A truncated *Bacillus subtilis* *dal* gene with a 3' *ssrA* gene tag regulates the growth and virulence of racemase-deficient *Listeria monocytogenes*

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Listeria monocytogenes (Lm) is a Gram-positive intracellular pathogen that can elicit strong cellular immunity. An attenuated strain (Lmdd) with deletions in two genes (*dal* and *dat*) required for D-alanine synthesis and viability has been shown to induce long-lived protective systemic and mucosal immune responses in mice when administered in the presence of the required amino acid. To bypass the necessity for exogenous D-alanine without compromising the safety of the original strain, the defect of Lmdd was complemented with a heterologous *Bacillus subtilis* *dal* gene, and the effects of truncating the upstream region of the gene on its transcription efficiency and of modifying its protein product with an *ssrA* tag at the 3'-terminus were examined. The strains with 551 bp and 80 bp upstream regions showed high levels of transcription and grew without D-alanine. The strains with the shortest upstream regions, 48 bp and 18 bp, showed greatly decreased levels of transcription and failed to grow in the absence of D-alanine. Addition of an *ssrA* tag to the longer genes resulted in a somewhat altered growth pattern in media and a reduced plaque size on L2 fibroblasts. These bacteria contained low levels of racemase protein and reduced free pools of D-alanine. One of the strains tested further, Lmdd/pA80S, was rapidly cleared from the spleens of infected mice but nevertheless induced a strong immune response that protected mice against challenge by wild-type *L. monocytogenes*. These bacteria can thus induce immune responses in mice comparable to the original Lmdd strain, but without the need for exogenous D-alanine, and may have use as a live vaccine vector against infectious diseases and cancers.

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INTRODUCTION

An effective cellular immune response is critical for survival against intracellular bacterial diseases, viral infections and cancer, and therefore the development of safe vaccines capable of inducing strong cellular immunity continues to be a major challenge for clinical medicine. We are exploring the use of an attenuated strain of *Listeria monocytogenes* (Lm) as a novel live vaccine vector for this purpose. *L. monocytogenes* is a Gram-positive facultative intracellular pathogen that has long served as a model for understanding innate and cell-mediated immunity against infection (Harty *et al.*, 2000; Pamer, 2004).

The merit of *L. monocytogenes* as a potential vaccine vector results from the close interface of its unique life cycle and the cellular immune system of its host. The micro-organism can enter phagocytic cells through Fc receptors or type I macrophage scavenger receptors (Dunne *et al.*, 1994; Suzuki *et al.*, 1997) and can invade non-phagocytic cells using the

bacterial surface proteins InlA and InlB (Gaillard *et al.*, 1991, 1996). Although the majority of engulfed organisms are killed in the phagosomal compartment of phagocytes (de Chastellier & Berche, 1994), a small fraction of the organisms escape that vacuole by means of the virulence factors listeriolysin O (LLO) and phosphatidylinositol-specific phospholipase C (PI-PLC) (Portnoy *et al.*, 1988; Smith *et al.*, 1995) and colonize the host cell cytosol. Proteins secreted by the organism in this milieu can be accessed directly by the MHC class I pathway of antigen processing and presentation (Ada, 1990; Braciale *et al.*, 1987). As a result, mice infected with a sublethal dose of wild-type bacteria develop long-lasting protective immunity, mediated predominantly by CD8⁺ T-cells, with little production of antibody (Finelli *et al.*, 1999; Kaufmann, 1993; Pamer, 2004). Since the natural route of infection by *L. monocytogenes* is by ingestion of contaminated foods, these infections activate the mucosal immune system (Marzo *et al.*, 2002).

These properties of *L. monocytogenes* have made it attractive as a potential live vaccine vector, and recombinant strains expressing foreign antigens have successfully protected mice

Abbreviations: Cam, chloramphenicol; i.v., intravenously; LLO, listeriolysin O; Sm, streptomycin.

against infection with lymphocytic choriomeningitis virus (Goossens *et al.*, 1995; Shen *et al.*, 1995), *Mycobacterium tuberculosis* (Miki *et al.*, 2004), papilloma virus (Jensen *et al.*, 1997; Kadish & Einstein, 2005) and influenza virus (Ikonomidis *et al.*, 1997) and against tumour challenge (Brockstedt *et al.*, 2005; Bruhn *et al.*, 2005; Gunn *et al.*, 2001; Yoshimura *et al.*, 2006). Nevertheless, several issues, such as anti-vector immunity and safety, need to be addressed before *L. monocytogenes* can be considered as a vaccine vector for human use. Unlike the situation with viral vectors, existing antilisterial immunity does not appear to diminish the therapeutic capacity of recombinant *L. monocytogenes* (Bouwer *et al.*, 1999; Starks *et al.*, 2004; Stevens *et al.*, 2005). However, safety remains an important concern, since wild-type *L. monocytogenes* poses a serious risk to neonates, infants, pregnant women, the elderly and immunocompromised individuals (Gellin & Broome, 1989). An ideal vaccine strain of *L. monocytogenes* should be attenuated and avirulent, but still retain immunogenicity. We previously generated a conditional lethal strain, *L. monocytogenes* *daldat* (Lmdd), attenuated by virtue of deletions in two genes necessary for synthesis of D-alanine, a rare amino acid required for peptidoglycan and lipoteichoic acid formation (Thompson *et al.*, 1998). Brief administration of D-alanine at the time of immunization was adequate to allow minimal but sufficient bacterial replication for the induction of an Lm-specific immune response in mice.

