Construction and Characterization of a *Salmonella enterica* Serovar Typhimurium Clone Expressing a Salivary Adhesin of *Streptococcus mutans* under Control of the Anaerobically Inducible nirB Promoter

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Attenuated *Salmonella enterica* serovar Typhimurium has been used for targeted delivery of recombinant antigens to the gut-associated lymphoid tissues. One potential problem associated with this vaccine approach is the likelihood of in vivo instability of the plasmid constructs caused by constitutive hypereexpression of the heterologous immunogen. The aim of this study was to generate and characterize an expression system encoding the saliva-binding region (SBR) of *Streptococcus mutans* antigen I/II adhesin, either alone or linked with the mucosal adjuvant cholera toxin A2/B subunits (CTA2/B), under the control of the inducible nirB promoter. This promoter is activated in an anaerobic environment and within macrophages, which are the primary antigen-presenting cells involved in phagocytosis and processing of *Salmonella*. The gene encoding the chimeric SBR-CTA2/B was amplified by PCR using primers containing appropriate restriction sites for subcloning into pTETnirB, which contains the nirB promoter. The resulting plasmid was introduced into serovar Typhimurium by electroporation. Production of the SBR-CTA2/B chimeric protein under anaerobic conditions was verified by enzyme-linked immunosorbent assay of whole-cell lysates on plates coated with G\(_{\text{M1}}\) ganglioside and developed with antibodies to SBR. Similar procedures were followed for cloning the gene encoding SBR in serovar Typhimurium under nirB control. Anaerobic expression of SBR was confirmed by Western blotting of whole-cell lysates probed with anti-SBR antibodies. The resulting serovar Typhimurium strains were administered by either the oral or the intranasal route to mice, and colonization was assessed by microbiologic analysis of dissociated spleens, Peyer’s patches (PP), and nasal tissues. High numbers of the recombinant strains persisted in PP and spleen for at least 21 days following oral challenge. A single intranasal administration of the *Salmonella* clones to mice also resulted in the colonization of the nasal tissues by the recombinant bacteria. *Salmonella* were recovered from nasal lymphoid tissues, superficial lymph nodes, internal jugular lymph nodes, PP, and spleens of mice for at least 21 days after challenge. This study provides quantitative evidence for colonization by *Salmonella* strains expressing a recombinant protein under the control of the inducible nirB promoter in PP or nasal tissues following a single oral or nasal administration of the bacteria, respectively.

Soluble proteins are usually ineffective immunogens when given perorally due to their breakdown by low pH in the stomach and by digestive enzymes in the gut where their uptake is generally poor. One way to overcome these problems is through the use of live attenuated *Salmonella enterica* serovar Typhimurium strains as foreign antigen delivery systems (10, 19). These serovar Typhimurium strains are genetically engineered to express protein antigens from other virulent organisms against which mucosal immunity is desired. Orally administered *Salmonella* strains can actively invade enterocytes but preferentially enter the lymphoid inductive sites of the Peyer’s patches (PP) through specialized microfold (M) cells (3, 14). Salmonellae are capable of replicating and persisting in the PP and thus presumably serve as a source of immunogen production at these mucosal inductive sites. They often take residence in macrophages not only in the PP but also in various other organs, such as the spleen, the liver, and regional lymph nodes, and consequently may also induce systemic immune responses (19).

Although the oral route is the traditional mode of *Salmonella* infection, recent evidence indicates the effectiveness of the intranasal (i.n.) route of immunization with a *Salmonella*-based vaccine for the induction of a mucosal immune response (11, 12). In fact, it has been reported that the i.n. route was as effective as the intragastric (i.g.) route in inducing mucosal immunoglobulin A (IgA) and serum IgG antibody responses to a cloned heterologous immunogen, despite the use of a smaller (10-times-less) inoculum (11). The nasal lymphoid tissue (NALT) also contains M cells (16), and presumably, serovar Typhimurium enters these inductive sites in a way similar to the invasion of the PP. Active invasion of the inductive sites is an essential attribute of live antigen delivery systems even for i.n. immunizations, since a substantial mucosal and serum antibody response to a cloned heterologous antigen was induced when invasive serovar Typhimurium, but not noninvasive *Escherichia coli*, was used as the vector for i.n. immunization (G. Hajishengallis, E. Harokopakis, T. E. Greenway, and S. M. Michalek, Abstr. 97th Gen. Meet. Am. Soc. Microbiol. 1997, abstr. E-91, 1997).

