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A Stably Engineered, Suicidal Strain of *Listeria monocytogenes* Delivers Protein and/or DNA to Fully Differentiated Intestinal Epithelial Monolayers

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Abstract: We have generated a recombinant stable, suicidal *Listeria monocytogenes* strain (rsΔ2) capable of delivering antigens as protein or DNA into nondividing intestinal epithelial cells. The rsΔ2 strain was generated by inserting a cell wall hydrolysin gene, "*ply118*" together with its associated holin gene from a *Listeria*-specific phage, into the attenuated *L. monocytonegenes* genome of strain Δ2. The *hol118/ply118* gene was placed under the control of the *Listeria* promoter P*actA*, inducing bacteria to undergo autolysis in eukaryotic cells. The rsΔ2 strain had normal growth rate in rich bacterial growth medium, but its replication in eukaryotic cells was limited, and its autolysis was used to deliver its contents to the cytoplasm of eukaryotic cells. The delivery potential of rsΔ2 was explored using engineered shuttle vectors designed to facilitate expression of a transgene, either in rsΔ2 (driven by P*hly*) or in the mammalian cell (driven by P_{CMV}), or both (using our engineered dual *Listeria* and mammalian expression vector, pDuLX). The luciferase reporter was used to demonstrate that pDuLX vector allowed delivery of both protein and DNA to dividing Caco-2 human epithelial cells. As expected, nondividing fully differentiated Caco-2 monolayers were resistant to transfection with Lipofectamine, which can be explained by lack of access to the cell nucleus. We demonstrated that when Caco-2 monolayers were treated with rsΔ2, the bacteria were able to deliver a significant quantity of luciferase protein. By implication the bacteria were also able to deliver DNA, but expression driven by the eukaryotic promoter in host Caco-2 cells was not observed. When the rsΔ2 strain was taken up by Caco-2 cells, there was little or no bacterial growth, whereas the control Δ2 strain was viable and grew by approximately three log cycles within the Caco-2 cells. A small mass of protein or DNA was delivered by the Δ2 strain perhaps because some bacteria died, but despite the level of growth the mass of protein delivered to dividing Caco-2 cells by the Δ2 strain was considerably less than that delivered by the rsΔ2 strain. We concluded that the *Listeria* delivery system has prospects for oral vaccination using antigens synthesized by the bacterium itself.

Keywords: *Listeria monocytogenes*; protein delivery; vaccine; antigen delivery; bacterial vaccine; gene delivery

Introduction

Oral delivery of macromolecules (e.g., proteins and nucleic acids) for prophylactic or therapeutic purposes such as vaccination or gene therapy would be attractive due to ease of administration and increased patient compliance. A number of significant challenges remain to be overcome, including appropriate cellular uptake and trafficking, and avoidance of degradation in the gut lumen. One approach is to use live recombinant microorganisms (bacteria and viruses) as vehicles for delivery of immunogenic proteins.[1,2]

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(1) Rocha, C. D.; Caetano, B. C.; Machado, A. V.; Bruna-Romero, O. Recombinant viruses as tools to induce protective cellular immunity against infectious diseases. *Int. Microbiol.* **2004**, *7*, 83–94.

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Bacterial vectors offer some advantages over viral vectors. They would be inexpensive to manufacture, and bacteria can provide a costimulatory adjuvant effect, or could be engineered to stimulate immunity by signaling through Toll-like receptors in a deliberate manner. In addition, antibiotics could be used to kill the bacteria in the event of an adverse response. Several bacterial strains have been developed and tested as vectors for DNA vaccinations or gene transfer purposes. Invasive entero-pathogens such as *Salmonella* spp, *Shigella flexneri*, invasive *Escherichia coli*, and *Listeria monocytogenes* have shown the most potential as vectors for delivering either protein or DNA (for reviews, see refs 3, 4.

The present study is focused on *Listeria monocytogenes,* which has recently received increasing attention as a bacterial vector for heterologous antigen delivery. This is mainly due its capacity to induce strong cell-mediated immunity which is required against infectious and neoplastic diseases.[5−8] The unique intracellular life cycle of *L. monocytogenes* has made it possible to engineer this species for cytoplasmic transfer of plasmid DNA and the eukaryotic expression of exogenous genes,[4,9−12] an approach which has potential for DNA vaccination and gene therapy.[13,14]

Listeria monocytogenes is a facultative intracellular bacterium responsible for severe infections, listeriosis, not only in humans but many animal species. This Gram-positive bacterium is capable of invading, surviving and replicating in the majority of mammalian host cells including nonphagocytic and phagocytic cells. Due to its intracellular parasitism, *L. monocytogenes* has been used for decades as a model for investigation of T cell-mediated immunity. The infection of *Listeria* is initiated by the interaction between listerial surface proteins such as internalin A and E-cadherin, its specific binding site on the intestinal cell surface. After internalization, the bacterium produces virulence factors including listeriolysin O (LLO) and two phospholipases (PlcA, PlcB) which facilitate escape from membrane bound vacuoles into the cytoplasm, i.e. before the vacuoles containing the bacterium, or the endosomal system, becomes fused with a destructive lysosome.

The free cytoplasmic *L. monocytogenes* replicates and forms polarized actin tails following expression of the surface protein ActA. This induced actin polymerization provides cytoplasmic motility that propels the bacterium toward the cell plasma membrane and forms a pseudopod-like structure, resulting in cell-to-cell spread. The membrane-bound protrusions are engulfed by neighboring cells.[15−17]

It has been suggested that *L. monocytogenes* is a safer vehicle than Gram-negative bacteria due to its lack of surface lipopolysaccharide (LPS). In addition, a *Listeria*-based delivery system can be administrated repeatedly because anti-*Listeria* immunity has no impact on the delivery potentials of *L. monocytogenes,* as shown in animal models.[18,19] A preclinical trial investigating the safety of the attenuated

(2) Ogra, P. L.; Faden, H.; Welliver, R. C. Vaccination strategies for mucosal immune responses. *Clin. Microbiol. Rev.* **2001**, *14*, 430–445.

(3) Gentschev, I.; Dietrich, G.; Spreng, S.; Pilgrim, S.; Stritzker, J.; Kolb-Maurer, A.; Goebel, W. Delivery of protein antigens and DNA by attenuated intracellular bacteria. *Int. J. Med. Microbiol.* **2002**, *291*, 577–582.

(4) Weiss, S.; Krusch, S. Bacteria-mediated transfer of eukaryotic expression plasmids into mammalian host cells. *Biol. Chem.* **2001**, *382*, 533–541.

(5) Ikonomidis, G.; Paterson, Y.; Kos, F. J.; Portnoy, D. A. Delivery of a viral antigen to the class I processing and presentation pathway by Listeria monocytogenes. *J. Exp. Med.* **1994**, *180*, 2209–2218.

(6) Soussi, N.; Milon, G.; Colle, J. H.; Mougneau, E.; Glaichenhaus, N.; Goossens, P. L. Listeria monocytogenes as a short-lived delivery system for the induction of type 1 cell-mediated immunity against the p36/LACK antigen of Leishmania major. *Infect. Immun.* **2000**, *68*, 1498–1506.

(7) Frankel, F. R.; Hegde, S.; Lieberman, J.; Paterson, Y. Induction of cell-mediated immune responses to human immunodeficiency virus type 1 Gag protein by using Listeria monocytogenes as a live vaccine vector. *J. Immunol.* **1995**, *155*, 4775–4782.