To obviate the dependence on exogenous D-alanine without significantly compromising the safety associated with the original strain, we developed several new second-generation attenuated strains of D-alanine-independent Lmdd. These strains carried either an IPTG-inducible or a recombinase-sensitive *Bacillus subtilis* racemase (*dal*) gene whose product could transiently complement the D-alanine deficiency of Lmdd (Li *et al.*, 2005; Zhao *et al.*, 2005). In this study we describe a different attenuation system that combines a truncated racemase gene promoter with an *ssrA* tag at the 3' terminus of the *B. subtilis* *dal* gene. The *ssrA* tag encodes a short peptide sequence at the C-terminus of the nascent racemase chain, which can stimulate its proteolysis by C-terminal-specific proteases (Gottesman *et al.*, 1998; Karzai *et al.*, 1999; Keiler *et al.*, 1996). This system resulted in an attenuated strain that retained good immunogenicity, without requiring D-alanine administration.

METHODS

Bacterial strains and cell growth conditions. *L. monocytogenes* *daldat* (Lmdd) is an attenuated strain of wild-type *L. monocytogenes* 10403S (Lm⁺) and was grown in brain heart infusion broth (BHI; Difco) at 30 °C supplemented with 200 µg D-alanine ml⁻¹ as described previously (Thompson *et al.*, 1998). In order to use standardized and known quantities of bacteria, many experiments were performed with thawed cultures prepared from 3–4 h growth of 1:10- or 1:20-diluted overnight cultures. Bacteria were stored at –80 °C in BHI. *E. coli* strains used for molecular cloning were grown in Luria-Bertani (LB) medium at 30 °C with agitation. Antibiotics were used at the following concentrations: ampicillin,

100 µg ml⁻¹; chloramphenicol (Cam), 10 µg ml⁻¹; streptomycin (Sm), 50 µg ml⁻¹. Tissue culture cells were grown in Dulbecco's modified Eagle medium (DMEM; Mediatech) supplemented with 10% fetal bovine serum and 2 mM L-glutamine (Mediatech) at 37 °C in a 5% CO₂/air atmosphere. Plasmid pKSV7-*Bsdal* contains the *B. subtilis* racemase gene (*dal*) with 551 upstream base pairs.

Construction of plasmids with the *B. subtilis* *dal* gene containing various lengths of upstream region, –551, –80, –48 and –18 bp, before the racemase start codon. The structures of plasmids are shown in Fig. 1. The *B. subtilis* *dal* gene with 551 bp upstream region was amplified from pKSV7-*Bsdal* using the 5' primer 5'-GCTCTAGAGCTTTGAATTAATAACAATTTG-3' (*Xba*I site underlined here and in the following) and 3' primer 5'-GCGTCTAGATTATTATGCATAATCTGGAACATCATATGGATAATTGCTTATATTACCTGCAATAAAGG-3' (HA epitope in bold) to yield a 1772 bp product containing the long upstream region, *dal* coding sequence, an HA (YPYDVPDYA) tag, a downstream translation termination sequence, and *Xba*I sites at the 5' and 3' termini. Ligating the *Xba*I-digested *B. subtilis* *dal* gene fragment into the *Xba*I site of shuttle vector pKSV7 generated the plasmid designated pK551. A PCR product with an *ssrA* tag added onto the 3' end of HA on the *B. subtilis* *dal* gene was generated using the earlier 5' primer and 3' primer 5'-GCGTCTAGATTATTAGGACGCGAAAGCTAGGTTTTGTTTTCTTTGCCAGTTGCATAATCTGGAACATCATATGGATAATTGC-3' (*ssrA* italicized), and ligated into pKSV7 to form pK551S. In like manner, additional constructs in pKSV7 were pK80 (5' primer 5'-GCTCTAGACGTTAGACATCGTTTCCCTTAGC-3'), pK80S, pK48 (5' primer 5'-GGGTCTAGATTAGCATGATATGTAAATG-3'), pK48S, pK18 (5' primer 5'-GCGTCTAGAAAGCTAGGAAGTGTCGTAATG-3') and pK18S. Those constructs that carried the *ssrA* tag were generated using the 3' primer above that contained both the HA and *ssrA* sequences. Those without *ssrA* used the 3' primer above that expressed HA only. Constructs prepared in plasmid pAM401 were designated pA551, pA551S, pA80 and pA80S.

Lmdd was transformed with each of the plasmids by electroporation. The transformants were selected on BHI + D-alanine + Cam agar plates at 30 °C. The plasmid-containing transformants were further screened on BHI + Cam and BHI + Cam + D-alanine agar plates.

In vitro growth. For uniformity of growth, retention of plasmid and to generate equivalent starting cultures for most experiments, Lmdd constructs were routinely grown overnight in BHI + Cam + D-alanine liquid medium. One millilitre of overnight culture was added to 19 ml of BHI +/– Cam broth, adjusted to the same OD₆₀₀ value, aliquoted into multiple 1 ml cultures, and grown at 30 °C. Wild-type Lm⁺ and Lmdd in the presence of D-alanine (200 µg ml⁻¹) were controls. At various times, samples were taken for OD₆₀₀ measurement.

Plasmid stability in vitro. One millilitre of overnight culture was added to 19 ml BHI +/– Cam broth, and grown to OD₆₀₀ 0.8 at 30 °C. Bacteria were counted on BHI +/– Cam and BHI + D-alanine agar plates. The fraction of bacteria with plasmid was calculated as the percentage of those that were D-alanine-independent (growth on BHI plates) relative to the total number of viable bacteria (growth on BHI + D-alanine plates).

RNA and DNA isolation. Total RNA from strains of *L. monocytogenes* was prepared after suspending bacteria in RNeasy Protect Bacteria Reagent (Qiagen), lysozyme lysis, proteinase K digestion, and isolation using the RNeasy mini-kit (Qiagen) according to the manufacturer's protocols. RT controls were performed to assure the complete removal of genomic DNA. For isolation of total genomic DNA plus plasmid DNA, cells were treated with lysozyme, proteinase K and RNase, and then extracted with phenol/chloroform/isomyl alcohol and precipitated with ethanol.