The abilities of a live antigen delivery system to invade the appropriate host tissues and to persist there while continuing to produce the foreign immunogen are considered to be significant advantages for vaccine development. However, unreg-

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ulated hyperexpression of the foreign protein is usually toxic for the bacterial vector and may result in deletion of the cloned gene or loss of the plasmid from the vector. Although inducible promoters, such as trp and lac, can be used in vitro to control the expression of cloned antigens in recombinant bacteria, the requirement of exogenous inducers (e.g., isopropylthiogalactoside for the lac promoter) renders these approaches prohibitive for in vivo immunization. For the development of Salmonella-based vaccines, Chatfield and coworkers have utilized an in vivo inducible promoter, the nirB promoter (4), which is activated in anaerobic environments and inside eukaryotic cells including the macrophages (6). The nirB promoter was shown to direct stable expression of fragment C of tetanus toxin, which induced protective serum IgG antibodies against tetanus toxin challenge in orally vaccinated mice (4).

Our group has been interested in developing a mucosal vaccine against Streptococcus mutans-induced dental caries (9). One approach involves the delivery of the vaccine by an attenuated serovar Typhimurium vector. A 42-kDa saliva-binding region (SBR) from S. mutans surface antigen I/II is considered to be a reasonable target for immunological intervention against caries, since this protein segment appears to mediate the initial adherence of S. mutans to the saliva-coated tooth surfaces (5, 8). A previously constructed serovar Typhimurium clone expressing SBR, or SBR linked to the A2/B subunits of the mucosal adjuvant cholera toxin (CT), under the control of the bacteriophage T7 promoter, induced salivary IgA antibodies to SBR (11). However, repeated mucosal administrations and a booster immunization were required for the induction of substantial IgA antibody levels.

The objective of this paper was to place the expression of SBR and SBR-CTA2/B under the control of the nirB promoter in attenuated serovar Typhimurium and to characterize the heterologous immunogen expression and vector colonization in mucosal inductive sites following i.g. or i.n. immunization of mice. We hypothesized that the replacement of the T7 promoter by the nirB promoter in these SBR- and SBR-CTA2/B-expressing serovar Typhimurium delivery systems would prolong the viability of the clones.

**MATERIALS AND METHODS**

**Genetic construction.** A previously constructed plasmid, pSBR-CTA2/DA1 (7), was used as the template for PCR amplification of the gene segments encoding SBR or SBR-CTA2/B (Fig. 1). Primers were selected with the help of the Oligo 4.03 primer analysis program (National Biosciences Inc., Plymouth, Minn.). For the amplification of both SBR and SBR-CTA2/B gene segments, the upper primers containing an Apal restriction site was originally designed to start immediately upstream of the Shine-Dalgarno (SD) sequence in the vector containing SBR-CTA2/B. It was later redesigned to be further upstream from the translation start site (5′ position 3318, 5′-TAACGGGCCCAGATCTCGATCCCGCGAAA) in order to avoid mRNA secondary structure problems and to provide optimal expression of the SBR cloned antigen. The lower primers for PCR amplification were designed to contain the Nhel restriction site. Lower primer 1 (5′ position 4656, 5′-GCATAGCTAGCACAATAATCCCCATAAA) and lower primer 2 (5′ position 5364, 5′-GCCATAGCTAGCATAATACGCACTAA) were used to amplify the gene segments encoding SBR and SBR-CTA2/B, respectively. The PCR was conducted on an automated thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.) for 35 cycles with the following parameters: (i) denaturation, 95°C for 1 min; (ii) primer annealing, 56.5 or 55.9°C for SBR or SBR-CTA2/B
gene segment, respectively, for 1 min; and (iii) primer extension, 72°C for 3 min. The resulting PCR products (1.4 and 2.1 kb, corresponding to SBR and SBR-CTA2/B, respectively) were ligated with pGEM-T (Promega, Madison, Wis.) and transformed into competent E. coli JM109. Transformants were grown and plated on Luria-Bertani (LB) agar plates (1% tryptone, 0.5% yeast extract, 1% NaCl, 1.8% agar) containing isopropylthio-β-D-galactoside (IPTG), 5-bromo-4-chloro-3-indolyl-β-D-galactoside, and 50 μg of carbenicillin per ml. White colonies were used for plasmid preparations by means of the Wizard Miniprep DNA purification system (Promega), and the existence of appropriate inserts was verified by ApoI and NheI digestions followed by gel electrophoresis. The inserts generated by restriction enzyme digestion with ApoI and NheI were purified using the QIAEX II gel extraction kit (QIA Genetech, Hong Kong) cloning vector pTETn15 (kindly provided by S. Chatfield, Medeva Vaccine Research Group, London, United Kingdom), which contained ApoI and NheI restriction sites at the 5′ and 3′ ends of the DNA sequence encoding the fragment C of tetanus toxin, respectively, was restriction digested at these sites using appropriate enzymes. The isolated inserts encoding SBR or SBR-CTA2/B were ligated with the linearized pTETn15 vector (after removal of the sequence encoding fragment C) via the ApoI and NheI restriction sites. Upon purification, the resulting pSBRn15 and pSBR-CTA2n15 plasmids were introduced into serovar Typhimurium BRD509, an arco arn0 mutant attenuated vaccine strain (23), by means of electroporation. The resulting Salmonella clones were confirmed by plasmid analysis, which demonstrated the presence of plasmids having the anticipated size and by Western blotting of protein extracts using antibodies to SBR and CTB.

