/organ) and the low VLP expression achieved (<1fg/bacteria).

5 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 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 explanation is in agreement with the idea that a longer persistence of antigens
10 in the mucosa-associated lymphoid tissues is a key mechanism that underlies the immune responses elicited by *Salmonella* vaccine strains (34) and contrasts with the other suggestion that the initial amount of antigen that prime 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 (13, 25)). They
15 include the use of *in vivo* inducible promoters or balanced-lethal plasmid stabilisation systems, but to our knowledge codon optimization of heterologous antigens were not previously reported to induce plasmid stabilization.

Interestingly, the plasmid stability and the lower VLP expression were associated with a faster growth rate of the L1S expressing bacteria *in vitro*. It is assumed that the
20 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, thereby increasing the growth rate to the detriment of L1/VLP expression. This
25 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 sub-unit vaccine now in phase III clinical trials
30 (reviewed in (24)). A single nasal immunization with PhoP^c L1S induced similar serum and vaginal anti-HVP16 VLPs IgG titers than three sub-cutaneous injections with 1 µg purified HPV16 VLPs, or three nasal/aerosol immunizations with 5µg 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 human. Here we report that the safer oral route can be used as 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/aerosol 5µg VLP doses without adjuvant (2).

One of the major limitation for testing an HPV16 *Salmonella*-based vaccine in human 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. typhimurium* strains (χ4989, PhoP["] and aroA) whose attenuating mutations have been tested in *S. typhi* and shown to be safe in human (χ4632(30, 39), Ty800 (15) and CVD908-htrA (40)) can induce anti-VLP 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 our previous findings (3, 4). Whether expression of the PhoQ24 (3) may enhance 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 the oral route. This result is highly encouraging as the *S. typhi* vaccine strain that harbor Aro deletions (CVD908 htrA) has the best record of safety and immunogenicity in human (reviewed in (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 prophylaxy of HPV16 infections and associated lesions.

The generation of a prophylactic vaccine against cervical cancer that has a worldwide applicability has been our major aim since many years. Here we report on final improvements in the development of a *Salmonella*-based vaccine i.e. an acceptable selection marker and identification of a safe vaccine strain, that will now permit the testing of such a vaccine in women. The development of recombinant bacterial vaccine has usually required the use of selectable markers. Unfortunately, our attempt to use the aspartate β-semialdehyde dehydrogenase balanced-lethal vector-host system [Curtiss, 1990 #59] to stabilize L1S expression in PhoP^c has failed to induce any VLP-specific immune response (data not shown). We have therefore selected an antibiotic selectable marker i.e.

kanamycin, that has an established biosafety record and whose use has been approved by the FDA [Administration, 1994 #1522]. The kanamycin selectable marker will not give the organisms any selectable advantage outside the laboratory and this phenotype is already quite ubiquitous in nature. In fact, the widespread bacterial resistance to kanamycine has limited the use of this antibiotic in human medicine and the high substrate specificity of the enzyme warrants the absence of development of resistance or interference against modern antibiotic therapies. Interestingly, the presence of the kanamycin resistance gene has further improved the stability of the L1S expressing plasmid in *Phop^c in vivo*, but the immune responses induced by oral immunization with the three attenuated *Salmonella enterica* serovar Typhimurium harboring the kan L1S plasmid were similar to those obtained with the bacteria harboring the ampicillin L1S plasmid [Baud, 2004 #1439].

With the exception of the *Salmonella enterica* serovar Typhimurium *PhoP*- strain that was tested as a recombinant vaccine in human [Angelakopoulos, 2000 #1194], the *Salmonella*-based vaccines are attenuated *Salmonella enterica* serovar Typhi strains that were first developed as vaccines against typhoid fever (reviewed in [Levine, 2001 #1428]). Two of the candidate typhoid vaccines, Ty800 [Hohmann, 1996 #596] and CVD908 *htrA* [Tacket, 1997 #643] harbor attenuations in the same pathways (*PhoPQ* and *aro*, respectively) than the Typhimurium strains (*PhoP*- and *AroA*) and were selected here as potential vaccine carrier for HPV16VLP. In addition we have also tested the licensed vaccine Ty21a. In vitro analysis of the three recombinant strains revealed a similar VLP expression level and a relative plasmid instability in the sense that the kan L1S plasmid is less stable in the Typhi strains than it is in the Typhimurium strains, but it is more stable than the amp plasmid in *PhoPc* [Baud, 2004 #1439]. Plasmid instability in Typhi, but not in Typhimurium was also observed when an urease encoding plasmid was used [Angelakopoulos, 2000 #1194]. Attenuated *Salmonella enterica* serovar Typhi intranasally administered at high doses in mice elicit an array of immune responses similar to those observed in volunteers given the attenuated strains orally (reviewed in [Pasetti, 2003 #1404]). This supports the use of this small animal model to pre-clinically evaluate the immunogenicity and efficacy of live *Salmonella* vaccine candidates. We first used this model to investigate the in vivo stability of the kan L1S plasmid. Relatively high numbers of bacteria were recovered from the lung one week after immunization reflecting the superior lung targeting achieved with nasal vaccination under deep anesthesia [Balmelli,

1998 #930; Londono-Arcila, 2002 #1526], while almost no bacteria were recovered from spleen at this late time point in agreement with previous studies [Pasetti, 2000 #1494; Lee, 2000 #1530; Pickett, 2000 #1158]. Although the kan L1S plasmid was found in all Ty21a bacteria recovered, it was only present in few of the CVD908 htrA and Ty800 bacteria. To our knowledge, experiments with recombinant Ty800 in the nasal murine model were not previously reported, while plasmid stability was previously observed in both Ty21a [Gentshev, 2004 #1531] and CVD908 htrA [Londono-Arcila, 2002 #1526]. The particular instability of kan L1S in CVD908 htrA may be linked to the presence of a constitutive promoter in our plasmid, instead of the *in vivo* inducible nirB promoter which was generally used in other recombinant CVD908 htrA strains [Wang, 2001 #1533; Londono-Arcila, 2002 #1526; Ruiz-Perez, 2002 #1532]. The kan L1S instability in CVC908 htrA and in Ty800 may relate to the low immunogenicity of these bacteria against HVP16 VLPs. Our data clearly show that only the recombinant Ty21a kan L1S is able to induce high anti-HPV16 VLP antibodies and VLP-specific CD4+ T cell responses in the nasal murine mode. In contrast both CVD908 htrA and Ty800 induced higher anti-LPS and anti-Flagellin antibodies, as well as flagellin-specific CD4T cells responses (for Ty800) than Ty21a. This finding is in agreement with previous studies where CVD908 htrA and/or Ty800 and Ty21a were compared in the murine nasal model [Wang, 2001 #1533 or in human.