(8) Pan, Z. K.; Ikonomidis, G.; Lazenby, A.; Pardoll, D.; Paterson, Y. A recombinant Listeria monocytogenes vaccine expressing a model tumour antigen protects mice against lethal tumour cell challenge and causes regression of established tumours. *Nat. Med.* **1995**, *1*, 471–477.

(9) Dietrich, G.; Bubert, A.; Gentschev, I.; Sokolovic, Z.; Simm, A.; Catic, A.; Kaufmann, S. H.; Hess, J.; Szalay, A. A.; Goebel, W. Delivery of antigen-encoding plasmid DNA into the cytosol of macrophages by attenuated suicide Listeria monocytogenes. *Nat. Biotechnol.* **1998**, *16*, 181–185.

(10) Verch, T.; Pan, Z. K.; Paterson, Y. Listeria monocytogenes-based antibiotic resistance gene-free antigen delivery system applicable to other bacterial vectors and DNA vaccines. *Infect. Immun.* **2004**, *72*, 6418–6425.

(11) Pilgrim, S.; Stritzker, J.; Schoen, C.; Kolb-Maurer, A.; Geginat, G.; Loessner, M. J.; Gentschev, I.; Goebel, W. Bactofection of mammalian cells by Listeria monocytogenes: improvement and mechanism of DNA delivery. *Gene Ther.* **2003**, *10*, 2036–2045.

(12) Grillot-Courvalin, C.; Goussard, S.; Courvalin, P. Wild-type intracellular bacteria deliver DNA into mammalian cells. *Cell. Microbiol.* **2002**, *4*, 177–186.

(13) Miki, K.; Nagata, T.; Tanaka, T.; Kim, Y. H.; Uchijima, M.; Ohara, N.; Nakamura, S.; Okada, M.; Koide, Y. Induction of protective cellular immunity against Mycobacterium tuberculosis by recombinant attenuated self-destructing Listeria monocytogenes strains harboring eukaryotic expression plasmids for antigen 85 complex and MPB/MPT51. *Infect. Immun.* **2004**, *72*, 2014–2021.

(14) Shen, H.; Kanoh, M.; Liu, F.; Maruyama, S.; Asano, Y. Modulation of the immune system by Listeria monocytogenes-mediated gene transfer into mammalian cells. *Microbiol. Immunol.* **2004**, *48*, 329–337.

(15) Vazquez-Boland, J. A.; Kuhn, M.; Berche, P.; Chakraborty, T.; Dominguez-Bernal, G.; Goebel, W.; Gonzalez-Zorn, B.; Wehland, J.; Kreft, J. Listeria pathogenesis and molecular virulence determinants. *Clin. Microbiol. Rev.* **2001**, *14*, 584–640.

(16) Dussurget, O.; Pizarro-Cerda, J.; Cossart, P. Molecular determinants of Listeria monocytogenes virulence. *Annu. Rev. Microbiol.* **2004**, *58*, 587–610.

(17) Gordon, R. C. Listeria Monocytogenes infections. *Indian J. Pediatr.* **1995**, *62*, 33–39.

(18) Bouwer, H. G.; Shen, H.; Fan, X.; Miller, J. F.; Barry, R. A.; Hinrichs, D. J. Existing antilisterial immunity does not inhibit the development of a Listeria monocytogenes-specific primary cytotoxic T-lymphocyte response. *Infect. Immun.* **1999**, *67*, 253–258.

Listeria, as an oral vaccine vector, has been conducted in healthy adult humans, and no side effects were observed.[20]

In this study we have developed a *Listeria*-based delivery system that allows protein and plasmids to be released from a stably transformed strain of *Listeria* cells, after the bacteria have gained access to the cytoplasm of mammalian target cells. Our strategy has been designed to incorporate various safety features. Our work is based on a strategy first described by Werner Goebel and colleagues,[9] who deleted a cluster of three adjacent genes (*mpl, actA* and *plcB*), which are driven by a common promoter (P*actA*), to produce the attenuated strain Δ2. This strain lacks the ability to move within and between cells.

In this paper we report the construction of a suicidal *L. monocytogenes* strain (rsΔ2) that is capable of invading the mammalian cells through ligand/receptor interactions and, after autolysis, release the contents of the bacterial cell into the cytosol of the host cells. Previous strategies which have been used to achieve cytosolic release of DNA expression vectors have had disadvantages. These include obligatory treatment with antibiotics, which involves an additional treatment step, or the use of self-destructive plasmids, which may lead to intracellular toxicity.

For applications in oral vaccination we reasoned that it might be possible to deliver protein antigen or a DNA expression cassette. With this in mind we developed a dual expression vector which can be introduced into rsΔ2. This strategy allows both prokaryotic and eukaryotic transcription of an antigen from the same vector. The consequence is both delivery of exogenous proteins to the mammalian cell and the expression of endogenous proteins in the mammalian cell after *Listeria* infection. To the best of our knowledge this is the first report of a stably transformed, suicidal bacterial delivery system which can be used to replicate and deliver vaccines using shuttle vectors such as those we describe here.

Despite recent developments in delivery of DNA to the cytoplasm, the nuclear envelope still remains a major barrier to successful gene delivery into the nucleus. Association of nuclear localization sequences (NLS) with DNA has the potential to facilitate nuclear transfer of plasmid through the nuclear pore complex (NPC). Approaches to linking NLS to DNA involve covalent conjugation,[21−24] noncovalent conjugation[25−30] and binding of karyophilic proteins to target sequences in the plasmid.[31,32] We investigated a strategy designed to take advantage of the spontaneous activation of nuclear factor kappa-B (NF-κB) by *Listeria* infection. This has been observed in nonphagocytic and phagocytic cells.[33−35] By inserting repeated NF-κB binding sites into our expression vectors we created an opportunity for NF-κB to bind to the plasmid and provide NLS activity. According to the work of David Dean and colleagues this might be expected to improve the efficiency of gene delivery to the nucleus, and indeed this effect has been reported by Mesika et al.[36,37]

(19) Stevens, R.; Lavoy, A.; Nordone, S.; Burkhard, M.; Dean, G. A. Pre-existing immunity to pathogenic Listeria monocytogenes does not prevent induction of immune responses to feline immunodeficiency virus by a novel recombinant Listeria monocytogenes vaccine. *Vaccine* **2005**, *23*, 1479–1490.

(20) Angelakopoulos, H.; Loock, K.; Sisul, D. M.; Jensen, E. R.; Miller, J. F.; Hohmann, E. L. Safety and shedding of an attenuated strain of Listeria monocytogenes with a deletion of actA/plcB in adult volunteers: a dose escalation study of oral inoculation. *Infect. Immun.* **2002**, *70*, 3592–3601.

(21) Sebestyen, M. G.; Ludtke, J. J.; Bassik, M. C.; Zhang, G.; Budker, V.; Lukhtanov, E. A.; Hagstrom, J. E.; Wolff, J. A. DNA vector chemistry: the covalent attachment of signal peptides to plasmid DNA. *Nat. Biotechnol.* **1998**, *16*, 80–85.

(22) Zanta, M. A.; Belguise-Valladier, P.; Behr, J. P. Gene delivery: a single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 91–96.

(23) Ludtke, J. J.; Zhang, G.; Sebestyen, M. G.; Wolff, J. A. A nuclear localization signal can enhance both the nuclear transport and expression of 1 kb DNA. *J. Cell Sci.* **1999**, *112* (Part 12), 2033–2041.

(24) Ciolina, C.; Byk, G.; Blanche, F.; Thuillier, V.; Scherman, D.; Wils, P. Coupling of nuclear localization signals to plasmid DNA and specific interaction of the conjugates with importin alpha. *Bioconjugate Chem.* **1999**, *10*, 49–55.