Fig. 1. *B. subtilis dal* gene constructs in plasmids pKSV7 and pAM401. The *B. subtilis dal* gene (1170 bp in length), which encodes D-alanine racemase, was cloned with various lengths of upstream region (–551, –80, –48 and –18 bp) and an HA tag (27 bp) at its 3' terminus. In addition, an *ssrA* tag was added onto the 3' end of the HA tag prior to the termination codon of the gene. The constructs were cloned into an *Xba*I site on either plasmid. The final plasmids are named to indicate the plasmid backbone, the length of upstream region and the presence or absence of an *ssrA* tag. Thus, Lmdd/pK80S is *L. monocytogenes daldat* containing a pKSV7 plasmid having a *dal* gene insert with an 80 bp upstream region and an *ssrA* tag. Some plasmid genes not shown on pKSV7 are a *ts* origin of replication, an ampicillin resistance gene for selection in *E. coli* and a chloramphenicol resistance gene for selection in Gram-positive bacteria. Genes not shown on pAM401 are tetracycline and chloramphenicol resistance genes for Gram-negative selection and a chloramphenicol resistance gene for selection in Gram-positive bacteria.

Real-time PCR. cDNA was synthesized using the Superscript First-Strand Synthesis System kit according to the manufacturer's suggested protocol (Invitrogen). Two microlitres of cDNA was used in a 25 µl reaction mixture for real-time PCR amplification with Power SYBR Green PCR Master Mix (Applied Biosystems). The reactions were performed using an iQ 5 Cyclor Real-Time PCR detection system (Bio-Rad). Reaction conditions were optimized and data were analysed using iQ5 Optical system software. Primers were designed to amplify a 186 bp sequence from the racemase-encoding *dal* gene of *B. subtilis* and a 182 bp sequence from the *hly* gene of *L. monocytogenes*. The *dal* gene forward primer was 5'-AATTGAA-AGGGACCGACATC-3' and reverse primer, 5'-TTAATGGTTTC-GAGCCTTCC-3'. The internal control *hly* gene primers were 5'-GCAAGCTAGCTCATTTACATC-3' and 5'-ATTTCGGATAAA-GCGTGGTG-3'. *dal* gene amplification began with 3 min at 95 °C followed by 45 cycles of 95 °C for 10 s, 59.6 °C for 30 s and 78 °C for 6 s; *hly* gene amplification was 95 °C for 3 min followed by 45 cycles of 95 °C for 10 s, 58.4 °C for 30 s and 75 °C for 6 s. Melt curves were performed to ensure detection of the correct product. Products were initially analysed by agarose gel electrophoresis. Threshold cycles (C_T) (PCR cycles at which fluorescence first accumulates above background) were determined for each amplification. The C_T values from serial dilutions of cDNA were plotted against

log input of the genomic DNA copies isolated from our standard control, Lmdd[–] dat[–] Bsdal⁺, to generate a standard curve. Unknown samples were quantified by comparison of their C_T values with that curve.

Plasmid copy number in cells of the different *Listeria* constructs was determined using real-time PCR to measure the number of plasmid-bearing *dal* genes in preparations of total genomic DNA, relative to the number of *hly* chromosomal genes detected in those same preparations. We assumed that a single *Listeria* chromosome was present in each of the cells in our cultures.

D-Alanine determination. D-Alanine pools were examined by a spectrophotometric method that coupled the oxidative deamination of D-alanine by the enzyme D-amino acid oxidase to the reduction of the resulting product, pyruvate, in the presence of lactic dehydrogenase and NADH (Bergmeyer & Grassl, 1983). Catalase was present to prevent the alternative conversion of pyruvate to acetic acid and carbon dioxide by accumulated hydrogen pyroxide. The reaction was linear from 10 µg ml^{–1} to 500 µg ml^{–1} D-alanine. Prior to assay, cultures (20–100 ml) at OD₆₀₀ 0.8 were concentrated to 1 ml, lysed with lysozyme and three freeze–thaw cycles, and then extracted with perchloric acid followed by neutralization with potassium bicarbonate to remove protein.

Detection of *B. subtilis* racemase. Bacteria were grown from overnight cultures in BHI+Cam+D-alanine medium at 30 °C and samples were collected at various times for electrophoresis. Equivalent amounts of bacteria, based on total protein analysis (D_c protein assay; Bio-Rad), were lysed using lysozyme at 37 °C for 1 h as described elsewhere (Zhao *et al.*, 2005). Samples were electrophoresed in SDS-PAGE gel, and transferred to Immun-Blot PVDF membrane (Bio-Rad). Anti-HA monoclonal antibody was the primary antibody (Roche), followed by sheep anti-murine IgG linked with horseradish peroxidase (Amersham Biosciences). Bands were detected with the ECL Western Blotting Analysis System kit (Amersham Biosciences).

Analysis of plaque formation on L2 fibroblasts. Assays of plaque formation on mouse L2 fibroblast cell monolayers were performed as previously described (Sun *et al.*, 1990), with some modification. Briefly, L2 cell monolayers were grown to confluence in six-well tissue culture plates. Approximately 1×10^6 bacteria from a frozen stock culture were used to infect the monolayers for 1 h in DMEM. Monolayers were washed three times with PBS, and a DMEM/0.7% agar overlay containing 10 µg gentamicin ml⁻¹ was added. Plates were incubated for 3 days to allow plaque formation. At day 4 the overlay was removed, and the cells were fixed in PBS/10% paraformaldehyde for 60 min, stained with 0.1% crystal violet/20% ethanol for 5 min, washed and air-dried. The diameter of plaques in the monolayers was measured after 10 × amplification.