An embodiment of the present invention relates to synthesis of novel nucleic acid sequences based on HPV (Human Papilloma Virus) such as HPV 16, HPV 18, 45 31 etc., major capsid proteins by modifying their codons for optimum stability of the recombinant plasmid vector when transformed in the prokaryotic micro-organism for improved immunogenicity of the resulting prokaryotic micro-organism. The prokaryotic micro-organisms are preferably selected from a group consisting of *Salmonella sps*, *Escherichia coli*, *Shigella*, *Yersinia*, *Lactobacillus*, *Mycobacteria*, or *Listeria*, preferably *Salmonella sps*. Further, the attenuated strain of *Salmonella sps* is selected from a group consisting of *Salmonella enterica* serovar Typhimurium, *Salmonella typhi*, *Salmonella typhimurium* *Salmonella enterica* serovar dublin or *Salmonella enterica* serovar enteritidis. Additionally, the the *Salmonella* strain is preferably selected from a group consisting of *Salmonella enterica* serovar Typhimurium PhoP^C (CS022), *Salmonella enterica* serovar Typhimurium PhoP^C (CS015), *Salmonella enterica* serovar Typhimurium χ^{4989} (Δ cya Δ crp-cdt and Δ aroA (SL7207)), *Salmonella enterica* serovar Typhi, Ty21a, CVD908 htrA, Ty800.

Yet another embodiment of the present invention relates to a method for improving the immunogenicity of a prokaryotic micro-organism against Human Papillomavirus Type 16 (HPV16) comprising steps of:

- a. synthesizing a novel nucleic acid as shown in SEQ ID NO: 1 encoding antigenic HPV16 L1 protein by modifying codon usage,
- b. constructing recombinant plasmid vector harboring nucleic acid sequence as shown in SEQ ID No: 1 by replacing the original HPV16L1 gene in the plasmid vector pFS14nsdHPV16L1 ,pFS14nsdHPV16 kan L1S, or pFSnsd-kan HinS-HPV16L1,
- c. introducing the recombinant plasmid vector from step (b) into attenuated micro-organism to obtain a recombinant inoculum,
- d. administering recombinant inoculum in mice

Another embodiment relates to an attenuated strain of a prokaryotic micro-organism transformed with codon modified nucleic acid encoding HPV (Human Papillomavirus) major capsid protein and expressing the corresponding protein, wherein atleast one codon is modified. The HPV major capsid protein is selected from a group of HPV strains causing anogenital cancer, wherein the HPV major capsid protein is selected from a group consisting of HPV 6, HPV 11, HPV 16, HPV18, HPV 31 and HPV 45. The above said strain harbors a novel nucleic acid sequence SEQ ID NO: 1.

Yet another embodiment of the present invention relates to a novel nucleic acid having one or more modified codons for optimum stability of recombinant plasmid vector in *Salmonella* strains for improved immunogenicity of the resulting *Salmonella* strain. Preferably, 163 out of 506 original codons have been changed in the HPV16 L1 sequence in the present invention.

Yet another embodiment of the present invention provides a recombinant plasmid vector containing DNA sequences as shown in SEQ ID NO: 1 wherein the said recombinant plasmid vector is either pFS14nsd-HPV16L1S , pFSnsd-HPV16 kan L1S or pFSnsd-kan HinS-HPV16L1.

Another embodiment of the present invention relates to a mode of administration of the *Salmonella* based vaccine against HPV16 which may be selected from group consisting of oral, intra-nasal, vaginal or rectal. One more embodiment of the invention provides the use of attenuated strain of a prokaryotic microorganism in the preparation of a medicament for the prophylactic or therapeutic treatment of papillomavirus infection and associated risk of cancer.

Preferred embodiments of the present invention are now described by way of example and not limitation with reference to the accompanying drawings. Embodiments of the invention

EXAMPLES

Example 1

5 Plasmid constructions and bacterial strains used

The L1S gene was synthesized by Microsynth, Buchs, Switzerland. The open reading frame 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, 10 pFS14nsd HPV16-L1S was introduced by electroporation (37) into the attenuated *Salmonella enterica* serovar Typhimurium strains PhoP^c, (CS022 (27)) and PhoPⁿ (CS015(26)), both a kind gift from John Mekalanos, Boston, USA, x⁴989 (*Acya Acrp*, (4)), x⁴990 (*Acya Acrp-cdt*, (4)) and *AaroA* (SL7207 (16)), a kind gift from Irene Cortesy-Theulaz, Lausanne, CH.

15 HPV16 L1 and VLP analysis

Expression of L1 in *Salmonella* lysates was analyzed by Western blot as previously described 15 (31) using the anti-HPV16 L1 mAb, CAMVIR-1 (Anawa). Data were normalized to the content in bacteria as measured by the OD600 of the cultures. The HPV16 VLP content was measured by a sandwich ELISA as previously described (4) 20 using two monoclonal antibodies that recognize conformational epitopes on HPV16 VLPs, H16E70 and H16 V5, (9). 20

Immunization of mice, analysis of the immune response and recovery of *S. typhimurium*

Six-week old female BALB/c mice from Iffa Credo, France were used in all experiments. Twenty JLLI of bacterial inoculum were administered orally (10^{8-9} CFU) or 25 intranasally (10^{67} 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 *Salmonella enterica* serovar Typhimurium was determined in organs from euthanized mice as previously described (31).

30 Design of an HPV16 L1 nucleotide sequence with codons optimized for translation in *Salmonella*.

The codons used for translation of major endogenous proteins in *Salmonella enterica* serovar. Typhimurium (7, 14) were considered to design an optimized L1 open

reading frame (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* (see Fig. 1-SEQ ID NO: 1). This included all the codons of the original L1 sequence which are rarely found in *Salmonella* (136) and some (27/72) of the less frequently used codons. The L1 orf
5 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 *Salmonella 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 here below) and Table 1 summarizes the different strains
10 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 blot (see Fig. 2A). Surprisingly, expression of L1 in the bacterial cultures was not improved with the new L1S sequence but rather decreased by
15 two-fold (Fig. 2B). This finding was confirmed when the amounts of VLPs produced in the two recombinant strains were compared by sandwich ELISA (see 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 50ml LB with a single colony was compared (ca. 7 hours for PhoP^c L1S and ca. 15 hours for PhoP^c L1). This may suggest that the optimized codon usage of L1
20 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*.