(25) Aronsohn, A. I.; Hughes, J. A. Nuclear localization signal peptides enhance cationic liposome-mediated gene therapy. *J. Drug Targeting* **1998**, *5*, 163–169.

(26) Collas, P.; Alestrom, P. Nuclear localization signals enhance germline transmission of a transgene in zebrafish. *Transgenic Res.* **1998**, *7*, 303–309.

(27) Branden, L. J.; Mohamed, A. J.; Smith, C. I. A peptide nucleic acid-nuclear localization signal fusion that mediates nuclear transport of DNA. *Nat. Biotechnol.* **1999**, *17*, 784–787.

(28) Subramanian, A.; Ranganathan, P.; Diamond, S. L. Nuclear targeting peptide scaffolds for lipofection of nondividing mammalian cells. *Nat. Biotechnol.* **1999**, *17*, 873–877.

(29) Liang, S. H.; Clarke, M. F. The nuclear import of p53 is determined by the presence of a basic domain and its relative position to the nuclear localization signal. *Oncogene* **1999**, *18*, 2163–2166.

(30) Balicki, D.; Putnam, C. D.; Scaria, P. V.; Beutler, E. Structure and function correlation in histone H2A peptide-mediated gene transfer. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 7467–7471.

(31) Kaneda, Y.; Iwai, K.; Uchida, T. Increased expression of DNA cointroduced with nuclear protein in adult rat liver. *Science* **1989**, *243*, 375–378.

(32) Vacik, J.; Dean, B. S.; Zimmer, W. E.; Dean, D. A. Cell-specific nuclear import of plasmid DNA. *Gene Ther.* **1999**, *6*, 1006–1014.

(33) Hauf, N.; Goebel, W.; Fiedler, F.; Kuhn, M. Listeria monocytogenes infection of Caco-2 human epithelial cells induces activation of transcription factor NF-kappa B/Rel-like DNA binding activities. *FEMS Microbiol. Lett.* **1999**, *178*, 117–122.

(34) Rose, F.; Zeller, S. A.; Chakraborty, T.; Domann, E.; Machleidt, T.; Kronke, M.; Seeger, W.; Grimminger, F.; Sibelius, U. Human endothelial cell activation and mediator release in response to Listeria monocytogenes virulence factors. *Infect. Immun.* **2001**, *69*, 897–905.

(35) Hauf, N.; Goebel, W.; Fiedler, F.; Sokolovic, Z.; Kuhn, M. Listeria monocytogenes infection of P388D1 macrophages results in a biphasic NF-kappaB (RelA/p50) activation induced by lipoteichoic acid and bacterial phospholipases and mediated by IkappaBalpha and IkappaBbeta degradation. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 9394–9399.

Table 1. Strains of *Listeria monocytogenes* Used in This Study

strains	locus	ref
EGD (wt)	*L. monocytogenes* Sv 1/2a EGT wild-type	37
Δ2	attenuated strain of *L. monocytogenes* Sv 1/2a; Δ(*mpl,actA plcB*); this strain has restricted mobility in mammalian cells	37
rsΔ2	recombinant suicidal strain of *L. monocytogenes*: derived from the Δ2 strain by targeting an expression cassette of the cell-wall holin and endolysin pair *hol118/ply118* to the *orfXYZ/orfBA* locus by homologous recombination; the *hol118/ply118* genes were expressed under the control of the *actA* promoter	this study

Table 2. List of Plasmids

plasmids	characteristics	ref
pLSV1	Em^r, ori^{ts} pE194	39
p3L	Amp^r, Tet^r, ori pBCE16, pCMV	this study
p3L-Luc	P3L:Luc	this study
pLH8	pLSV:P*actA-ply118*/orf*XYZ*	this study
pDuLX	Amp^r, Tet^r, ori pBCE16, PCMV/P*hly*, NF-κB site(5x)	this study
pDuLX-Luc	pDuLX:*luc*	this study
pDuLX-Luc-ΔCMV	pDuLX-Luc lacking the CMV promoter	this study

Experimental Section

Bacterial Strains and Plasmids. All *Listeria* strains (Table 1) were maintained in brain−heart infusion (BHI) broth, and *E. coli* DH5α was grown in Luria−Bertani (LB) medium at 37 °C. Antibiotics were added at the following concentrations when required: ampicillin (Amp), 100 μg/mL erythromycin (Em), 5 μg/mL, tetracycline (Tet) 15 μg/mL. For determination of P*hly* activity, *Listeria* strains were grown in BHI pretreated with 0.2% activated charcoal (BHIC). The plasmids constructed in this study are listed in Table 2

Construction of pLH8 Knock-in Plasmid Vector. Chromosomal DNA from the attenuated *L. monocytogenes* was prepared as described by Flam et al.[38] A 2.1 kb fragment comprising an orfXYZ region was used to provide the homologous region for subsequent allelic exchange. This fragment was amplified by polymerase chain reaction (PCR) using the primers 5′-GATGAATTCCAAGGTATTGATGGT T-3′ (*Eco*RI site is underlined) and 5′-GTCCGGA TC-CATTAGATCTTTACG-3′ (*Bam*HI site is underlined) and ligated into pUC18, yielding the plasmid pUH. A fusion gene coding the *actA* promoter region of *Listeria* and a fragment encoding the cDNA of genes for the holin and hydrolysin *hol118/ply118* of listerial bacteriophage A118 was also amplified by PCR from the plasmid p3LGFP118[9] using the primers 5′-CCTAGGATATCGCAAAAAGCTC-3 and 5′-CGA AAATGGATATCCAAGCTC-3′ (*Eco*RV sites are underlined) and cloned into the *Eco*RV linearized pUH, generating pUH8. The cassette of the homologous region with *hol118/ply118* genes regulated by *actA* promoter was excised from pUH8 by double digest with *Eco*RI and *Bam*HI and then ligated into the pLSV1 shuttle vector,[39] leading to the pLH8 knock-in vector (Figure 1A).

Generation of the Suicidal *L. monocytogenes* Strain (rsΔ2). pLH8 was introduced into *L. monocytogenes* strain Δ2 by electroporation as described by Stewart et al.[40] For screening, the electroporants were grown on BHI agar plates containing erythromycin (5 μg/mL) at permissive temperature (30 °C). Selection of the first homologous recombination was carried out after incubating the electroporants onto the BHI plates with erythromycin at the nonpermissive temperature (42 °C). Selected recombinants were subsequently grown in BHI broth, without the antibiotics, at 30 °C, to excise the plasmid from the listerial genome by a second homologous recombination. Putative clones were screened by PCR to confirm the chromosomal integration of the autolysis element and the loss of the pLSV1 backbone.

Construction of Expression Vectors. pDuLX and pDuLX-Luc. The promoter region *hly* was amplified from the *Listeria* chromosome by PCR with the primers 5′-TTCC AAGCT-TAAAGTGACTTTTATG-3 and 5′-CACTAAGCTTCTA-CATTTTTTAACC-3′ (*Hin*dIII site is underlined) and cloned into *Hin*dIII-digested pcDNA3.0 (Invitrogen), yielding p3p*hly*. Correct orientation of the insert was confirmed by restriction fragment analysis. A 73 bp oligonucleotide construct consisting of five repeats of the NF-κB binding site, 5′-GCTGGGACTTTCC-3′, and *Bam*HI restriction site was synthesized and inserted into *Bgl*II/*Nru*I-digested p3p*hly* plasmid, giving rise to pn3p*hly*. A 5.0 kb DNA fragment containing the tetracycline resistance gene and a Gram-positive bacteria origin of replication was isolated from the shuttle vector p3LGFP (comprising pBCE16 and pcDNA 3) by *Sal*I digestion and cloned into the *Stu*I-digested pn3p*hly*, yielding the duplex plasmid pDuLX. For pDuLX-Luc constructions, the luciferase cDNA was isolated from pC-MVLuc and subcloned into *Bam*HI/*Xho*I-digested pDuLX.