Generation of rabbit IgG anti-SBR antibodies. To quantify the expression of SBR by the recombinant serovar Typhimurium clones using a sandwich enzyme-linked immunosorbent assay (ELISA), rabbit IgG antibody specific for SBR was generated. Recombinant SBR, which contains a six-His-residue tag (derived from the vector) at its C terminus, was inductively expressed with IPTG and purified from cell lysates of E. coli BL21(DE3)(pSBR) (11). SBR was purified from the cell lysates by a nickel-charged affinity chromatography column (Novagen, Madison, Wis.) according to the manufacturer’s instructions and eluted with imidazole. The quality of the purification was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 12.5% polyacrylamide gel. The amount of purified SBR was quantified by the bicinchoninic acid protein determination assay (Pierce, Rockford, Ill.), using bovine serum albumin as the standard. Purified SBR was then used to hyperimmunize rabbits in order to obtain IgG antibody against SBR. All animal work was performed according to the National Institutes of Health guidelines, and protocols were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee. On day 0, 100 μg of protein emulsified in complete Freund’s adjuvant was given subcutaneously, and on days 14, 28, and 42, the same amount of SBR was given with incomplete Freund’s adjuvant. On day 56, blood was collected via cardiac puncture, and serum was collected after centrifugation. The resulting Salmonella clones were confirmed by plasmid analysis, which demonstrated the presence of plasmids having the anticipated size and by Western blotting of protein extracts using antibodies to SBR and CTB.

Optimization of the expression of SBR in the recombinant serovar Typhimurium clones. The chimeric SBR protein was cloned into plasmid pBRD509(SBR-CTA2/Bear) under in vitro anaerobic conditions was determined by assaying whole-cell lysates on plates coated with GM1 ganglioside under in vitro anaerobic conditions subsequently conjugated to horseradish peroxidase (HRP) (1). The optical density at 450 nm (OD450) in which the sequence encoding fragment C was processed as described above. Each was treated to load an equivalent amount of soluble extracts on the sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel (24). Proteins were resolved by electrophoresis from each lane and subsequently transferred to nitrocellulose membranes. Rabbit IgG anti-SBR antibodies followed by goat anti-rabbit IgG antibody was used to detect the presence of SBR or SBR-CTA2/B (from the SBR-CTA2/B clone). A serovar Typhimurium clone, BRD509(pTETn15), which carried an unrelated antigen (fragment C of tetanus toxin) was similarly processed and used as a negative control.

