

Bacteria as gene delivery vectors for mammalian cells

Commentary

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Abbreviations

dap diaminopimelate

GFP green fluorescent protein

Introduction

Gene transfer can occur from bacteria to a very broad host range of recipient cells. It has been reported between phylogenetically remote bacteria genera [1], between bacteria and yeast [2] and from bacteria to plants [3]. Recently, as will be reviewed below, several laboratories have reported that bacteria can also transfer functional genes into mammalian cells. Plasmid transfer has been described after either *in vivo* administration of attenuated *Shigella* or *Salmonella* or after *in vitro* co-incubation of *Shigella* or *Listeria* with phagocytic or nonphagocytic cells. We have used a nonpathogenic bacteria, an invasive *Escherichia coli* deficient in cell-wall synthesis, to study this type of transfer *in vitro*. This process is known as abortive or suicidal invasion as the bacteria have to lyse for DNA transfer to occur (Figure 1).

The bacterial species that can transfer genes to professional and nonprofessional phagocytes have in common the ability to invade these cells. This property is shared by a broad range of Gram-positive and Gram-negative bacteria and results from the interaction between bacterial ligands and host cell receptors. The plasmid DNA released by intracellular bacteria is transferred from the cytoplasm to the nucleus resulting in cellular expression of the transfected gene(s). Bacterial transfer of DNA can result in stimulation of humoral and cellular immune responses after *in vivo* administration. Gene delivery by abortive invasion of eukaryotic cells by bacteria may be valuable for *in vivo* and *ex vivo* gene therapy and for cancer therapy. We will describe the different bacterial vectors that have proven to be effective in gene delivery, either *in vivo* or *in vitro*.

In vivo gene transfer in animal models

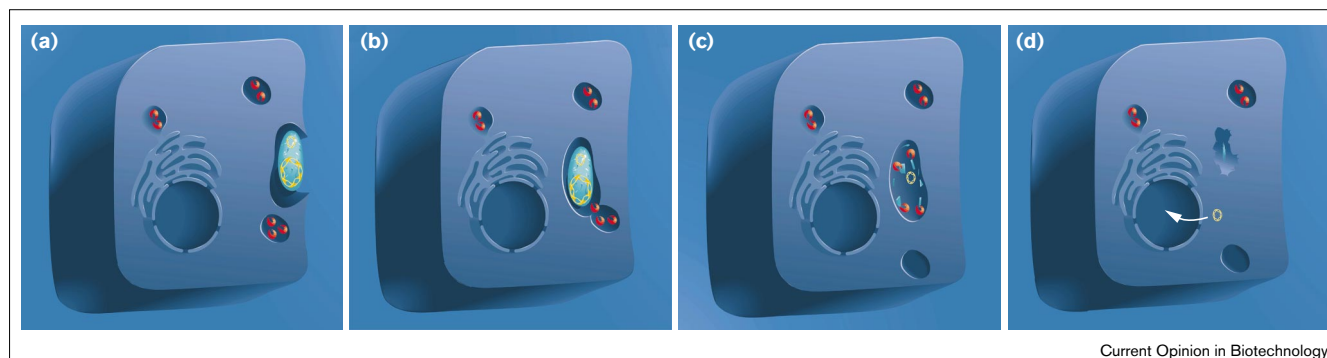
The ability of *Shigella* to enter intestinal epithelial cells and to escape from the endocytic vesicle was exploited as a method to direct plasmid DNA into the cell cytoplasm [4]. A diaminopimelate (dap) auxotroph mutant (dap⁻) of *Shigella*, which undergoes lysis upon entry into mammalian cells because of impaired cell-wall synthesis, was transformed with plasmid pCMVβ, which directs the synthesis

of *E. coli* β-galactosidase under the control of the pCMV eukaryotic promoter. Forty eight hours after a 90 minute co-incubation of this suicidal bacterial vector with cultured baby hamster kidney (BHK) cells, 1–2% of cells expressed β-galactosidase. Similar results were obtained with the murine cell line P815. Splenocytes from mice inoculated twice intra-nasally with 10⁶–10⁷ bacteria four weeks apart, proliferated after a three day culture *in vitro* in the presence of β-galactosidase. Anti-β-galactosidase antibodies were detected in the sera of the mice [5]. These results provided the first evidence that *in vivo* delivery of plasmid DNA by *Shigella* could lead to stimulation of an immune response to the plasmid-encoded antigen.