(36) Mesika, A.; Grigoreva, I.; Zohar, M.; Reich, Z. A regulated, NFkappaB-assisted import of plasmid DNA into mammalian cell nuclei. *Mol. Ther.* **2001**, *3*, 653–657.

(37) Mesika, A.; Kiss, V.; Brumfeld, V.; Ghosh, G.; Reich, Z. Enhanced intracellular mobility and nuclear accumulation of DNA plasmids associated with a karyophilic protein. *Hum. Gene Ther.* **2005**, *16*, 200–208.

(38) Flamm, R. K.; Hinrichs, D. J.; Thomashow, M. F. Introduction of pAM beta 1 into Listeria monocytogenes by conjugation and homology between native L. monocytogenes plasmids. *Infect. Immun.* **1984**, *44*, 157–161.

(39) Wuenscher, M. D.; Kohler, S.; Goebel, W.; Chakraborty, T. Gene disruption by plasmid integration in Listeria monocytogenes: insertional inactivation of the listeriolysin determinant lisA. *Mol. Gen. Genet.* **1991**, *228*, 177–182.

(40) Egan, P.; Cheers, C. Relationship between colony-stimulating activity and interferon production during infection. *Immunology* **1990**, *70*, 191–196.

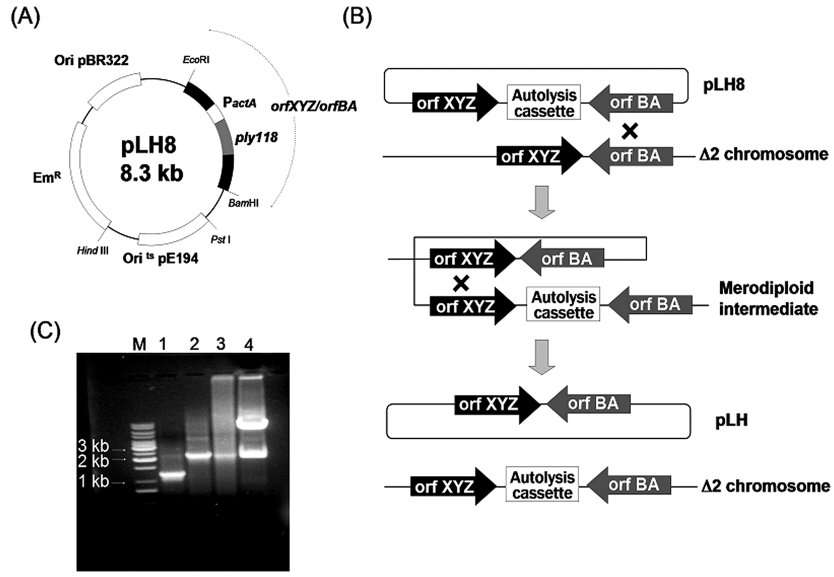

Figure 1. Construction of rsΔ2, a suicidal strain of *Listeria monocytogenes*. (A) The plasmid pLH8 comprising a temperature-sensitive (ts) origin from pE194 was generated for homologous recombination by cloning the 2.1 kb orf*XYZ*/P*act*A/*hol118/ply118* fragment into the plasmid pLSV1, as described in the Experimental Section. (B) Schematic diagram of the construction, by homologous recombination of the stable suicidal strain of *Listeria monocytogenes* rsΔ2. (C) Agarose gel confirming the integration of the autolysis cassette (P*act*A/*hol118/ply118* fragment) into the *L. monocytogenes* genome by PCR. Lane M, DNA maolecular weight markers; lanes 1−3, PCR products from *L. monocytogenes* Δ2 genome, rsΔ2 genome and pLH8, respectively; lane 4, the plasmid pLH8 digested by *Bam*HI/*Eco*RI liberating the autolysis cassette.

Cell Culture, Transfection, and Infection. The human colon adenocarcinoma cell line Caco-2 (ATCC HTB37) (kindly provided by Peter Gibson, Monash University, Australia) was propagated in antibiotic-free Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) supplemented with 10% fetal calf serum (FCS; Gibco-BRL) at 37 °C in a humidified 5% CO_2 atmosphere. For transfections, cells were seeded 24 h prior to transfection into a 6-well plate at a density of 8×10^5 cells per well with 2 mL of complete culture medium. On the following day the medium was replaced with 1 mL of Opti-MEM (Gibco-BRL) containing Lipofectamine (Invitrogen)/DNA complexes. After 4 h of incubation, the medium was replaced with 2 mL of complete medium. Forty-eight hours after transfection, cells were harvested and assayed for luciferase activity. For examination of luciferase activity resulting from transduction with *Listeria*, the growth medium was renewed 24 h prior to the introduction of bacteria. After washing three times with phosphate-buffered saline (PBS), the cells were infected with logarithmically growing *L. monocytogenes* at the ratio of 10 bacteria per cell, and then incubated at 37 °C for 60 min in DMEM containing 2% FCS. After incubation, extracellular bacteria were removed by washing the cells three times with PBS and cultured in DMEM containing 2% FCS and 10 μg/mL gentamycin (Sigma) for 30 min to avoid reinfections.

Differentiated Caco-2 Cell Culture. In order to induce differentiation, Caco-2 cells were seeded at a density of 1×10^5 cells/mL on 6.5 mm cell culture inserts in apical (TranswellPermeable Supports, pore size 3 μm, Corning, Sydney, Australia) and basolateral sides (ThinCerts pore size 3 μm, Greiner bio-one, Sydney, Australia). The inserts were placed into 24-well tissue culture plates and cultured for up to 28 days at the same culture condition (described above) but without the antibiotics. Cell culture medium was changed every second day for the first 7 days and then every day until the cells were fully differentiated (day 28). Samples, in triplicate, were taken on day 3 and day 28, harvested and analyzed for luciferase activity 2 or 24 h after infection.

Intracellular Bacterial Survival and Growth Assay. Caco-2 cells were infected and treated as described above. At selected time points, cells were washed three times with PBS and lysed with ice-cold sterile distilled water (applied for 5 min). Lysates were removed from the wells, diluted 10-fold serially in PBS, and plated onto BHI agar plates. Plates were incubated at 37 °C overnight, and colony forming units (CFU) were counted.

Determination of Luciferase Activity. The luciferase assay was performed using the luciferase reporter assay system (Promega). Briefly the medium was removed from the plates, and the cells were washed with PBS. Cells were then lysed with 200 μL of cell lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10% glycerol, and 1% Triton X-100). Luciferase assays were carried out on cell lysate as follows. Twenty microliters of the cell lysate was added to 100 μL of the luciferase assay buffer, swirled briefly, and placed in a luminometer (Fluostar BMG). The signal was integrated for 30 s with a 2 s delay and was reported in relative light units (RLU).