Bacterial proliferation in spleen. To examine the virulence of Lmdd constructs in mice, animals were infected intravenously (i.v.) with 0.2 LD₅₀ of bacteria (i.e. 1.4×10^7 of Lmdd in the presence of D-alanine, 4×10^6 of Lmdd/pA80S or 2×10^3 of Lm⁺). Viable bacteria in spleen were determined at days 1, 3, 5 and 7 post-infection. The organ was homogenized in 3 ml Hanks' buffered salt solution, and splenocytes were lysed in H₂O and dilutions plated on BHI+D-alanine+Sm or BHI+Sm. Since the wild-type strain from which Lmdd was derived is streptomycin-resistant, inclusion of this antibiotic allowed selection against possible contaminant organisms.

Primary T-cell responses and protection induced by Lmdd/pA80S. Six- to eight-week-old BALB/c (H-2^d) female mice were infected i.v. with 2×10^3 of Lm⁺, 1.4×10^7 of Lmdd +/− 20 mg D-alanine, or 4×10^6 of Lmdd/pA80S. At 8 days after infection, spleens were homogenized as above, splenocytes were collected, red blood cells removed with ACK lysis buffer, and the washed lymphocytes stained with anti-CD8a-FITC, anti-CD11a-PE (eBiosciences) and LLO₉₁₋₉₉-MHC I tetramers labelled with APC. IFN-γ intracellular staining was performed according to methods described elsewhere (Pearce *et al.*, 2004). Flow cytometry was performed using a FACSCalibur flow cytometer (Becton Dickinson) and the data were analysed using FlowJo software (Tree Star Inc.). For the protection studies, mice were infected as above with 0.2 LD₅₀ of each strain, challenged 21 days later with 2×10^4 wild-type *L. monocytogenes*, and sacrificed at day 3 postchallenge. Spleens and livers were removed, homogenized as above, and bacterial content enumerated.

RESULTS

D-Alanine-independent strains of Lmdd that express a *B. subtilis* *dal* gene with various lengths of 5' upstream region and 3' *ssrA* tags

The growth and viability of Lmdd, the *dal dat* double deletion strain of *L. monocytogenes*, are absolutely dependent on an exogenous source of D-alanine, both *in vitro* and *in vivo* (Thompson *et al.*, 1998). To obviate this requirement for an external source of the amino acid and to produce a

potentially more useful vaccine vector, we generated several plasmids that carried the *B. subtilis* *dal* gene; this gene encodes racemase, one of the two D-alanine-synthesizing enzymes used by *L. monocytogenes*. Each plasmid carried a *dal* gene with a different length of upstream region: 551 bp, 80 bp, 48 bp or 18 bp. The *dal* genes were also modified by addition of an *ssrA* tag at the 3'-terminus. These *dal* gene sequences were inserted into two plasmids used for gene transfer in *L. monocytogenes*: pKSV7, a shuttle vector with a temperature-sensitive origin of replication (Smith & Youngman, 1992), and pAM401, whose replication is not temperature sensitive (Wirth *et al.*, 1986) (Fig. 1). As shown previously, *B. subtilis* racemase is able to complement the growth defect of Lmdd (Li *et al.*, 2005; Zhao *et al.*, 2005).

The growth *in vitro* of each of these strains was examined. Growth of constructs prepared in the pKSV7 vector is shown in Fig. 2(a). Those constructs with the shorter upstream regions, 48 bp and 18 bp, either grew very slowly or failed to grow at all at 30 °C in the absence of D-alanine. The two constructs with the longer upstream regions, 551 bp and 80 bp, grew well in the absence of D-alanine, although they grew more slowly than Lmdd+D-alanine during the 6 h culture. There was little effect on growth of the *ssrA* tag at the 3' ends of the genes. However, *ssrA* did affect the growth of constructs prepared with plasmid pAM401 (Fig. 2b). Both Lmdd/pA551S and Lmdd/pA80S grew somewhat more slowly than their counterparts without the 3' tag.

To examine this property further, the strains with the two longer upstream regions were plated on solid BHI agar medium and allowed to grow at 23 °C, 30 °C and 37 °C. The presence or absence of *ssrA* on the pKSV7 plasmid constructs did not differentially affect colony size during growth of the bacteria at any temperature (data not shown). Conversely, Lmdd carrying the pAM401 constructs did reveal significant effects of *ssrA*. These differences were small when bacteria were grown at 37 °C, but were most distinct, as shown in Fig. 3, when growth of the bacteria was at 23 °C. Although the 551 bp and the 80 bp upstream regions allowed similar rates of colony growth, if either of the *dal* genes was tagged with *ssrA*, colony growth was severely delayed. After 24 h incubation, colonies of Lmdd/pA551 and Lmdd/pA80 were obvious (Fig. 3a) while those of Lmdd/pA551S or Lmdd/pA80S were barely detectable. By 96 h, the slow-growing bacteria had almost caught up with their counterparts, so that at this time there was little difference in colony size between constructs with or without the *ssrA* tags (Fig. 3c).

The cell morphology of individual bacteria with *ssrA*-tagged *dal* genes was also distinctive. After growth of Lm⁺, Lmdd+D-alanine, Lmdd/pA80 (not shown in figure) and Lmdd/pA80S in liquid medium followed by Gram staining, all bacteria except Lmdd/pA80S had the morphology of short rods (Fig. 4). Conversely, the Lmdd/pA80S cultures contained mainly long rods as well as large numbers of incompletely septate bacteria.

Fig. 2. Growth kinetics of Lmdd containing the various *B. subtilis dal* gene constructs with different lengths of upstream region and with or without an *ssrA* tag. Overnight cultures were grown in BHI + Cam + D-alanine. After dilution the cultures were grown in BHI + Cam for 6 h at 30 °C. Similar results were seen if the antibiotic was absent. Controls were Lm⁺ or Lmdd in the presence of 200 µg D-alanine ml⁻¹. (a) pKSV7-containing bacteria. These bacteria were diluted 1 : 10 prior to regrowth. (b) pAM401-containing bacteria. These bacteria were diluted 1 : 20 prior to regrowth; they grew somewhat more rapidly than the pKSV7-containing bacteria.