RESULTS

Expression of recombinant proteins. The gene sequences encoding the S. mutans adhesin SBR or the chimeric SBR-CTA2/B were PCR amplified and placed into vector plasmid pTETn15 from which the sequence encoding fragment C was removed. The restriction maps of the plasmids purified from the resulting clones indicated a 2.3-kb pTETn15 vector and a 1.4-kb fragment representing SBR or a 1.5-kb fragment encoding the SBR-CTA2/B fragment as predicted. The expression of SBR by the serovar Typhimurium BRD509(pSBRn15B) clone was enhanced under anaerobic growth conditions at 37°C (11.3 μg/g 109 cells, in comparison to 0.1 μg/g 109 cells under aerobic conditions) (Table 1); at 37°C, a similar 10-fold difference in SBR production was observed between the anaerobic and aerobic growth conditions. The highest production of SBR was seen in cultures grown at 37°C under anaerobic conditions. The greatest production of chimeric SBR-CTA2/B by the serovar Typhimurium BRD509(pSBR-CTA2/Ber) clone was seen in cultures grown at 30°C under anaerobic conditions (Table 1). Furthermore, the production of SBR-CTA2/B was similar whether grown with or without antibiotic selection.
Results obtained from Western blots probed with rabbit IgG antibody against recombinant SBR also indicated that the production of SBR or SBR-CTA2/B was efficiently induced under anaerobic but not aerobic growth conditions (Fig. 2). When assessed by ELISA, a very low level of expression was observed by the clones when cultured under aerobic growth conditions (Table 1). As expected, no expression of SBR was seen under any conditions with the control strain carrying pTET-20.

### TABLE 1. Inducible expression of SBR or SBR-CTA2/B in serovar Typhimurium clones

<table>
<thead>
<tr>
<th>Clone</th>
<th>Protein amount (µg/10⁶ cells) with growth temp (°C) and condition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anaerobic</td>
</tr>
<tr>
<td>pSBRnirB</td>
<td>11.3</td>
</tr>
<tr>
<td>pSBR-CTA2/BnirB</td>
<td>39.8</td>
</tr>
<tr>
<td>pSBR-CTA2/BnirB/</td>
<td>30.1</td>
</tr>
</tbody>
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* a Detected with a sandwich ELISA using plates coated with rabbit IgG anti-SBR and developed with rabbit Ig anti-SBR-HRP.
* b Detected with GSα ELISA. 
* c GM1 ganglioside was used for coating, and rabbit IgG anti-SBR-HRP was used for detection.
* d Cultures were grown without antibiotic selection.
* e Data are the means of duplicate determinations.

Intestinal colonization of serovar Typhimurium following i.e. administration. The Salmonella clones were found to persist in the PP and spleen for at least 3 weeks after their oral administration to mice. Comparable degrees of colonization were observed in the PP and spleen for both serovar Typhimurium BRD509(pSBR-CTA2/BnirB) and BRD509(pSBRnirB) clones which peaked at day 10 (Fig. 3A). In the spleens, the colonization of the serovar Typhimurium BRD509(pSBR-CTA2/BnirB) clone peaked at day 5 with 348 colonies recovered from the organs removed from the animals (Fig. 3B). However, the colonization of the serovar Typhimurium BRD509(pSBRnirB) clone did not peak until day 10 (450 CFU). Western blotting verified that SBR was expressed by both clones isolated either from PP or from spleens for at least 21 days (Fig. 4A). The Salmonella clones expressing SBR or the chimeric SBR-CTA2/B under the control of the T7 temperature-sensitive promoter produce an enormous amount of recombinant protein in vitro when transferred from 30 to 37°C, but at the expense of cell viability (11). These clones lose most of their viability after transfer to 37°C and overnight incubation. As expected from the in vitro observations, the serovar Typhimurium BRD509(pGP1-2/pSBR-CTA/B) and BRD509(pGP1-2/pSBR) clones were not recovered from either tissue at any time point tested.

### DISCUSSION

Stable expression of cloned antigens is an important aspect in the development of live vaccines carrying heterologous recombinant protein. Since overexpression of the cloned protein in vivo often results in rapid loss of the plasmid, one way to overcome this possibility is to use an environmentally inducible promoter for the induction of protein expression (4, 21). One promoter that is regulated by the host’s environment is the nirB promoter. It has been shown to induce the production of various immunogens in serovar Typhimurium under anaerobic conditions (4, 20, 21) or inside eukaryotic cells (6). In vivo evidence suggests that the nirB promoter can be a highly efficient expression system for live vaccine delivery.