Statistical analysis. The significance of the differences in expression levels was evaluated using one-way analysis

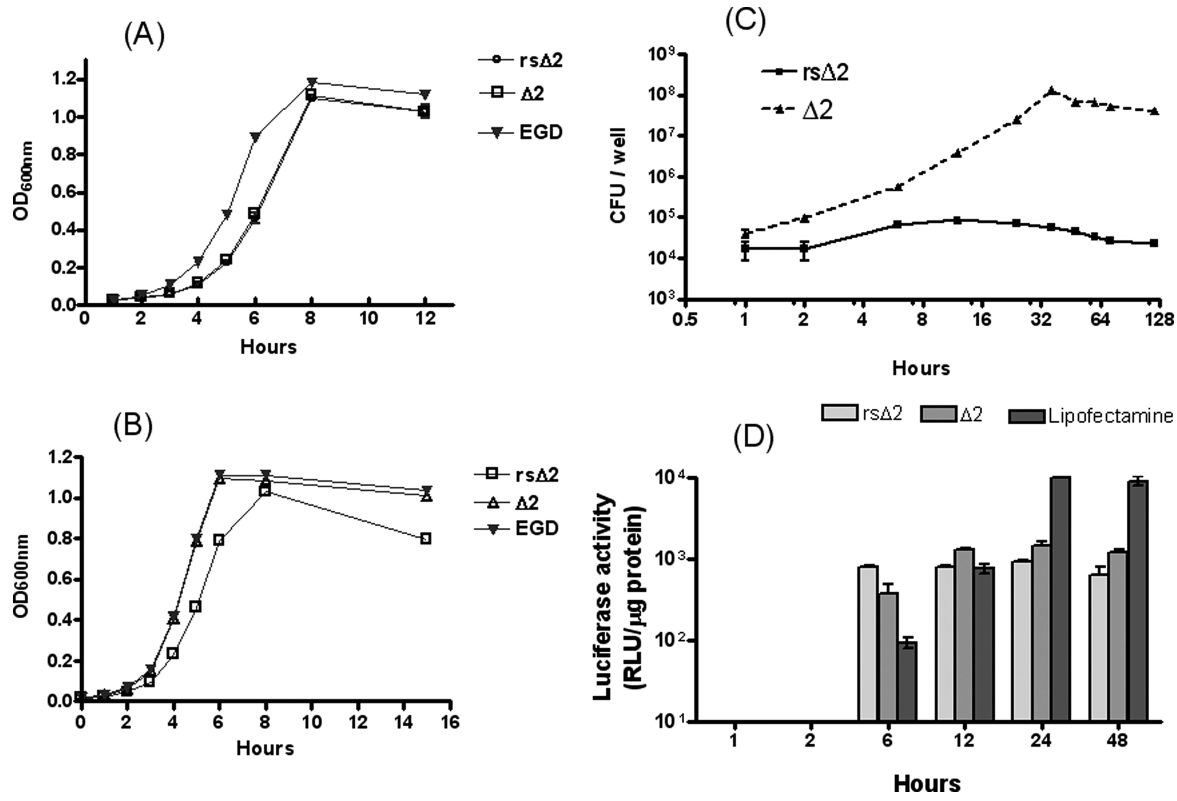

Figure 2. Growth of *Listeria monocytogenes* rsΔ2 in culture medium, human Caco-2 cells, and transfection of Caco-2 cells with a luciferase reporter gene. Growth kinetics of *Listeria monocytogenes* strains in (A) BHI and (B) BHIC. Overnight cultures of *Listeria* strains, rsΔ2, Δ2 and the wild type EGD, were diluted 1:100 in fresh medium and grown for 12 h (BHI) or 16 h (BHIC) at 37 °C and 200 rpm. The absorbance at OD_{600nm} was measured generally every 60 min during growth phase ($n = 3$). (C) Intracellular growth of *L. monocytogenes* strains rsΔ2 and Δ2 in Caco-2 cells after transduction. Bacterial growth was assayed by plating out bacteria extracted from Caco-2 cells. Data were expressed as the mean number of bacterial CFUs ± standard error ($n = 3$) extracted from Caco-2 cells at each time point over a 120 h. (D) Luciferase expression resulting from delivery of a mammalian expression plasmid to dividing Caco-2 cells (3 days after plating at 20% confluency). Caco-2 cultures were transfected with the shuttle vector p3LLuc, which contains an episomal CMV-*luc* expression cassette, using either rsΔ2, Δ2 or Lipofectamine as the mammalian cell transduction system.

of variance (ANOVA). Where $p < 0.01$ Dunnett's multiple comparison was carried out to compare all samples with untreated controls (GraphPad Prism 5).

Results and Discussion

Construction of a Recombinant Suicidal *L. monocytogenes* Strain, rsΔ2. To construct a strain of *L. monocytogenes* capable of undergoing programmed autolysis within the host cells, a fusion gene consisting of the promoter *actA* and *hol118/ply118* (the autolysis cassette) retrieved from plasmid p3LGFP118 was integrated into the *Listeria* chromosome. This was achieved without disrupting any of the bacterial genes essential for the growth of the organism (Figure 1). Since the integration of foreign DNA at the open reading frame "XYZ" (orf*XYZ*) of the *Listeria* chromosome has no influence on bacterial growth,[41] a copy of orf*XYZ* was inserted into pUC18 for subsequent homologous recombination. The autolysis cassette was amplified from p3LGFP118 and cloned into *Eco*RV site of orf*XYZ*/pUH to form integration element, and then the entire integration element fragment was subcloned into the shuttle vector, pLSV-1, generating pLH8 (Figure 1A).

This was then delivered into the competent *L. monocytogenes* by electroporation. A strain with chromosomal integration of autolysis cassette was positively selected using a series of growth conditions (see Experimental Section). The presence of orf*XYZ* in the strain was confirmed by PCR (Figure 1C). The strain thus generated was designated as rsΔ2 (recombinant suicidal *Listeria monocytogens* Δ*2*). The growth rate of rsΔ2 strain as compared to its parental strains, Δ2 and the wild type strain *Listeria monocytogenes* (EGD), in a standard rich medium such as brain−heart infusion broth (BHI) was comparable (Figure 2A) and not significantly

(41) Shen, H.; Slifka, M. K.; Matloubian, M.; Jensen, E. R.; Ahmed, R.; Miller, J. F. Recombinant Listeria monocytogenes as a live vaccine vehicle for the induction of protective anti-viral cell-mediated immunity. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 3987–3991.

different in charcoal-treated BHI (BHIC) (Figure 2B), reaching stationary phase after 4−6 h in all cases.

Intracellular autolysis of the suicidal mutant within the mammalian cells was tested using the human colon carcinoma Caco-2 cell line as a model. This cell line is a commonly used colon carcinoma which divides freely in plate-based culture, but can be used to generate a differentiated polarized monolayer using filter culture, which closely resembles the epithelium of the small intestine. Experiments to investigate the intracellular growth of *Listeria* in dividing Caco-2 cells indicated that growth of the rsΔ2 strain was restricted by *Listeria*-specific hydrolysin-mediated lysis (Figure 2C). After infection with rsΔ2 the CFU/well appeared to double over 12 h, during which time uptake may have been progressing, but total CFU/well then declined to levels similar to 1 h over the next day, and continued to decline over the next few days. In contrast the parental Δ2 strain, which was not suicidal, grew logarithmically in Caco-2 cells after infection over 24 h by almost four log cycles (Figure 2C).