Since the survival of the Lmdd constructs in the absence of D-alanine depends on the presence of the *B. subtilis dal* gene-expressing plasmids, we anticipated that every live cell would harbour a plasmid. As expected, when cultures containing the pAM401-based plasmids were grown in media without D-alanine and then plated in the absence of D-alanine, all bacteria formed colonies. Lmdd itself cannot form colonies under these conditions (Thompson *et al.*, 1998). Therefore we were surprised to observe that this was not the case for the pKSV7 constructs. After growth in D-alanine-free liquid media, only 21–35 % of the Lmdd

harbouring the temperature-sensitive pKSV7-based plasmids formed colonies on D-alanine-free plates (compared to colony formation on D-alanine-containing plates). This was observed regardless of the temperature of growth of the cultures or the plates, between 23 °C and 37 °C. We infer that in these cultures, Lmdd that had lost their plasmids were able to grow on D-alanine released from the plasmid-containing bacteria. Because of this plasmid instability, we focused most of our efforts to devise a more useful vaccine strain on the stable pAM401-containing constructs.

Fig. 3. Growth of Lmdd/pA80S (lanes 1), Lmdd/pA80 (lanes 2), Lmdd/pA551S (lanes 3) and Lmdd/pA551 (lanes 4) on BHI + Cam plates at 23 °C. About 100 c.f.u. from overnight cultures grown as indicated in Fig. 2 were plated in each lane. Photographs were taken at the indicated times.

Fig. 4. Photomicrographs showing that Lmdd/pA80S forms long rods and chains during growth at 30 °C in BHI. The culture of Lmdd was supplemented with 200 µg D-alanine ml⁻¹ (Lmdd + D). Bacteria in cultures of Lmdd/pA80 had the same appearance as Lmdd + D (not shown). The bacteria were Gram stained prior to photography (100 × magnification).

Effects on transcription of the *B. subtilis dal* gene

In order to examine the effects on gene expression of the *dal* gene modifications introduced into each of the plasmids, we examined the level of *dal* gene transcription by real-time RT-PCR. A strain of Lmdd that contained only one integrated copy of the *B. subtilis dal* gene and one natural chromosomal copy of the haemolysin (*hly*) gene of *Listeria* (strain Lmdal[−] dat[−] Bsdal⁺) served as the control for these tests. The ratio of mRNA molecules (*dal/hly*) transcribed from these two genes in these bacteria was found to be 1·08 in this assay. In cells containing plasmids that express the *B. subtilis dal* gene, and only one chromosomal *hly* gene, the *dal/hly* ratio should be higher. The results of RT-PCR of each of the plasmid-bearing constructs are shown in Table 1. In the pKSV7 series, the *dal/hly* ratio for bacteria carrying the full-length upstream region, Lmdd/pK551, was over 40. Truncation of the upstream sequence from 551 bp to 80 bp had no detectable effect, while further shortening to 48 bp reduced the ratio of *dal/hly* transcripts to 23·5, and additional shortening to 18 bp reduced the ratio to 5·3. To determine the transcription efficiency from each plasmid in the constructs, plasmid copy number was ascertained and used to calculate mRNA copies per plasmid template (Table 1). These data show that decreasing the upstream region from 551 bp to 80 bp decreased the number of *dal* transcripts per plasmid template by approximately 20 %. Further truncation to 48 bp and 18 bp had still more drastic effects on transcription efficiency.

In the pAM401 series, transcription of the two longer genes that allowed D-alanine-independent growth, with or without *ssrA* modification, was examined. Lmdd/pA551 and Lmdd/pK551 showed similar absolute levels of *dal* gene transcription (41·7 and 46·0 *dal/hly* ratios). Reduction of the upstream region of the *dal* gene from 551 bp to 80 bp resulted in a small decrease in the absolute number of

transcripts (6 %) but again a significant reduction of transcripts per plasmid template (29 %). Transcripts per plasmid template also decreased when Lmdd/A551S was compared with Lmdd/A80S (12 %).

To summarize these results, shortening the upstream region of the *B. subtilis dal* gene below 80 bp led to striking reductions in transcription of the gene. The truncation from 551 bp to 80 bp had a smaller effect on the total number of transcripts in the cell, but when examined on the basis of transcription per plasmid, the decrease was significant. The presence of *ssrA* on the 3' end of the *dal* gene did not appear to affect transcription, but did lead to an increased plasmid copy number. As shown later, *ssrA*-tagging of the *dal* gene was associated with a decrease in D-alanine pool size. Plasmid number may increase in these cells in an attempt to accommodate this change in D-alanine pool size through a feedback mechanism.

Racemase expression and the effect of *ssrA*

Eubacteria utilize tmRNA, encoded by the *ssrA* gene, to allow the release of ribosomes stalled on stop-codon-deficient mRNA (Keiler *et al.*, 2000). The mechanism generates a short peptide tag at the C-terminus of the abortive protein product which functions as a signal for enhanced proteolysis. If the *ssrA* sequence that was placed at the 3' end of the *B. subtilis dal* gene functions in this way, we would expect to find lower quantities of racemase, the product of the *dal* gene, in cells that harbour this modified gene. Cultures of Lmdd/pA551, Lmdd/pA551S, Lmdd/pA80 and Lmdd/pA80S were grown at 30 °C, diluted into fresh medium and sampled at several subsequent time points. Fig. 5 shows Western blots from two experiments. In both, the amount of HA-tagged racemase was significantly lower for the strains possessing the *ssrA* sequence at the 3' end of the *dal* gene. The *ssrA* effect appeared to be greater in the early exponential phase of growth than at later time points.