Inventor has 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).
25 The stability of the L1S- and L1- encoding plasmids was first compared *in vitro*. For this purpose the percentage of bacteria still harboring the L1- or L1S- encoding plasmids was compared during four successive O/N cultures in the absence of antibiotic selection (see 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
30 absence of antibiotic selection. The stability of the L1S-encoding plasmid was further examined *in vivo* after nasal and oral immunization of mice (see Table 2). In contrast to the original L1-encoding plasmid (4), the L1S encoding plasmid was completely stable for at

least two weeks in the organs close to the sites of infection/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 none were detected harboring the L1 plasmid. We should also note that there is no evidence of higher invasiveness or persistence of the L1S harboring bacteria, despite the faster growing capacity of these bacteria observed *in vitro*.

Anti-HPV16 VLP systemic and mucosal antibody production following nasal or oral vaccination with PhoP^c L1S.

The final aim was to test whether expression of the HPV16 L1S gene would improve the immunogenicity of the HPV16 VLP antigen in *Salmonella enterica* serovar Typhimurium. We therefore immunized groups of female BALB/c mice via the nasal or oral route with the PhoP^c L1S strain. The anti-VLP antibody titers measured in serum and vaginal secretions of the mice 4 to 6, 8 and 24 weeks after a single immunization are shown in Fig. 4. A single nasal vaccination induced high and long-lasting anti-HPV16 VLP IgG titers 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, but a major improvement is that they are one to two orders of magnitude higher than those achieved with a single 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 or three oral doses of PhoP^c L1S (data not shown) did not increase the immune responses.

Anti-HPV16 VLP systemic antibodies following nasal or oral vaccination with differently attenuated *Salmonella enterica* serovar Typhimurium strains expressing the HPV16 L1S gene.

Inventor have previously shown that nasal vaccination of mice with differently attenuated *Salmonella enterica* serovar Typhimurium strains expressing the original L1 encoding plasmid induced only low 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 %4989, ^4990, PhoPⁿ and AroA (see Table 1 for precise attenuations, abbreviations and references). The anti-HVP16 VLP IgG titers measured in mice 7 weeks after a single nasal or oral vaccination with

these new recombinant strains are shown in Fig. 5. In contrast to our previous observation, all the new recombinant strains induced consistent anti-HPV16 VLP humoral 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 (see Fig. 4). As expected, oral vaccination was less immunogenic, with the exception of the AroA L1S strain, which induced similar anti-HPV16 VLP IgG titers after both routes of vaccination.

Example 2

Plasmid constructions and bacterial strains used

In plasmid, pFS14nsd HPV16-L1S [Baud, 2004 #1439], the ampicillin resistance coding sequence was replaced by a kanamycin resistance coding sequence as follow. A SacII-XbaI fragment containing the kanamycin coding sequence and promoter was generated by PCR using pET-9a (Novagen) plasmid DNA as template. The primers used were a 25-mer located 54 nucleotides upstream from the first ATG of kanamycin and containing a SacII restriction site (underlined): 5'-GGGCCGCGGTGGTCATGAACAATAA-3', and a 28-mer containing a XbaI restriction site (underlined): 5'-GGGTCTAGAAGCTGTCAAACATGAGAAT-3'. Another SacII-XbaI large fragment containing the entire pFS14nsd HPV16-L1S plasmid sequence, but devoid of the ampicillin resistance gene, was generated by inverse PCR with Expand High Fidelity PCR (Roche Molecular Biochemicals) with the following primers: a 28-mer located 92 nucleotides upstream from the ATG of ampicillin and containing a SacII site (underlined) 5'-GGGCCGCGGTTTGTAGAAACGCAAAAAG-3, and a 28-mer containing a XbaI site (underlined) and the stop codon of ampicillin (bold) 5'-GGGTCTAGATCCTAACTGTCAGACCAAG-3'. The two SacII-XbaI fragments were ligated together to generate plasmid pFS14nsd-kan³-HPV16-L1S. The new plasmid was introduced by electroporation [Schödel, 1990b #242] into the attenuated *Salmonella enterica* serovar Typhimurium strains PhoP^c, (CS022 [Miller, 1990 #181]) and PhoP^r (CS015 [Miller, 1989 #178]), and Δ aroA (SL7207 [Hoiseth, 1981 #123]), as well as into the attenuated *Salmonella enterica* serovar Typhi Ty800 [Hohmann, 1996 #596] CVD908htrA [Tacket, 1997 #643], and Ty21a ([Germanier, 1975 #103], Berna biotech, Switzerland).

HPV16 L1 and VLP analysis

Expression of L1 in *Salmonella* lysates was analyzed by Western blot as

previously described [Nardellihaefliger, 1997 #742] using the anti-HPV16 L1 mAb, CAMVIR-1 (Anawa). Data were normalized to the content in bacteria. The HPV16 VLP content was measured by a sandwich ELISA as previously described [Benyacoub, 1999 #1050] using two monoclonal antibodies that recognize conformational epitopes on HPV16 VLPs, H16E70 or H161A and H16 V5, [Christensen, 1996 #1053].

Immunization of mice, analysis of anti-HPV16 VLP antibodies and recovery of *Salmonella*

Six-week old female BALB/c mice from Iffa Credo, France were used in all experiments. Twenty μ l of bacterial inoculum were administered orally (10^9 CFU) or intranasally (10^{7-9} CFU) under anesthesia as previously described [Hopkins, 1995 #381; Nardellihaefliger, 1997 #742]. Sampling of blood and vaginal washes as well as determination of anti-HPV16 VLP antibody titers by ELISA were performed as reported earlier [Hopkins, 1995 #381; Nardellihaefliger, 1997 #742]. Recovery of *Salmonella enterica* serovar Typhimurium or Typhi was determined in organs from euthanized mice as previously described [Nardellihaefliger, 1997 #742].