Preliminary Evaluation of DNA Delivery by *L. monocytogenes* rsΔ2 and Validation of the Expression Plasmids. *L. monocytogenes* rsΔ2 and its parental strain Δ2 (as control) were used as agent to transfect the shuttle vector p3L-Luc into the E-cadherin expressing Caco-2 cells (E-cadherin required for *Listeria invasion*), at a MOI of 10:1. The reporter plasmid p3L-Luc, which contained a eukaryotic expression cassette, was electroporated into the *L. monocytogenes* prior to transfections. For comparison the Caco-2 cells were transfected with 0.5 μg of p3L-Luc conventionally using Lipofectamine (Figure 2D). DNA-transfer efficiency was determined by the levels of luciferase activity within the dividing Caco-2 cells at predetermined time points. As shown in Figure 2D using 3-day old cultures, at the early time of 6 h postinfection, rsΔ2 demonstrated the highest luciferase activity, 3- and 8-fold higher than Δ2 and Lipofectamine, respectively, resulting from PLY118-mediated release of plasmids.

The luciferase activity 24−48 h after infection with rsΔ2 was lower than that detected after transfection with Δ2 and Lipofectamine. We postulate that further incubation of Caco-2 cells allows Δ2 to replicate within the cytoplasm resulting in the amplification of the cytosolic plasmids. In the presence of this high bacterial load some release of plasmids into the cytoplasm would occur due to a small amount of spontaneous bacterial lysis. This would allow access of DNA to the nucleus during Caco-2 cell division. Since luciferase has a short half-life, the lower activity found with rsΔ2 when compared to other treatments after 24 h and 48 h could be explained by degradation of the protein. Transfection of 7-day-old confluent Caco-2 cell adherent plate cultures resulted in a 100-fold decrease in luciferase expression, compared with those in 3-day-old cultures (not shown). This can be explained by the lower frequency of cell divisions, which restricted the transfer of DNA into daughter cell nuclei.

The experiments carried out on 3-day adherent plate cultures indicate that plasmids are released into cytosol after bacterial lysis. Transfection of 3-day cultures is efficient using all delivery systems including Lipofectamine. The reduction in expression levels in 7-day cultures (referred to above) indicates that the mammalian expression vector p3L-Luc requires the cells to be dividing to gain access to the cell nucleus. In the experiments using adherent plate-cultured Caco-2 cells, the Δ2 strain, which is able to replicate within the cytoplasm, showed a noticeable gene transfer activity after extended incubation of the Caco-2 cells. This finding is in agreement with previous reports in which nonphagocytic cells were used for transfections.[4,11,12,42] One explanation is that nonphagocytic cells may not have effective bactericidal activity, which allows rapid intracellular replication of the bacteria resulting in excess plasmid release due to the incidence of spontaneous lysis.

Taken together the data shown in Figure 2 indicate that (i) the eukaryotic expression vectors were active when the DNA gained access to cell nuclei during mitosis, (ii) the rsΔ2 strain was suicidal, and (iii) the suicidal bacterial strain can deliver material to mammalian cells. However the aim of the work is to deliver protein and or DNA to nondividing cells. To investigate this aim we extended our work by investigating transfection of differentiated Caco-2 monolayers grown using filter-culture (described below). We believe this is a more realistic model of the challenge faced in oral vaccine delivery. Gene expression following attempted transfection of differentiated Caco-2 cells with Lipofectamine is negligible, as reported elsewhere.[43,44] Oral vaccination using bacterial delivery systems could be achieved by the delivery of recombinant protein antigens or eukaryotic expression plasmids. We reasoned that transfection of nondividing cells may require active processes for intracellular trafficking and delivery to the nucleus. Thus even if delivery of plasmids to the cytoplasm is achieved, the access of plasmids to the nucleus is likely to be limited. With this in mind we established a combined expression system that simultaneously delivers recombinant protein and expression plasmid into mammalian cells. We constructed a novel dual expression vector (using serial promoters for a single gene), named pDuLX, which we used in association with rsΔ2 (Figure 3). We also excised the CMV promoter, which is active in mammalian cells, to produce the vector pDuLX-ΔCMV, which should only allow expression in prokaryotic cells.

(42) Hense, M.; Domann, E.; Krusch, S.; Wachholz, P.; Dittmar, K. E.; Rohde, M.; Wehland, J.; Chakraborty, T.; Weiss, S. Eukaryotic expression plasmid transfer from the intracellular bacterium Listeria monocytogenes to host cells. *Cell. Microbiol.* **2001**, *3*, 599–609.

(43) Mortimer, I.; Tam, P.; MacLachlan, I.; Graham, R. W.; Saravolac, E. G.; Joshi, P. B. Cationic lipid-mediated transfection of cells in culture requires mitotic activity. *Gene Ther.* **1999**, *6*, 403–411.

(44) Uduehi, A. N.; Moss, S. H.; Nuttall, J.; Pouton, C. W. Cationic lipid-mediated transfection of differentiated Caco-2 cells: a filter culture model of gene delivery to a polarized epithelium. *Pharm. Res.* **1999**, *16*, 1805–1811.

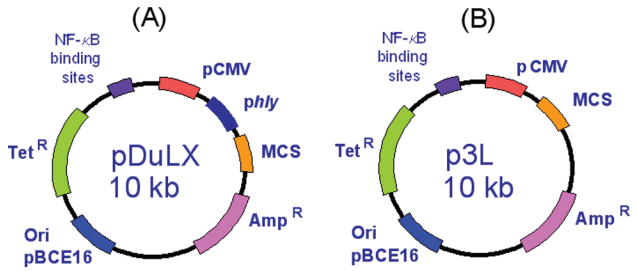

Figure 3. Schematic representations of modified shuttle/expression vectors. (A) pDuLX: Dual expression vector for protein expression in both *Listeria* and mammalian cells. This vector contains five NF-κB-binding sites which may enhance delivery of the plasmid to the mammalian cell nucleus. (B) p3L: this vector is analogous to pDuLX but lacks the *hly* promoter and NF-κB-binding sites. Key: P*hly*, listeriolysin O promoter from *Listeria monocytogenes*; pCMV, immediate-early promoter of hCMV; Ori pBCE16, replication region for *Listeria monocytogenes*; Tet, tetracycline (selection marker); Amp, ampicilin (selection marker); MCS, multiple cloning sites; NF-κB-binding site.

Listerial P*hly* Activity Is Induced during Growth in BHIC Medium. In wild-type *Listeria,* P*hly* controls the transcription of the *hly* gene encoding "listeriolysin O". which is regulated by the pleiotropic regulatory activator A (PrfA) in response to several environmental factors such as nutritional stress and temperature. Such stresses have been reported to have a positive effect on the regulation of P*hly*. This promoter is remarkably active at the early stage of intracellular life cycle and remains weak in a nutrient-rich medium such as BHI. It has also been shown that growth of *Listeria* in charcoal-treated BHI can induce the expressions of genes which are under regulation of PrfA. Therefore, *Listeria* strains, rsΔ2 and Δ2, transformed with pDuLX-Luc and p3L-Luc, were grown in BHI or BHIC to determine the functionality of P*hly* cloned into pDuLX. Luciferase activity was easily detected when *L. monocytogenes* rsΔ2/pDuLX-Luc was grown in BHI, but not when the bacteria contained p3L-Luc. A significant increase in luciferase activity (per unit mass of total protein) was detected when *L. monocytogenes* rsΔ2/pDuLX-Luc was grown in BHIC (Figure 4A). Again no luciferase was detected during growth of rsΔ2/p3L-Luc. Since the rsΔ2 strain was constructed by chromosomal integration of *hol118/ply118*, under the control of P*actA,* which is also regulated by PrfA, charcoal induction could potentially activate P*actA* resulting in the autolysis of LM rsΔ2 during cultivation. We found no such an effect on the growth of rsΔ2 strain in BHIC medium (Figure 2A).