Table 1. Transcription of the *B. subtilis dal* gene in *Listeria* constructs

Transcription was assessed using real-time RT-PCR to measure the ratio of the number of *B. subtilis dal* gene transcripts to the number of transcripts from the *L. monocytogenes hly* gene (which is present on the *Listeria* chromosome in a single copy). The control for this experiment was Lmdal[−] dat[−] Bsdal⁺, which harbours single chromosomal copies of both genes. Among Lmdd/pKSV7 constructs, the *dal/hly* ratio showed significant differences when compared to K551 (*t* test: **P*<0·01, ***P*<0·001). Among Lmdd/pAM401 constructs, the *dal/hly* ratio was not significantly different when compared to A551 (*P*>0·05).

	Lmdal [−] dat [−] Bsdal ⁺	Lmdd/pKSV7				Lmdd/pAM401			
		K551	K80	K48	K18	A551	A551S	A80	A80S
<i>dal/hly</i> ratio	1·08	46·0	47·7	23·5*	5·3**	41·7	34·9	39·4	33·8
<i>dal/hly</i> ratio per plasmid†		2·09	1·64	0·73	0·17	3·48	0·96	2·46	0·85

†Plasmid copy number was determined via real-time PCR by measuring the copy number of *dal* genes relative to the number of *hly* chromosomal genes, based on the assumption of a single *Listeria* chromosome per cell in our cultures. The *dal* gene copy numbers (=plasmid copy number) were: K551, 22; K80, 29; K48, 32; K18, 32; A551, 12; A551S, 36; A80, 16; A80S, 40.

Fig. 5. Effect of an *ssrA* tag on the abundance of *B. subtilis* racemase in cultures of *Listeria* constructs. Bacteria were diluted into BHI + Cam + D-alanine medium and grown at 30 °C for the indicated times. Cell pellets were lysed and equal amounts of protein were separated by SDS-PAGE and analysed by Western blotting using a mouse monoclonal anti-HA antibody. Results of two independent experiments (a and b) are shown.

D-Alanine pools

The slightly lower level of *dal* gene transcripts in the cells with 80 bp constructs, and the much lower level of racemase detected by Western blotting in cells with *ssrA*-tagged *dal* genes, suggested that D-alanine levels in these cells may be affected. To determine whether this was the case, cultures of various strains of *Listeria* were grown at 30 °C to late exponential phase (OD_{600} 0.8, to obtain sufficient numbers

of bacteria to achieve reliable data using the spectrophotometric assay), and cell lysates were prepared and assayed for D-alanine content. Three independent experiments were performed with similar results, one of which is presented in Table 2. D-Alanine pools were found to be slightly larger in our wild-type *Listeria* than in *Escherichia coli* (Mengin-Lecreux *et al.*, 1982), not unexpected since the latter has a much thinner peptidoglycan layer.

Lmdd/pA551 and Lmdd/pA80 contained similar levels of D-alanine as wild-type *Listeria*. However, the D-alanine pools in the bacteria carrying the *ssrA*-tagged *dal* genes were 33 % lower. In two other experiments, the reductions varied from 39 % to 63 %. Since the cultures from which the samples were obtained were in the late exponential phase of growth, it is possible that a greater reduction of the pools would be seen in early exponential phase, when the racemase enzyme appears to be at its lowest level according to Western blot analysis. As controls for these experiments, Table 2 verifies that Lmdd grown in the presence of D-alanine (and then washed) contains very high levels of D-alanine, which are depleted by additional growth in the absence of the amino acid for 20 or 45 min.

Table 2. Free D-alanine in cytosol of *Listeria* constructs

Bacteria were grown at 30 °C in BHI medium or BHI plus 200 μ g D-alanine ml^{-1} . For Lmdd, the bacteria were first grown to OD_{600} 0.7 in the presence of D-alanine, washed once with PBS (Lmdd + D-alanine), and then culture was continued for 20 min or 45 min in BHI in the absence of D-alanine. Samples of all other bacteria were collected at OD_{600} 0.8. Lmdal[−] dat[−] Bsdal⁺ is Lmdd with a single chromosomal copy of the *B. subtilis* *dal* gene. The bacterial pellets were subjected to lysozyme lysis at 37 °C for 1 h, followed by measurement of intracellular soluble D-alanine as described in Methods. Three independent experiments were performed with similar results.

Bacterium	$10^{-4} \times$ Molecules D-alanine per cell
Lm ⁺	191
Lmdal [−] dat [−] Bsdal ⁺	151
Lmdd + D-alanine	254
Lmdd − D-alanine (20 min)	90
Lmdd − D-alanine (45 min)	71
Lmdd/pA551	194
Lmdd/pA551S	129
Lmdd/pA80	174
Lmdd/pA80S	116

Plaques produced by Lmdd constructs on L2 fibroblast monolayers

We sought to determine whether the properties of the new Lmdd constructs described thus far would affect their ability to interact with host cells, that is, to infect, multiply and spread in eukaryotic cells, or affect their virulence and ability to elicit an immune response in mice. An *in vitro* test, infection of monolayers of murine L2 fibroblasts, leads to growth of the micro-organism, its cell-to-cell spread and visible plaque formation (Sun *et al.*, 1990). Fibroblast monolayers were infected with Lm⁺, Lmdd +/− D-alanine, Lmdd/pA551, Lmdd/pA551S, Lmdd/pA80 and Lmdd/

Table 3. Plaque size produced by Lmdd constructs grown on monolayers of mouse L2 fibroblasts

Monolayers of L2 fibroblasts were infected as described in Methods. Plaque formation was visualized after 96 h incubation at 37 °C. Plaque diameters from 20 random plaques per sample were measured. Values are presented as the mean percentage $\pm$ SEM, relative to Lmdd + D-alanine.