Neutralization assays

Neutralizations assays were performed with secreted alkaline phosphatase (SEAP) HPV16 pseudoviruses as described in detail in [Pastrana DV, 2004 #1429]. Briefly, optiprep-purified SEAP HPV16 pseudoviruses diluted 2000-fold were incubated on ice for 1 h with two-fold serial serum dilutions, and the pseudovirus-antibody mixtures were used to infect 293TT cells for 3 days. The SEAP content in 10 μ l of clarified cell supernatant was determined using the Great ESCAPE SEAP Chemiluminescence Kit (BD Clontech). Neutralization titers were defined as the reciprocal of the highest serum dilution that caused at least a 50% reduction in SEAP activity (100% SEAP activity ranging from 50 to 100 relative light units).

Proliferation assays.

Splenocytes were isolated by mechanical dissociation as previously described [Balmelli, 2002 #1357]. CD4⁺ T cells were purified by magnetic antibody cell sorting (MACS) with anti-CD4⁺-coated microbeads (Miltenyi Biotec, Galdbach, Germany) according to manufacturer's instructions. CD4⁺ T cells from naïve or immunized mice were incubated for three days with medium alone or Concavalin A (2.5 μ g/ml,) as negative

and positive controls as well as with HPV16 VLPs (2µg/ml, GMP preparation) and Flagellin (10µg/ml, purified from Ty21a) then 0.5µCurie of 3H thymidine was added over night and incorporation was measured in cpm.

Expression of HPV16L1S and plasmid stability in a kanamycin resistant PhoP^c strain.

A kanamycin resistant plasmid expressing HPV16 L1S was constructed by replacing the ampicillin resistant gene in the original pFSnsdHPV16 L1S [Baud, 2004 #1439] by a kanamycin selectable gene. An inverse PCR strategy was used to amplify the entire plasmid flanking the ampicillin resistant gene sequence and the resulting fragment was ligated to a kanamycine resistance encoding sequence (see material and method for details). The resulting plasmid was designated pFSnsd-kan L1S and electroporated in PhoP^c to yield PhoP^c-kanL1S (see Table 1A) for abbreviations and references of the strains used in this study). As expected, *in vitro* expression of HPV16 VLPs in this new strain was similar to the one measured in the ampicillin resistant strain, PhoP^c-L1S (ca 10µgVLPs/10¹¹ CFU, [Baud, 2004 #1439]). Similar growth rates were also observed between the two strains, with about 7 hours to reach mid-log phase and plasmid stability was very high with almost 100% of the bacteria still harboring the plasmid after four consecutive over-night cultures in absence of antibiotic. The stability of the kan L1S plasmid was increased *in vivo*, as compared to the ampicillin plasmid, with all the bacteria harborig the kan L1S plasmid two weeks after oral immunization and this, in all organs examined (see Table 2A).

Anti-HPV16 VLPs humoral responses after oral immunization with PhoP^c, PhoP⁻ and AroA carrying the kan L1S encoding plasmid

The kan L1S plasmid was further introduced in two attenuated *Salmonella enterica* serovar Typhimurium strains, PhoP⁻ and AroA (see Table 1A), whose attenuating mutations in *Salmonella enterica* serovar Typhi vaccine strains have already been shown to be safe in human. Three groups of 5 to 10 mice were immunized once by the oral route with the three recombinant *Salmonella enterica* serovar Typhimurium strains and the HPV16 VLPs specific antibody responses in serum and vaginal washes are compared at 6 weeks post-immunization (see Fig. 6). Interestingly, the anti-VLP antibody titers induced by the AroA kan L1S strain appear as high as those induced by PhoP^c kan L1S and by the former PhoP^c L1S strain [Baud, 2004 #1439]. These antibody titers were stable for at least

four months (data not shown). In contrast the anti-VLPs antibody titers induced by PhoP⁻ kan L1s are lower, as previously reported with PhoP⁻ L1S [Baud, 2004 #1439]. HPV16 VLP specific IgG and IgA were also induced in vaginal secretions of the mice immunized with PhoP^c and Aroa kan L1S, while none were detected after PhoP⁻ kan L1S. These data
5 obtained in the mouse model using oral immunization suggest that recombinant *Salmonella enterica* serovar Typhi harboring Δ aro deletions may be more immunogenic than those harboring Δ PhoP/phoQ deletions.

Expression and stability of the kan L1 S plasmid in three *Salmonella enterica* serovar Typhi vaccine strains

10 We introduced the kan L1S plasmid into three *Salmonella enterica* serovar Typhi vaccine strains which were available to us, i.e. Ty800 (Δ PhoP/phoQ, [Hohmann, 1996 #596]), CVD908-htrA (Δ aroC, Δ aroD, htrA, [Tacket, 1997 #643]) and Ty21a [Germanier, 1975 #103], the licensed typhoid vaccine strain (see Table 1A for abbreviations and references). In vitro expression of VLPs was similar in the three strains
15 (20 to 30 μ g VLP/10¹¹ CFU). The stability of the kan L1S plasmid in the three vaccine strains in absence of antibiotics was first examined in vitro (see Fig.7). In contrast to the previous results with *Salmonella enterica* serovar Typhimurium strains, a lower stability of the kan L1S plasmid was observed in the vaccine strains. A slow loss of plasmid occurred after the second over-night, but after five consecutive overnights, the plasmid
20 was still retained in 9, 7 and 18 % of the bacteria in Ty21a kan L1S, Ty800 kan L1S and CVD908-htrA kan L1S, respectively. *Salmonella enterica* serovar Typhi vaccines strains will only undergo limited rounds or replication in human and they are not invasive by the oral route in mice. Mice can transiently be infected if the bacteria are administered at high doses by the nasal route as shown with recombinant CVD908htrA [Pickett, 2000 #1158]
25 [Pasetti, 2000 #1494]. We have thus examined the stability of the kan L1S plasmid in *Salmonella* recovered from the lung and spleen of mice one week after intranasal immunization with 10⁹ CFU of CVD908-htrA kan L1S and Ty21a kan L1S or 10⁷ CFU of Ty800 kan L1S. This latter strain had to be administered at a lower dose because it was otherwise lethal to the mice, in agreement with previous findings after intraperitoneal
30 injection of such PhoPQ deleted bacteria with hog gastric mucin in mice [Baker, 1997 #659]. Interestingly, all the bacteria recovered from lung after Ty21a kan L1S immunization harbored the kan L1S while after Ty800 kan L1S and CVD908-htrA kan

L1S immunizations few (7.9 and 0.8 %, respectively) harbored the kan L1S plasmid (see Table 3). This indicates that in the nasal murine model the kan L1S plasmid is more stable in Ty21a than in the other *Salmonella enterica* serovar Typhi vaccine strains tested.