Listerial P*hly* Activity Is Induced during Growth in Caco-2 Cells. Luciferase expression driven by our pDuLX-Luc vector after either *Listeria*-mediated transduction or lipofection of Caco-2 cells was examined initially using cells cultured on regular adherent plates. As shown in Figure 4B, luciferase activity was detected in 3-day-old cultures of Caco-2 cells as early as 1 h after infections with rsΔ2/pDuLX-Luc and Δ2/pDuLX-Luc, while luciferase expression was not observed until 12 h with Lipofectamine. The results can be compared with the previously described data obtained using p3L-Luc. Results with pDulX-Luc demonstrate that P*hly* promoter is functional and either is already activated when the bacteria are in culture or is immediately activated upon infection, leading to bacteria-driven expression, which can be detected after 1−2 h. Host cell expression driven by pCMV is likely to be responsible for much of the luciferase detected after 6 h, as exemplified by the Lipofectamine transfections. This is consistent with the expected time scale for gene expression after transfection of dividing cells, which normally reaches a peak at 24−48 h for luciferase.

Luciferase has a short half-life in living cells so that the activity of the prokaryotic protein product is likely to be lost over the first few hours. Seven-day-old cultures of Caco-2 cells were more confluent, implying a lower proportional rate of cell division. The time course of luciferase expression in these cells was similar to that observed with cells in log phase of growth, but the lower mass of luciferase detected (typically 10% of that expressed in 3-day-old cultures in many cases) likely reflects the relative lack of opportunity for uptake of plasmid into daughter cell nuclei (Figure 4C). Luciferase activity after 1−2 h, thought to be due to prokaryotic expression, was again evident when the 7-day cultures were treated.

Kinetics and Extent of Luciferase Expression in Differentiated Epithelium after *Listeria*-Mediated Transfection. To investigate the effects of cell differentiation on *Listeria*-based transfections, Caco-2 cells were transfected both apically and basolaterally at different stages of differentiation during "filter-culture". Using this culture method Caco-2 cells adopt the differentiated, polarized phenotype of small intestinal epithelia, develop microvilli, and stop dividing within 2−4 weeks. The Caco-2 cell line was used as a model primarily because of this well-known characteristic of spontaneous differentiation.[33,45,46] In addition its NF-κB activity is inducible upon *Listeria* infection, which could be useful for evaluating the influence of NF- kB on nuclear delivery of mammalian expression plasmids.

The levels of luciferase activity at different stages of Caco-2 differentiation after apical transfection over a time period 2−24 h are shown in Figure 5, with either *Listeria* (rsΔ2 or Δ2) or Lipofectamine. After apical exposure, as differentiation of the Caco-2 cells progressed, differences were observed in extent and the time-course of expression. The cells were plated onto the filter wells at approximately 70% confluence, so it was expected that mitosis would still be evident at day 3. This was confirmed by transfecting the cultures with DuLX-Luc using Lipofectamine (Figure 5A), which generated highly significant levels of luciferase activity 24 h after transfection. By contrast the cells which had been cultured for 28 days were resistant to transfection with

(45) Goldfine, H.; Wadsworth, S. J. Macrophage intracellular signaling induced by Listeria monocytogenes. *Microbes Infect.* **2002**, *4*, 1335–1343.

(46) Daniels, J. J.; Autenrieth, I. B.; Goebel, W. Interaction of Listeria monocytogenes with the intestinal epithelium. *FEMS Microbiol. Lett.* **2000**, *190*, 323–328.

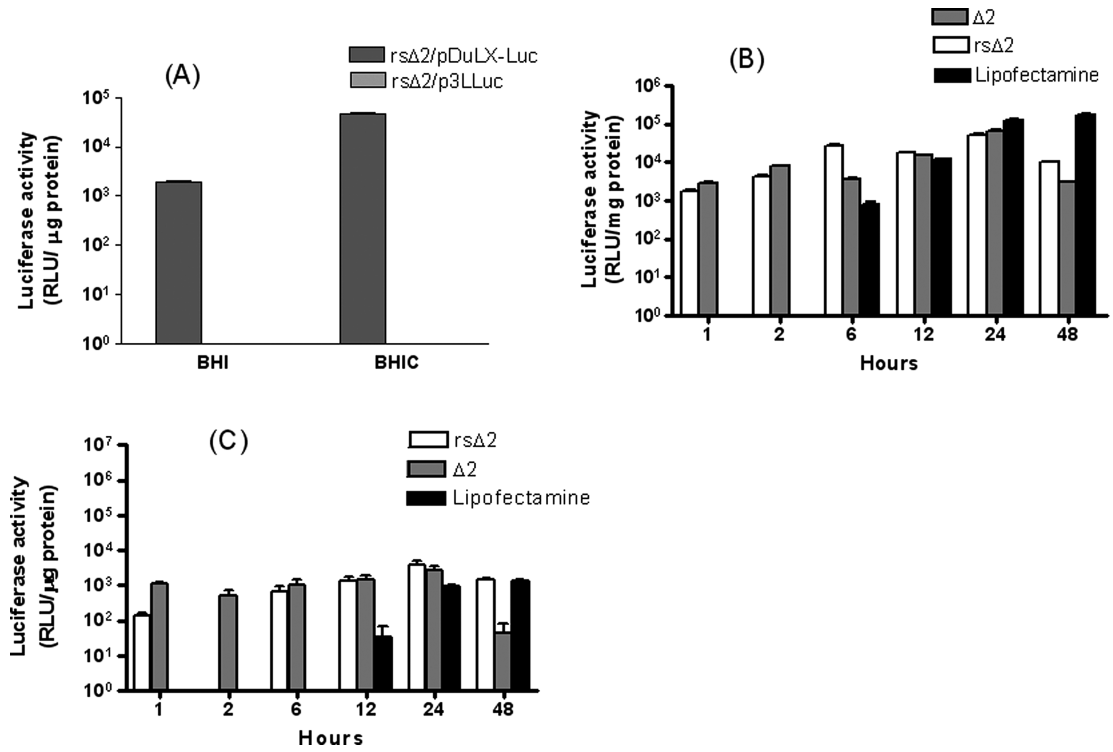

Figure 4. Functionality of the dual expression vector pDuLX. (A) Luciferase expression in *Listeria* rsΔ2 strain transformed with pDuLX-Luc or p3L-Luc. Growth of *Listeria* in BHIC caused a 50-fold upregulation of luciferase expression driven by P*hly* in *Listeria*. No expression was detected in bacteria containing p3L-Luc. (B, C) Kinetics of dual luciferase expression of luciferase from pDuLX-Luc after transduction of plate-cultured Caco-2 cells with bacteria or Lipofectamine. Caco-2 cultures were exposed, 3 days (B) or 7 days (C) after plating at 20% confluency, to *Listeria* rsΔ2 or Δ2 transduced with pDuLX-Luc, or pDuLX-Luc complexed with Lipofectamine. After transduction with *Listeria*, luciferase protein produced under the control of the *hly* promoter could be detected in the Caco-2 cells after 1 h. Mammalian gene expression caused by Lipofectamine was not detected until 6 h in 3-day-old cultures or 12 h in 7-day-old cultures.