In the present study, we have placed genes encoding 5.
*mutans* SBR or a chimeric protein comprising SBR linked to CTA2/B subunits (SBR-CTA2/B) under the control of the anaerobic inducible promoter *nirB*. The expression of these recombinant proteins was efficiently induced under anaerobic growth conditions at 30 or 37°C. However, more SBR production by the serovar Typhimurium BRD509(pSBR*nirB*) clone was detected at 37 than at 30°C. In contrast, the production of chimeric protein by the serovar Typhimurium BRD509(pSBR-CTA2/B*nirB*) clone was higher at the lower temperature. The difference in the SBR-CTA2/B production at different temperatures may be explained by the possible higher proteolytic activity at the higher temperature, which would degrade a portion of SBR and dissociate it from the chimeric molecule. Therefore, dissociated SBR fragments could not be detected by the *G* *M*1 ELISA but could still be detected in a sandwich ELISA using plates coated with IgG anti-SBR antibody. Evidence for the production of free SBR by the serovar Typhimurium BRD509(pSBR-CTA2/B*nirB*) clone further proves this hypothesis. While the total production of SBR (detected by sandwich ELISA) by the serovar Typhimurium BRD509 (pSBR-CTA2/B*nirB*) clone was similar to the production of SBR detected by *G* *M*1 ELISA (representing the SBR associated with CTA2/B) at 30°C (31.2 and 39.8 μg/10¹⁰ cells, respectively), the level of total SBR production detected was much higher than the SBR content of SBR-CTA2/B detected by *G* *M*1 ELISA at 37°C (99.3 and 26.0 μg/10¹⁰ cells, respectively).

Another important aspect of the live vaccine delivery system is the need to prolong the presence of the antigens at the local immune inductive sites in order to elicit long-lasting immune responses. The natural route of entry into the host by live *Salmonella* strains is through invasion of enterocytes or the M cells overlying the lymphoid tissues of PP in the gut-associated lymphoid tissues (13, 14). Attenuated *Salmonella* vaccine strains are able to survive within macrophages and thereby provide a source of cloned immunogen to antigen-presenting cells. Professional antigen-presenting cells then are able to present the antigen peptide to lymphocytes to induce their differentiation and to elicit appropriate immune responses. In our *Salmonella* delivery system, bacteria were recovered from PP and spleens of the animals for at least 21 days after i.g. challenge, and the bacterial colonies recovered from the mice

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**FIG. 3.** Viable serovar Typhimurium recovered from PP (A) and spleens (B) of mice after i.g. infection. No serovar Typhimurium organisms were recovered from uninfected controls. Dilutions of homogenized tissues were plated on LB medium supplemented with 50 μg of carbenicillin (Cb) per ml or on BS medium. Results are shown as geometric means for two mice at any given time point.

**FIG. 4.** Western blot of crude extracts of SBR expressed by serovar Typhimurium recovered from murine tissues 21 days after the animals were infected with 10¹⁰ CFU of bacteria i.g. (A) or 10⁹ CFU of bacteria i.n. (B). Isolates were cultured in LB broth containing 50 μg of carbenicillin per ml at 37°C. (A) Lanes 1 and 2, extracts from clones pSBR-CTA2/B*nirB* and pSBR*nirB*, respectively, recovered from PP; lanes 3 and 4, extracts from clones pSBR-CTA2/B*nirB* and pSBR*nirB*, respectively, grown from freeze cultures; lane 5, extract from a clone carrying unrelated pTE15 plasmid. (B) Lanes 1 and 2, extracts from clones pSBR-CTA2/B*nirB* and pSBR*nirB*, respectively, recovered from the NALT; lanes 3 and 4, extracts from clones pSBR-CTA2/B*nirB* and pSBR*nirB*, respectively, recovered from superficial cervical lymph nodes; lanes 5 and 6, extracts from clones pSBR-CTA2/B*nirB* and pSBR*nirB*, respectively, grown from freeze cultures; lane 9, extract from the clone carrying the unrelated pTE15 plasmid. The blots were probed with a rabbit IgG anti-SBR antibody directly conjugated to HRP. Numbers to the left of each panel indicate molecular masses in kilodaltons.
were still capable of expressing the immunogen. Persistent antigenic stimulation in the PP is expected to result in the generation of long-lasting secretory IgA responses to SBR in the various mucosal compartments including the oral cavity where immunity against \textit{S. mutans} is desired. Alternatively, a state of immunologic unresponsiveness known as oral tolerance could be generated, when soluble antigens are given orally. T-cell responses, especially T helper type 1 (Th1) responses, are more affected than B-cell responses by oral tolerance (17). Like other intracellular bacteria, \textit{Salmonella} induces a cell-mediated immune response, and the response generally elicits a cytokine profile corresponding to the Th1 response. Gamma interferon is important for the clearance of \textit{Salmonella} and is the characteristic response elicited by this infection (22). Recombinant \textit{Salmonella} strains are thought to be able to abrogate the effect of oral tolerance on the immune response to the cloned protein by inducing host cellular immune response. Therefore, the persistent antigenic stimulation provided by our recombinant \textit{Salmonella} system is unlikely to induce tolerance.