Humoral and cellular immune responses induced by *Salmonella enterica* serovar Typhi vaccine strains expressing HPV16 VLPs in the intranasal murine model.

The immunogenicity of the three recombinant *Salmonella enterica* serovar Typhi strains was evaluated in groups of 5 to 10 female BALB/c mice after administration of two monthly spaced intranasal doses (10^9 CFU for CVD908-htra and Ty21a and 10^7 CFU for Ty800 recombinant strains). The IgG titers against the heterologous HPV16 VLP antigen as well as against two homologous antigens, LPS and flagellin, were measured in serum for eight weeks (see Fig 8). Interestingly, high anti-VLP IgG titers were only induced after two immunizations with Ty21a kan L1S, while low or barely detectable titers were induced by the two others strains. In contrast, Ty800 kan L1S and CVD908htra kan L1S induced higher anti-LPS and anti-flagellin IgG titers than the Ty21 kan L1S ($p < 0.01$ at week 8), indicating that the two former recombinant strains have successfully infected the mice and induced humoral responses against the homologous antigens.

T helper responses participate in the generation and maintenance of high antibody titers, therefore cellular immune responses against flagellin and HPV16 VLPs were also examined. Eight weeks after immunization, the mice were killed and antigen specific proliferation was measured with $CD4^+$ T cells purified from the spleen (see Fig.9). HPV16 VLP stimulation of $CD4^+$ T cells was only significantly induced after two intranasal vaccination with Ty21a kan L1S ($p < 0.001$) in agreement with the induction of anti-HPV16 VLPs titers. In contrast, a significant flagellin specific stimulation of $CD4^+$ T cells was only measured after vaccination with Ty800 kan L1S ($p < 0.001$).

HPV16-neutralizing titers are induced in serum and genital secretions of mice immunized with Ty21a kan L1S alone or primed with a subcutaneous VLP dose.

An additional group of 5 female BALB/c mice received two nasal doses of Ty21 kan L1S (ca. 10^9 CFU) at week 0 and 4 and sampling of blood and vaginal secretions was performed at week 8. Anti-HPV16 VLP and HPV16-neutralizing antibody titers were determined by ELISA and the SEAP HPV16 pseudovirion neutralization assay, respectively, in both serum and vaginal secretions (see Fig 10A). As previously reported after immunization with recombinant *Salmonella enterica* serovar typhimurium L1S

strains the HPV16-neutralizing titers were slightly lower than the anti-VLP ELISA titers [Baud, 2004 #1439]. In addition, we show here that anti-HPV16 VLP IgGs and IgAs were induced in vaginal secretions and that those secretions contain HPV16-neutralizing antibodies (see Fig.10-B). We also investigated the effect of a subcutaneous (s.c.) priming with 1µg purified VLPs on the immune response induced by a sub-optimal immunization with Ty21a kan L1S (i.e. a single 10⁹ CFU nasal dose). The data shows (Fig11) that indeed a s.c. priming with 1µg VLPs resulted in a significant increase in the serum anti-VLP IgG titers following a single nasal boosting with Ty21a kan L1S (p < 0.01 as compared to no VLP priming). In contrast, there is no statistical differences in the anti-VLP vaginal IgG induced while anti-VLP vaginal IgA were only induced when both VLP and Ty21a kan L1S were administered. *Neutralizing titers to be determined.*

REFERENCES

1. Andre, S., B. Seed, J. Eberle, W. Schraut, A. Bultmann, and J. Haas. 1998. Increased immune response elicited by DNA vaccination with a synthetic gp120 sequence with optimized codon usage. J. Virol. 72:1497-1503.
2. Balmelli, C, R. Roden, A. Potts, J. Schiller, P. De Grandi, and D. Nardelli-Haeffliger. 1998. Nasal immunization of mice with human papillomavirus type 16 virus-like particles elicits neutralizing antibodies in mucosal secretions. J. Virol. 72(10):8220-8229.
3. Baud, D., J. Benyacoub, V. Revaz, M. Kok, F. Ponci, M. Bobst, R. Curtiss III, P. De Grandi, and D. Nardelli-Haeffliger. 2004. Immunogenicity against human papillomavirus type 16 virus like particles is strongly enhanced by the PhoP^c phenotype in *Salmonella typhimurium*. Infect. Immun. 72:750-756.
4. Benyacoub, J., S. Hopkins, A. Potts, S. Kelly, J.-P. Kraehenbuhl, R. Curtiss, P. De Grandi, and D. Nardelli-Haeffliger. 1999. The nature of the attenuation of *S.typhimurium* strains expressing human papillomavirus type 16 virus like particles determines the specific antibody responses in nasally immunized mice. Infect. Immun. 67:3674-3679.
5. Berg, O. G., and C. G. Kurland. 1997. Growth rate -optimised tRNA abundance and codon usage. J. Mol. Biol. 270:544-550.
6. Bosch, F. X., A. Lorincz, N. Munoz, C. J. Meijer, and K. V. Shah. 2002. The causal relation between human papillomavirus and cervical cancer. J. Clin. Pathol. 55:244-265.
7. Burns, D. M., and I. R. Beacham. 1985. Rare codons in *E.coli* and *S.typhimurium* signal sequences. FEBS lett. 189:318-324.