Lipofectamine (Figure 5B). This suggests that the poor nuclear access in nondividing cells cannot be rescued after Lipofectamine treatment by making use of NF-κB binding sites. We do not know whether NF-κB is activated on lipofection, but plasmid delivery to the nucleus is clearly very limited and luciferase levels were not significantly different from untreated cells (i.e., background noise of the assay). The bacterial delivery systems also resulted in significant levels of gene expression when day 3 filter-cultures were examined 24 h after exposure to rsΔ2 or Δ2. This luciferase activity was thought to be due to transfection of Caco-2 cells with plasmid released from the *Listeria* because the activity developed after 24 h and was 10−20 times higher than that normally achieved by protein expressed in the bacteria. The levels of expression after treatment with the bacterial delivery systems were variable, but this may be due to differences in the extent of cell division occurring on the filters on day 3. We did not consider this to be an important result and did not study this exhaustively. The more important data is the performance of the delivery systems in 28-day filter-cultures (Figures 5B and 6). Levels of luciferase activity were low so using Dunnett's multiple comparison test we compared the luciferase activities for each test system with that of control (untreated cells). When apical treatment was used, only the Caco-2 cells infected with rsΔ2/pDuLX-Luc had luciferase activity significantly higher than controls. Luciferase activity was significant 2 and 24 h after exposure ($p < 0.01$), which suggests that the activity results from delivery of luciferase expressed in the *Listeria* cells. This conclusion is supported by the observation that, although the activity determined after treatment with rsΔ2/p3L-Luc appeared to be higher, it was not significant. Treatment with either plasmid by lipofection was also ineffective, suggesting that plasmids cannot gain access to the nucleus. We also applied treatment to the basolateral surface of differentiated monolayers making use of filter inserts with wide pore dimensions which we could invert in the well plates. One reason for trying this approach is that we reasoned that the apical surface might display limited endocytosis, whereas the basolateral surface is expected to be more involved in vesicular trafficking. In the basolateral treatment experiments we also tested a plasmid which was designed to express luciferase only in *Listeria*. This plasmid rsΔ2/pDuLX-Luc-ΔCMV was derived by excising the CMV promoter from rsΔ2/pDuLX-Luc. Figure 6 shows that the pattern of expression after basolateral treatment was similar to apical, but in this case the activity following treatment with rsΔ2/pDuLX-Luc-ΔCMV was also

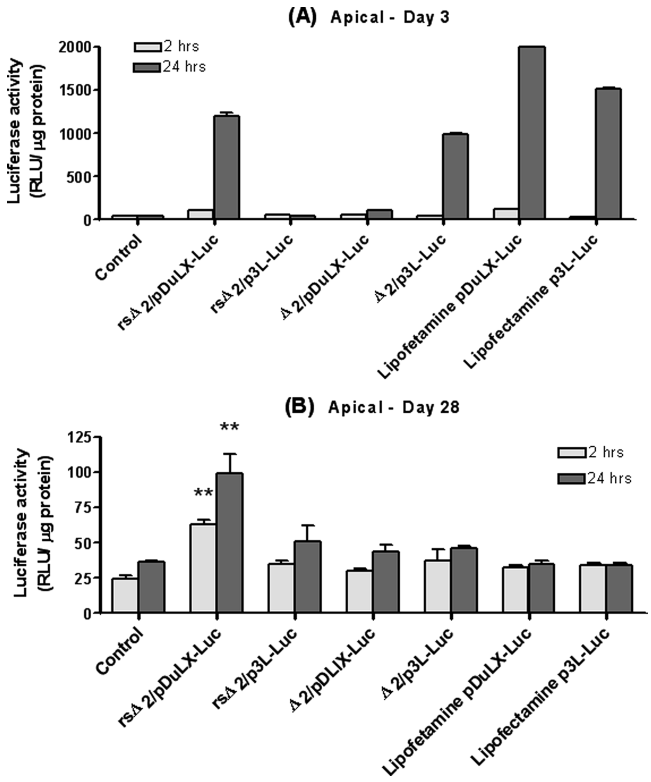

Figure 5. Luciferase expression (RLU/μg total protein) in (A) immature (3-day) and (B) fully differentiated (28-day) filter-cultured Caco-2 cells after transduction by way of the apical surface with *Listeria* rsΔ2 or Δ2, or Lipofectamine, carrying a dual (pDuLX-Luc) or mammalian only (p3L-Luc) luciferase expression plasmid. Experiments were carried out in duplicate wells in each of three replicate experiments. Luciferase expression is shown normalized for total cell protein as a function of time 2 h or 24 h after the initial transduction event. (In panel B, relating to the fully differentiated cells, significance is denoted by the asterisks, ** $p < 0.01$.)

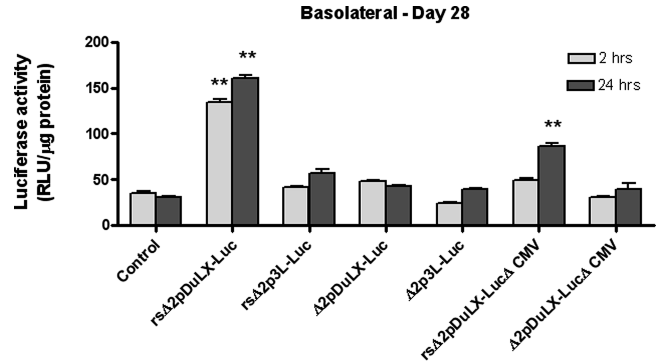

Figure 6. Luciferase expression (RLU/μg total protein) in fully differentiated (28-day) filter-cultured Caco-2 cells after transduction by way of the basolateral surface with *Listeria* rsΔ2 or Δ2, carrying a dual (pDuLX-Luc), mammalian only (p3L-Luc), or *Listeria* only (pDuLXΔcmv) luciferase expression plasmid. Experiments were carried out in duplicate wells in each of three replicate experiments. Luciferase expression is shown normalized for total cell protein 2 h or 24 h after the initial transduction event. Significance is denoted by the asterisks ** $p < 0.01$.

significant. This lends further support to the conclusion that the rsΔ2 strain is able to deliver and release its contents but that only protein translated in *Listeria* is detected.

In summary our results suggest that the suicidal rsΔ2 strain of *Listeria moncytogenes* derived in this study has the potential to deliver its contents to the cytoplasm of mammalian cells. At present we can deliver protein in detectable amounts, but the delivery of DNA expression plasmids is unproductive, due to lack of access to the host cell nucleus. Although the Δ2 strain is able to gain access to mammalian cells and replicate within them, the delivery of protein and presumably DNA plasmids is less efficient than delivery from the suicidal rsΔ2 strain. Our work indicates that rsΔ2 has the potential to deliver protein vaccines to the oral epithelium, which may make possible an inexpensive method for oral vaccination.

Our work correlates well with the recent immunological evaluation of *Listeria*-based vaccine delivery system reported by Goebel and colleagues.[11,47] In the latter study the bacteria were rendered suicidal using a plasmid-based approach which compares closely with our work, although the *Listeria* were administered intravenously in the Goebel study. A response was evident when ovalbumin protein was delivered by the Listeria but not when an ovalbumin mammalian expression vector was delivered. The likely explanation for this result is that the plasmid DNA vaccine was not able to enter the nucleus of somatic cells in vivo in the absence of an active nuclear trafficking system. Our work and the work of the Goebel group emphasize that a more sophisticated construct will be needed to enable expression of antigens from DNA expression vectors.

Abbreviations Used

NLS, nuclear localization sequence; NPC, nuclear pore complex; NF-κB, nuclear factor kappa B; BHI, brain heart infusion; BHIC, charcoal-treated BHI; Luc, luciferase; RLU, relative luminescent unit; MOI, multiplicity of infection; CFU, colony forming unit.

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(47) Loeffler, D. I.; Schoen, C. U.; Goebel, W.; Pilgrim, S. Comparison of different live vaccine strategies in vivo for delivery of protein antigen or antigen-encoding DNA and mRNA by virulence-attenuated Listeria monocytogenes. *Infect. Immun.* **2006**, *74*, 3946–3957.