In our intestinal colonization study, \textit{Salmonella} strains expressing SBR or SBR-CTA2/B under the control of the temperature-sensitive T7 promoter were used as controls, and no colonies were recovered from the PP or spleens following plating of samples on either LB selective or BS plates. However, these clones have been shown to induce high levels of serum IgG and secretory IgA specific immune responses against SBR after an initial and booster oral immunization (11). It is possible that the clones carrying the T7 promoter colonize the intestine for a short time and express a high amount of the cloned antigen which is sufficient for the priming and boosting of immune responses against the foreign protein. However, the toxic effect of the overexpression of the foreign protein by these clones suppresses further growth of the bacteria, and bacteria die within 24 h. The results obtained from our intestinal colonization study indicate that recombinant \textit{Salmonella} strains expressing cloned proteins under the control of the \textit{nirB} promoter persist for at least 21 days following a single i.g. administration of the live vaccine. This colonization could result in a different pattern of immune response to the cloned antigen than that seen with the \textit{Salmonella} strains expressing the cloned genes under the control of the T7 promoter.

Nasal passages are another important port of entry of antigen for the induction of mucosal immune responses. NALT

![Figure 5](http://iai.asm.org/fig/5.jpg)

**FIG. 5.** Viable serovar Typhimurium recovered from NALT (A), superficial cervical lymph nodes (B), or internal jugular lymph nodes (C) of mice after i.n. infection. No serovar Typhimurium organisms were recovered from uninfected controls. At any given time point, tissues were pooled from three mice infected with \(10^6\) CFU of the clones carrying pSBR-CTA2/\textit{nirB}, pSBR\textit{nirB}, or the control pTET\textit{nir15} plasmid. Dilutions of homogenized tissues were plated on LB medium supplemented with 50 \(\mu\)g of carbenicillin (Cb) per ml or on BS medium. The results are representative of two separate experiments.
possesses lymphoid cell accumulations and has cellular structures similar to those of PP in the intestinal lumen, such as the M cells overlying these structures. Since Salmonella is able to actively invade M cells in the intestinal tissue, it is thought that it can also effectively colonize the nasal tissue via a similar mode of invasion. A Salmonella vaccine strain can then also deliver the antigen of interest to the nasal inductive sites and provide a source of antigen stimulation. The nasal mucosa is drained by the superficial cervical lymph nodes, which then drain to the posterior cervical lymph nodes (15, 24). When particulate antigen such as Salmonella is taken up by M cells in the NALT, secretory IgA and systemic immune responses can be evoked. Previous studies suggest that the i.n. immunization is effective in generating mucosal and systemic immune responses to cloned antigens under the control of a temperature-sensitive promoter (11). In our study, we examined the colonization potential of our Salmonella vaccine strains in nasal tissues following i.n. administration. A recent study has shown that, after i.n. immunization, Salmonella can be recovered from the lungs, cervical lymph nodes, PP, and spleen of infected mice (2). In our study, Salmonella was recovered from the NALT, superficial cervical lymph nodes, internal jugular lymph nodes, PP, and spleen of mice. To our knowledge, this is the first study that demonstrates the recovery of Salmonella from the NALT. Our data suggest that the bacteria colonized the NALT within 1 day after challenge with the bacteria and then disseminated through the draining lymph nodes within 5 days after the challenge. The Salmonella traveled to the PP and spleen and reached peak numbers on day 10 after challenge.

In this study, we placed the gene encoding S. mutans adhesin SBR with and without linking it to the A2/B subunits of CT under the control of an anaerobically inducible nirB promoter. We have shown that Salmonella expressing a cloned protein under the control of the nirB promoter persists for at least 21 days in lymphoid tissues following a single i.g. immunization. This result was in contrast to that seen with similar constructs under the control of the T7 promoter, where no Salmonella bacteria could be isolated after i.g. immunization. Following i.n. immunization, the Salmonella clones under the control of the nirB promoter were shown to colonize and persist for at least 21 days in nasal lymphoid tissues, as well as in PP and spleen of infected animals. Current studies are under way to determine the in vivo immunogenicity of the cloned SBR or SBR-CTA2/B in salmonel-like Typhimurium under the control of the nirB promoter and to determine the ability of the immune response induced against SBR to inhibit the colonization of S. mutans on the tooth surface.

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