8. Cardenas, L., U. Dasgupta, and J. D. Clements. 1994. Influence of strain viability and antigen dose on the use of attenuated mutants of *Salmonella* as vaccine carriers. *Vaccine* 12(9):833-40.
9. Christensen, N. D., J. Dillner, C. Eklund, J. J. Carter, G. C. Wipf, C. A. Reed, N. M. Cladel, and D. A. Galloway. 1996. Surface conformational and linear epitopes on HPV-16 and HPV-18 LI virus-like particles as defined by monoclonal antibodies. *Virology*, 223(1): 174-84.
10. Covone, M. G., M. Brocchi, E. Palla, Dias, da, Silveira, W, R. Rappuoli, and C. L. Galeotti. 1998. Levels of expression and immunogenicity of attenuated *Salmonella enterica* serovar typhimurium strains expressing *Escherichia coli* mutant heat-labile enterotoxin. *Infect. Immun.* 66(1):224-31.
11. Curtiss, R., J. O. Hassan, J. Herr, S. M. Kelly, M. Levine, G. G. Mahairas, D. Milich, D. Peterson, F. Schodel, J. Srinivasan, C. Tacket, S. A. Tinge, and R. Wright. 1994. Nonrecombinant and recombinant avirulent *Salmonella* Vaccines, p 340-351. In G. P. e. a. Talwar (ed.), *Recombinant and Synthetic Vaccines*. Narosa Publishing House, New Delhi, India.
12. Demi, L., A. Bojak, S. Steck, M. Graf, J. Wild, R. Schirmbeck, H. Wolf, and R. Wagner. 2001. Multiple effects of codon usage optimization on expression and immunogenicity of DNA candidate vaccines encoding the human immunodeficiency virus type 1 Gag protein. *J. Virol.* 75:10991-11001.
13. Garmory, H., K. A. Brown, and R. W. Titball. 2002. *Salmonella* vaccines for use in humans: present and future perspectives. *FEMS Microbiol. Rev.* 26:339-353.
14. Grosjean, H., and W. Fiers. 1982. Preferential codon usage in prokaryotic genes: the optimal codon-anticodon interaction energy and the selective codon usage in efficiently expressed genes. *Proc. Natl. Acad. Sci. USA* 86:7077-7081.
15. Hohmann, E. L., C. A. Oletta, K. P. Killeen, and S. I. Miller. 1996. Phop/phoQ-deleted *Salmonella typhi* (ty800) is a safe and immunogenic single-dose typhoid fever vaccine in volunteers. *J. Infect. Dis.* 173(6): 1408-1414.
16. Hoiseth, S. K. and B. A. D. Stocker. 1981. Aromatic-dependent *Salmonellatyphimurium* are non-virulent and effective as live vaccines. *Nature* 291:238-239.
17. Hopkins, S., J.-P. Kraehenbuehl, F. Schodel, A. Potts, D. Peterson, P. De Grandi, and D. Nardelli-Haeffliger. 1995. A recombinant *Salmonella typhimurium* vaccine induces local immunity by four different routes of immunization.

Infect. Immun. 63:3279-3286.

18. Kirnbauer, R., J. Taub, H. Greenstone, R. Roden, M. Durst, L. Gissmann, D. R. Lowy, and J. T. Schiller. 1993. Efficient self-assembly of human papillomavirus type 1 LI and L1-L2 into virus-like particles. *J. Virol.* 67:6929-6936.
- 5 19. Koutsky, L. A., D. A. Ault, C. M. Wheeler, D. R. Brown, E. Barr, F. B. Alvarez, L. Chiacchierini, and K. Jansen. 2002. A controlled trial of a human papillomavirus type 16 vaccine. *N. Engl. J. Med.* 347:1645-1651.
- 10 20. Leder, C, J. A. Kleinschmidt, C. Wiethe, and M. Muller. 2001. Enhancement of capsid gene expression: preparing the human papillomavirus type 16 major structural gene L1 for DNA vaccination purposes. *J. Virol.* 75(19):9201-9.
21. Levine, M. M., C. O. Tacket, and M. B. Sztein. 2001. Host-Salmonella interaction: human trials. *Microbes Infect.* 3:1271-1279.
22. Levine, M. M., G. C. Woodrow, J. B. Kaper, and G. S. Cobon. 1997. Attenuated *Salmonella* as a live vector for expression of foreign antigens. Dekker, New York.
- 15 23. Liu, J. W., K. N. Zhao, F. G. Gao, G. R. Leggatt, J. P. Fernando, and I. H. Frazer. 2002. Polynucleotide viral vaccines: codon optimisation and ubiquitin conjugation enhances prophylactic and therapeutic efficacy. *Vaccine* 20:862-869.
24. Lowy, D. R. and I. H. Frazer. 2003. Chapter 16: Prophylactic human papillomavirus vaccines. *J. Natl. Cancer Inst. Monogr.* 31:111-116.
- 20 25. Mastroeni, P., J. A. Chabalgoity, S. J. Dunstan, J. Maskell, and G. Dougan. 2001. *Salmonella*: immune responses and vaccines. *Vet. J.* 161:132-164.
26. Miller, S. L., A. M. Kukral, and J. J. Mekalanos. 1989. A two-component regulatory system (*phoP phoQ*) controls *Salmonella typhimurium* virulence. *Proc. Natl. Acad. Sci. USA* 86:5054-5058.
- 25 27. Miller, S. L., and J. J. Mekalanos. 1990. Constitutive expression of the *phoP* regulon attenuates *Salmonella* virulence and survival within macrophages. *J. Bacteriol.* 172:2485-2489.
28. Nardelli-Haeffliger, D., J. Benyacoub, R. Lemoine, S. Hopkins-Donaldson, A. Potts, F. Hartmann, J.-P. Kraehenbuhl, and P. De Grandi. 2001. Nasal vaccination with attenuated *Salmonella typhimurium* strains expressing the Hepatitis B nucleocapsid: Dose response analysis. *Vaccine*, 19: 2854-2861
- 30 29. Nardelli-Haeffliger, D., R. Roden, C. Balmelli, A. Potts, J. Schiller, and P. De Grandi. 1999. Mucosal but not parenteral immunization with purified human papillomavirus type 16 virus-like particles induces neutralizing titers of antibodies