Bacterium	Plaque size
Lmdd + D-alanine	100 $\pm$ 1.13
Lmdd - D-alanine	0
Lm ⁺	101.4 $\pm$ 1.04
Lmdd/A551	97.4 $\pm$ 1.04
Lmdd/A551S	86.1 $\pm$ 1.22*
Lmdd/A80	95.8 $\pm$ 1.09
Lmdd/A80S	73.9 $\pm$ 0.81*

*There was a significant difference in plaque size between the *ssrA*-tagged and non-tagged forms of these constructs when compared to that produced by Lmdd + D-alanine ($P < 0.001$).

pA80S, and the presence and diameter of plaques produced were measured. Table 3 shows that Lm⁺ and Lmdd + D-alanine produced comparable, large plaques, while Lmdd in the absence of D-alanine failed to produce any plaques. Lmdd/pA551 and Lmdd/pA80 produced slightly smaller than normal diameter plaques. The plaques that resulted from infection with Lmdd/pA551S and Lmdd/pA80S, which carried *ssrA*-tagged *dal* genes, were reduced in size by 14 % and 26 %, respectively, relative to Lmdd + D-alanine.

Accelerated clearance of Lmdd/pA80S from spleens of mice

Following systemic infection of mice, *L. monocytogenes* spreads intracellularly and intercellularly, with spleen and liver being two of the primary target organs (Unanue, 1997). To assess the safety of the new constructs, the number of bacteria found in these organs of infected host animals was examined at various times after infection. Groups of mice infected i.v. with about 0.2 LD₅₀ of bacteria (1.4×10^7 of Lmdd + D-alanine, 4×10^6 of Lmdd/pA80S or 2×10^3 of wild-type Lm) were examined at various time points up to 7 days post-infection for total viable bacteria in spleen. As indicated in Fig. 6, wild-type Lm multiplied in this organ, reaching a maximum number of organisms at day 3, and was then eliminated by host immune defences by day 7. In contrast, Lmdd + D-alanine and Lmdd/pA80S failed to replicate significantly and their numbers fell continuously. They were cleared from the organ in about 3 days. Thus Lmdd/pA80S had a similar low virulence in mice as the original Lmdd (+D-alanine) strain, despite the fact that the former was able to satisfy its own need for D-alanine.

Fig. 6. Survival of bacteria in spleen. Mice were infected i.v. with about 0.2 LD₅₀ of bacteria (1.4×10^7 Lmdd + 20 mg D-alanine, 4×10^6 Lmdd/pA80S or 2×10^3 Lm⁺). Viable bacteria in spleen were determined at days 1, 3, 5 and 7. Splenocytes were lysed in H₂O and dilutions were plated on BHI + D-alanine + Sm or BHI + Sm agar plates. Sm was included to limit the growth of contaminants. The values at each time point are the mean $\pm$ SEM from at least six mice per curve in two individual experiments. The day zero numbers represent the dose of the inocula.

Induction of LLO-specific CD8 T-cells

Listeriolysin O (LLO), a major virulence factor for *L. monocytogenes* pathogenesis, is responsible for lysing the phagocytic vacuole, allowing bacterial access to the cytosol (Gaillard *et al.*, 1986; Kathariou *et al.*, 1987). This protein also contains the dominant CD8⁺ T-cell epitope of *Listeria* in BALB/c mice (Wipke *et al.*, 1993). We therefore assessed the immune response of mice to Lmdd/pA80S by determining the level of activated (CD11a^{hi}) LLO-specific CD8⁺ effector T-cells in spleen following immunization. As shown in the FACS analysis presented in Fig. 7(a), Lmdd/pA80S elicited a similar percentage of LLO-tetramer⁺ CD8⁺ T-cells in spleen as was induced by wild-type Lm. Most of these splenocytes also functioned as effector cells, as evidenced by their expression of intracellular IFN- γ , shown in Fig. 7(b). Lmdd administered to mice without a supply of D-alanine generated few antigen-specific T-cells.

Protection against challenge by wild-type *L. monocytogenes*

To determine whether infection of mice with sublethal doses of Lmdd/pA80S could induce long-lasting protective immunity, as does immunization with wild-type *L. monocytogenes*, we injected mice i.v. with Lmdd +/– D-alanine (1.4×10^7), Lmdd/pA80S (4×10^6) or Lm⁺ (2×10^3), and subsequently challenged the mice with 2×10^4 of Lm⁺ 3 weeks later. Immune protection blocks the replication of the challenge *Listeria*. The levels of viable bacteria remaining at day 3 in the spleens (Fig. 8a) and livers (Fig. 8b) of mice immunized by Lmdd/pA80S were similar to those after

Fig. 7. Lmdd/pA80S elicits functional antigen-specific CD8⁺ T-cells during the primary T-cell response in vaccinated mice. BALB/c mice were infected i.v. with 1.4×10^7 Lmdd +/–D-alanine, 4×10^6 Lmdd/pA80S or 2×10^3 Lm⁺. At day 8 after immunization, activated splenic LLO-specific CD8⁺ T-cells were measured by tetramer analysis of gated CD8⁺ T-cells (a) and IFN-γ intracellular staining (b). Data represent at least two independent experiments.

immunization with wild-type Lm and Lmdd + D-alanine, with more than a six-log₁₀ reduction in bacterial numbers compared to the challenged naïve group. Prior immunization with Lmdd in the absence of D-alanine led to little protection (Thompson *et al.*, 1998).

DISCUSSION

L. monocytogenes has long been studied as a model for the induction of innate and cellular immunity. It is also a potential live vaccine vehicle for infectious diseases and cancer (Mackanness, 1962; Schafer *et al.*, 1992; Shen *et al.*, 1995; Starks *et al.*, 2004). However, the wild-type organism can cause listeriosis, a serious food-borne illness that poses a health threat especially for pregnant women and other immunocompromised individuals (Gellin & Broome, 1989). Live micro-organisms must be attenuated if they are to be used as human vaccine vectors but a balance between their safety and immunogenicity needs to be achieved since overattenuation can result in loss of the desired immune response.