- throughout the estrous cycle of mice. *J. Virol.* 74:9609-9613.
30. Nardellihaefliger, D., J. P. Kraehenbuhl, R. Curtiss, F. Schodel, A. Potts, S. Kelly, and P. Degrandi. 1996. Oral and rectal immunization of adult female volunteers with a recombinant attenuated salmonella typhi vaccine strain. *Infect. Immun.* 5 64(12):5219-5224.
 31. Nardellihaefliger, D., R. Roden, J. Benyacoub, R. Sahli, J. P. Kraehenbuhl, J. T. Schiller, P. Lachat, A. Potts, and P. Degrandi. 1997. Human papillomavirus type 16 virus-like particles expressed in attenuated *Salmonella typhimurium* elicit mucosal and systemic neutralizing antibodies in mice. *Infect. Immun.* 65(8):3328-3336.
 - 10 32. Narum, D. L., S. N. Kumar, W. O. Rogers, S. R. Fuhrmann, H. Liang, M. Oakley, A. Taye, B. K. Sim, and W. L. Hoffman. 2001. Codon optimization of gene fragments encoding Plasmodium falciparum merozoite proteins enhances DNA vaccine protein expression and immunogenicity in mice. *Infect. Immun.* 69:7250-7253.
 33. Pasetti, M. F., M. M. Levine, and M. B. Sztein. 2003. Animal models paving the 15 way for clinical trials of attenuated *Salmonella enterica* serovar Typhi live oral vaccines and live vectors. *Vaccine* 21:401-418.
 34. Roberts, M., S. N. Chatfield, and G. Dougan. 1994. *Salmonella* as Carriers of Heterologous Antigens, p. 27-58. CRC Press Inc.
 35. Schiller, J. T., and A. Hidesheim. 2000. Developing HPV virus-like particle vaccines to 20 prevent cervical cancer: a progress report. *J. Clinical Virol.* 19:67-74.
 36. Schodel, F. 1992. Prospects for oral vaccination using recombinant bacteria expressing viral epitopes. *Adv. Vir. Res.* 41:409-446.
 37. Schodel, F., G. Enders, M.-C. Jung, and H. Will. 1990b. Recognition of a hepatitis B virus nucleocapsid T-cell epitope expressed as a fusion protein with the subunit B of 25 *Escherichia coli* heat labile enterotoxin in attenuated salmonellae. *Vaccine* 8:569-572.
 38. Stradford, R., G. Douce, L. Zhang-Barber, N. Fairweather, J. Eskola, and G. Dougan. 2000. Influence of codon usage on the immunogenicity of a DNA vaccine against tetanus. *Vaccine* 19:810-815.
 39. Tacket, C. O., S. M. Kelly, F. Schodel, G. Losonsky, J. P. Nataro, R. Edelman, M. 30 M. Levine, and R. Curtiss. 1997. Safety and immunogenicity in humans of an attenuated salmonella typhi vaccine vector strain expressing plasmid-encoded hepatitis b antigens stabilized by the asd-balanced lethal vector system. *Infect. Immun.* 65(9):3383.
 40. Tacket, C. O., M. B. Sztein, G. A. Losonsky, S. S. Wasserman, J. P. Nataro, R.

Edelman, D. Pickard, G. Dougan, S. N. Chatfield, and M. M. Levine. 1997. Safety of live oral salmonella typhi vaccine strains with deletions in *htrA* and *aroC* and immune response in humans. *Infect. Immun.* 65(2):452-456.

41. Walboomers, J. M., M. V. Jacobs, M. M. Manos, F. X. Bosch, J. A. Kummer, K. V. Shah, P. J. Snijders, J. Peto, C. J. Meijer, and N. Munoz. 1999. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J. Pathol.* 189(1): 12-9.
42. Zhou, J., W. J. Liu, S. W. Peng, X. Y. Sun, and I. Frazer. 1999. Papillomavirus capsid protein expression level depends on the match between codon usage and tRNA availability. *J. Virol.* 73(6):4972-4982.

TABLE 1: Salmonella strains used in this study

Strains (attenuations)	Plasmid electroporated	Abbreviations	References
CS022 (PhoP ^c , <i>pho-42</i>)		PhoP ^c	(26)
"	pFS14nsd-HPV16L1	PhoP ^c L1	(31)
"	pFS14nsd-HPV16L1S	PhoP ^c L1S	This work
χ^{4989} ($\Delta cya \Delta crp$)			(4)
"	pFS14nsd-HPV16L1	χ^{4989} L1	(4)
"	pFS14nsd-HPV16L1S	χ^{4989} L1S	This work
χ^{4990} ($\Delta cya \Delta crp-cdt$)			(4)
"	pFS14nsd-HPV16L1	χ^{4990} L1	(4)
"	pFS14nsd-HPV16L1S	χ^{4990} L1S	This work
CS015(PhoP ^r , $\Delta phoPQ$)			(26)
"	pFS14nsd-HPV16L1	PhoP ^r L1	(4)
"	pFS14nsd-HPV16L1S	PhoP ^r L1S	This work
SL7207 ($\Delta aroA$)			(16)
"	pFS14nsd-HPV16L1	AroA L1	This work
"	pFS14nsd-HPV16L1S	AroA L1S	This work

TABLE 1 A: Salmonella strains used in this study

Strains (attenuations)	Plasmid electroporated	Abbreviations	References
<i>Salmonella enterica</i> serovar <i>Typhimurium</i>			
CS022 (PhoP ^c , <i>pho-42</i>)		PhoP ^c	(miller)
"	pFS14nsd-HPV16 L1S		Baud 04
"	pFS14nsd-kan ³ -HPV16 L1S	PhoP ^c kanL1S	This work
GL01 (PhoP ^c Δ asd)		GL01	(revaz)
"	PYA3342-HPV16L1	GL01L1	(REVAZ)
"	PYA3342-HPV16L1S	GL01L1S	This work
CS015 (PhoP ^c , Δ <i>phoPQ</i>)			(26)
"	pFS14nsd-HPV16 L1S	PhoP ^c L1	(4)
"	pFS14nsd-kan ³ -HPV16 L1S	PhoP ^c kanL1S	This work
SL7207 (Δ <i>aroA</i>)			(16)
"	pFS14nsd-HPV16 L1S	AroA L1S	Baud 04
"	pFS14nsd-kan ³ -HPV16 L1S	AroA kanL1S	This work
<i>Salmonella enterica</i> serovar <i>Typhi</i>			
Ty800 (Δ <i>phoPQ</i>)		Ty800	Boston..
"	pFS14nsd-kan ³ -HPV16 L1S	Ty800kanL1S	This work
CVD908-htrA (Δ <i>aroC</i> , Δ <i>aroD</i> , Δ <i>htrA</i>)		CVD908htrA	M levine
"	pFS14nsd-kan ³ -HPV16 L1S	CVD908htrAkanL1S	This work
Ty21a		Ty21a	Berna
"	pFS14nsd-kan ³ -HPV16 L1S	Ty21akanL1S	This work

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

Route or immunization	Organs analyzed	Means of total <i>Salmonella</i> recovered ^a		% of <i>Salmonella</i> 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 (log₁₀) of CFU/organ ± SEM; ^b data after nasal immunization with PhoP^c L1 are taken from [Benyacoub, 1999#1050].; ^c Not detectable

TABLE 2A: Recovery of *Salmonella* PhoP^c L1S-encoding plasmids carrying the ampiciline or the kanamycine resistant genes two weeks after oral immunization.

Route or immunization	Organs analyzed	Means of total <i>Salmonella</i> recovered ^a		% of <i>Salmonella</i> bearing the plasmid	
		PhoPc kan L1S	PhoP ^c L1S ^b	PhoPc kan L1S	PhoP ^c L1S ^b
oral	Peyer's patches	3.31 ± 0.23	2.38 ± 0.29	100	100
	Mesenteric lymph nodes	2.93 ± 0.41	2.67 ± 0.15	100	27
	Spleen	2.61 ± 0.13	2.46 ± 0.12	100	16

^a means (log₁₀) of CFU/organ ± SEM

^b data taken from Baud et al. 2004

TABLE 3: Recovery of different *Salmonella enterica* serovar Typhi carrying kan L1S plasmids one week after nasal immunization.

<i>Salmonella enterica</i> serovar Typhi strain	Organs analyzed	Means of total <i>Salmonella</i> recovered ^a	% of <i>Salmonella</i> bearing the plasmid
Ty21a kan L1 S	Lung	4.25 ± 0.06	100
	Spleen	ND	
CVD908 htrA kan L1 S	Lung	4.4 ± 0.07	0.8
	Spleen	1.55 ± 0.18	ND
Ty800 kan L1S	Lung	5.75 ± 0.05	7.9
	Spleen	ND	

^a means (log₁₀) of CFU/organ ± SEM

ND none detected (<1.3 CFU/organ)

We Claim:

1. A novel nucleic acid sequence as shown in SEQ ID NO: 1 encoding antigenic HPV16 L1 protein and having one or more modified codon compared to the wild-type sequence, wherein said modified sequence stabilizes a recombinant plasmid vector harboring said modified sequence when introduced in a prokaryotic microorganism.
2. A recombinant plasmid vector comprising nucleic acid sequence as claimed in claim 1.
3. A recombinant plasmid vector as claimed in claim 2, wherein said recombinant plasmid vector is selected from a group consisting of pFS14nsd-HPV16L1 S and pFSnsd-HPV16 kan L1S.
4. A prokaryotic microorganism comprising a recombinant plasmid vector as claimed in claim 2.
5. The prokaryotic microorganism as claimed in claim 4, wherein the prokaryotic microorganism is selected from a group consisting of *Salmonella sps*, *Escherichia coli*, *Shigella*, *Yersinia*, *Lactobacillus*, *Mycobacteria* and *Listeria*.
6. The prokaryotic micro-organism as claimed in claim 5, wherein said *Salmonella sps* is selected from a group consisting of *Salmonella enterica* serover *typhi*, *Salmonella enterica* serover *typhimurium*, *Salmonella enterica* serover *dublin* and *Salmonella enterica* serover *enteritidis*.
7. The prokaryotic microorganism as claimed in claim 6, wherein the strain of *Salmonella sps* is selected from a group consisting of *Salmonella enterica* serovar Typhimurium PhoP^C (CS022), *Salmonella enterica* serovar Typhimurium PhoP⁻ (CS015), *Salmonella enterica* serovar Typhimurium χ^{4989} (Δ cya Δ crp-cdt and Δ aroA (SL7207)), *Salmonella enterica* serovar Typhi Ty21a, CVD908 htrA, Ty800.

8. An attenuated strain of a recombinant prokaryotic microorganism comprising codon modified nucleic acid sequence encoding HPV (Human Papillomavirus) major capsid protein and expressing corresponding protein, wherein said modified nucleic acid sequence stabilizes a recombinant plasmid vector harboring said modified nucleic acid sequence when introduced in a prokaryotic microorganism.
9. The attenuated strain as claimed in claim 8, wherein the HPV major capsid protein is selected from a group consisting of HPV strains , HPV 16, HPV 18, HPV 31 and HPV 45.
10. The attenuated strain as claimed in claim 8, wherein said prokaryotic microorganism is selected from a group consisting of *Salmonella sps*, *Escherichia coli*, *Shigella*, *Yersinia*, *Lactobacillus*, *Mycobacteria*, and *Listeria*.
11. The attenuated strain as claimed in claim 10, wherein the *Salmonella sps* is selected from a group consisting of *Salmonella enterica* serovar Typhimurium PhoP^C (CS022), *Salmonella enterica* serovar Typhimurium PhoP (CS015), *Salmonella enterica* serovar Typhimurium χ^{4989} (Δ cya Δ crp-cdt and Δ aroA (SL7207)), and the *Salmonella enterica* serovar Typhi, Ty21a, CVD908 htrA, Ty800.
12. A method for producing a recombinant microorganism for use as vaccine comprising of:
 - a. synthesizing a novel nucleic acid as shown in SEQ ID NO: 1 encoding antigenic HPV16 L1 protein,
 - b. constructing recombinant plasmid vector harboring nucleic acid sequences as shown in SEQ ID No: 1 by replacing the original HPV16L1 gene in the plasmid vector pFS14nsdHPV16L1 or pFS14nsdHPV16 kan L1, and
 - c. introducing the recombinant plasmid vector into a prokaryotic microorganism to obtain a recombinant microorganism.
13. The method as claimed in claim 12, wherein the introduction of the recombinant plasmid vector is carried out by electroporation.

14. The method as claimed in claim 12, wherein the recombinant plasmid is either pFSI4nsdHPV16L1 S or pFSI4nsdHPV16 kan L1S.
15. The method as claimed in claim 12, wherein the prokaryotic microorganism is selected from a group consisting of *Salmonella* *sps*, *Escherichia coli*, *Shigella*, *Yersinia*, *Lactobacillus*, *Mycobacteria*, and *Listeria*.
16. The method as claimed in claim 12, wherein, the *Salmonella* *sps* is selected from a group consisting of *Salmonella enterica* serovar Typhimurium PhoP^C (CS022), *Salmonella enterica* serovar Typhimurium PhoP (CS015), *Salmonella enterica* serovar Typhimurium χ^{4989} (Δ cya Δ crp-cdt and Δ aroA (SL7207)) and the *Salmonella enterica* serovar Typhi, Ty21 a, CVD908 htrA, Ty800.
17. Use of attenuated strain of a prokaryotic microorganism of any one of the claims 4 to 10, in the preparation of a medicament for the prophylactic or therapeutic treatment of papillomavirus infection and associated risk of cancer.