L. monocytogenes has been attenuated in various ways. Genes altered for this purpose have included *actA* (Kocks *et al.*, 1992) and *actA* in combination with *plcB* (Angelakopoulos *et al.*, 2002; Peters *et al.*, 2003) or with *intA/B* (Brockstedt *et al.*, 2004); lipotease protein ligase *LplA1*

(O’Riordan *et al.*, 2003); *aro* (Stritzker *et al.*, 2004); and even psoralen-inactivated whole populations of recombinant *Listeria* (Brockstedt *et al.*, 2005). Our work has focused on a strain of *L. monocytogenes* in which the two genes responsible for D-alanine synthesis, *dal* and *dat*, have been deleted (Thompson *et al.*, 1998). These bacteria, Lmdd, are absolutely dependent on an exogenous supply of D-alanine for cell wall synthesis, both *in vitro* and *in vivo*. The strain was shown to effectively induce protective immunity against challenge by the wild-type organism itself or by other organisms expressing a common antigen, when infection was initiated with a small dose of D-alanine (Rayevskaya & Frankel, 2001; Thompson *et al.*, 1998).

In order to generate a derivative of Lmdd that would obviate its need for exogenous administration of the amino acid yet still retain attenuation, in this study we cloned a *B. subtilis* *dal* gene (which can complement the deficiency of Lmdd) with different lengths of upstream region into vectors pKSV7 and pAM401 and transformed the resulting plasmids into Lmdd. We found that a minimum 80 bp promoter length was necessary to allow D-alanine-independent growth of Lmdd (Fig. 2a). The promoterless construct, Lmdd/pK18, with only a Shine–Dalgarno sequence, and Lmdd/pK48 failed to grow. The number of *dal* gene transcripts in cells carrying these constructs was severely reduced (Table 1). Thus, the sequence between –48 bp and –80 bp

must contain important elements for the *dal* gene promoter. When comparing the number of *dal* gene transcripts generated from the two long promoter regions (551 bp and 80 bp) there was little or no difference in the total number of transcripts, although the number per plasmid copy was significantly reduced for the 80 bp construct.

A general mechanism used by many species of bacteria to rescue stalled ribosomes relies on the intervention of tmRNA encoded by the *ssrA* gene. This stable RNA molecule binds to ribosomes and its encoded peptide tag is cotranslationally added to the abortive polypeptide chain, targeting it for proteolysis (Karzai *et al.*, 2000). In Gram-positive bacteria, the proteolysis appears to be mediated by ClpXP protease (Wiegert & Schumann, 2001). We sought to utilize this salvage mechanism in our constructs to destabilize accumulated racemase, by intentionally tagging the *dal* gene at its 3' end with the *ssrA* gene. This had the effect of greatly decreasing the steady-state concentration of racemase in the cells (Fig. 5), presumably by enhancing its degradation. We showed that this in turn was associated with decreased pools of free D-alanine. This evidently restricted the overall growth rate of the bacteria (Fig. 3) and limited their ability to multiply, spread and form plaques in monolayers of L2 fibroblasts (Table 3).

The goal of these studies was to generate a strain of *L. monocytogenes* for use as a vaccine vector with reduced virulence but strong immunogenicity. Compared with wild-type *L. monocytogenes*, the approximate LD₅₀ of Lmdd/pA80S is increased by at least 3-log₁₀ (1×10^4 vs approximately 2×10^7). While the reduced length of upstream region of the *B. subtilis dal* gene from 551 bp to 80 bp had a fairly small effect on total transcription of the gene (Table 1), we have observed in some preliminary experiments a fourfold higher LD₅₀ for constructs that carried the longer upstream sequence (data not shown). A greater contribution to the attenuation of these bacteria was provided by the *ssrA* gene tag on the *dal* gene, which resulted in a greatly reduced concentration of racemase and a notable reduction of D-alanine pool size. Following infection of mice with 4×10^6 of Lmdd/pA80S, at day 3 there was a 4-log₁₀ decline in number of organisms in the spleen, whereas at day 3 after infection with 10^3 wild-type Lm⁺ there was a 2-log₁₀ increase (Fig. 6). These results attest to the attenuation of Lmdd/pA80S.

Despite early indications to the contrary (North *et al.*, 1981), experiments that temporally abrogate *Listeria* infection of mice with antibiotics revealed that 24 h of infection may suffice to generate a T-cell response of normal magnitude (Mercado *et al.*, 2000). Consistent with this, despite the short time that Lmdd/pA80S survives in animals, the strain was able to induce an effective immune response. A strong induction of LLO-specific CD8⁺ effector T-cells as shown by LLO-tetramer and IFN- γ intracellular staining was obtained (Fig. 7). Furthermore, a high level of protection against subsequent challenge by the wild-type organism was achieved (Fig. 8).

Fig. 8. Protection of immunized BALB/c mice against challenge with wild-type *L. monocytogenes*. Groups of three mice were immunized with 2×10^3 Lm⁺, 1.4×10^7 Lmdd +/–D-alanine, or 4×10^6 Lmdd/pA80S. Mice were challenged 3 weeks later with 2×10^4 Lm⁺ and the total number of viable bacteria in spleen (a) or liver (b) was determined by growth on BHI+Sm plates. Sm was included to limit the growth of contaminants. The detection limit was 10 c.f.u. in spleen and 100 c.f.u. in liver. Data are presented as means $\pm$ SEM. The data shown represent at least two independent experiments.

In summary, we have characterized a new vaccine system for *L. monocytogenes* based on Lmdd, a D-alanine-requiring mutant. The new strain Lmdd/pA80S satisfies the need for D-alanine through limited expression of a racemase gene. Immunization of mice with these bacteria resulted in an effective immune response that led to protection against subsequent challenge. Oral immunization of rhesus macaques with recombinant strains of these organisms revealed them to be a safe and effective inducer of cellular immune responses in this nonhuman primate model (R. Ruprecht and others, personal communication). Lmdd/pA80S may thus be a candidate as a live bacterial vaccine vector for oral immunization that meets the important medical need for vectors able to elicit cellular and mucosal immunity.

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