Attenuated *Salmonella* expressing heterologous antigens have been used as vectors for oral immunisation in mice, farm animals and humans. This approach to vaccine delivery resulted in efficient stimulation of mucosal and systemic immune responses [6]. An attenuated strain of *S. typhimurium* was used successfully as a vehicle for oral genetic immunisation [7]. In this study, oral administration of a non-virulent *aroA*⁻ auxotrophic mutant of *S. typhimurium* harbouring plasmids encoding *E. coli* β-galactosidase or truncated ActA and listeriolysin (two virulence factors of *Listeria monocytogenes*), each under the control of a eukaryotic promoter, led to excellent humoral and cellular immune responses in mice. The immunised mice were protected from a lethal challenge with virulent *L. monocytogenes*, even after a single oral administration. β-galactosidase activity was detected in splenic macrophages five weeks after oral administration of *S. typhimurium* carrying plasmid pCMVβ. Plasmid DNA transfer was studied *in vitro* after one hour since infection of mouse primary peritoneal macrophages with attenuated *S. typhimurium* carrying pCMVβ. In these experiments, evidence of synthesis of β-galactosidase by the infected macrophages was provided; tetracycline had to be added during the entire culture period to kill any viable intracellular bacteria and thus inhibit endogenous residual synthesis of β-galactosidase by bacteria.

In a similar study [8], oral administration of an attenuated *Salmonella* harbouring pCMVβ to mice generated humoral and cellular immune responses to β-galactosidase and a protective response against an aggressive murine fibrosarcoma transduced with the β-galactosidase gene. Expression of the transgene by antigen-presenting cells after per os administration was documented with a eukaryotic vector expressing the green fluorescent protein (GFP). Among the 19% of splenocytes expressing GFP 28 days after oral administration, 50% were dendritic cells and 30% expressed macrophage markers. This provides evidence of direct *in vivo* gene transfer by orally administered bacteria to antigen-presenting cells.

Figure 1



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Schematic representation of plasmid DNA transfer from an attenuated *dap⁻ E. coli* to a eukaryotic cell. (a) Entry of the bacteria in a primary vesicle of the host cell. (b) Fusion of the primary vesicle with the lysosomal compartment. (c) Bacterial lysis. (d) Escape of plasmid DNA from the vesicle to the cytoplasm and then to the cell nucleus.

In vitro gene transfer

Studies of gene transfer *in vitro* from bacteria to mammalian cells have allowed better understanding of the mechanism of gene transfer and the description of new bacterial vectors.

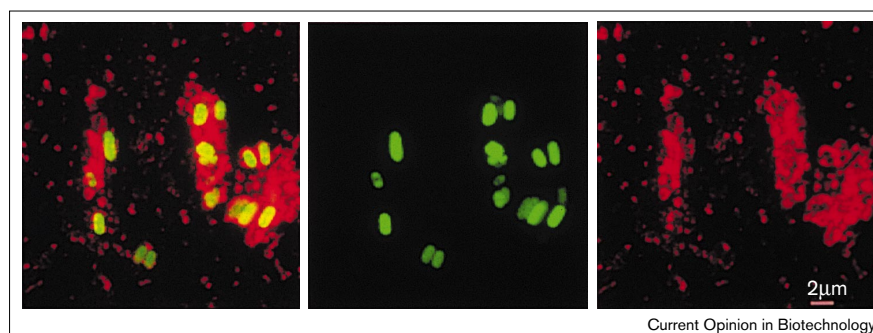
L. monocytogenes, a Gram-positive species lacking lipopolysaccharide, is able to invade a wide range of mammalian cell types. After internalisation, these bacteria rapidly escape from the primary vacuole into the cytosol where they replicate [9]. An attenuated strain, impaired in intra- and intercellular movements has been engineered to undergo self-destruction in the cell cytosol by production of a phage lysin under the control of the promoter of *actA*, which is preferentially activated in the cytosol [10]. This bacterial vector was able to deliver a plasmid carrying *gfp*, *cat* (the structural gene for a chloramphenicol acetyltransferase) or part of the *ova* (ovalbumine) gene under the control of the CMV promoter into the P388D macrophage cell line. Functional transfer was increased (from 0.001% to 0.2%) if there was lysis of the *Listeria* internalised in the cytoplasm, either by self-destruction following lysin production or after antibiotic treatment. Efficient expression of chloramphenicol acetyltransferase or antigen presentation was observed in P388D macrophages after

L. monocytogenes-mediated plasmid delivery. Plasmid DNA, recovered from macrophage clones cultured in selective medium for more than 12 weeks, was found to be integrated into the macrophage genome at a frequency of $\sim 10^{-7}$.

We and others have shown that mammalian cells can transiently express genes delivered intracellularly by strains of *Shigella flexneri* impaired in cell-wall synthesis [4,11]. This direct gene transfer was monitored by screening for production of nuclear β -galactosidase. The genes responsible for entry, intracellular mobility and cell-to-cell spread of *S. flexneri* are borne by the ~ 200 kb virulence plasmid pWR100. Transfer of the plasmid to *E. coli* confers the ability to enter epithelial cells on this otherwise extracellular bacterial species. With this invasive strain of *E. coli*, we have shown that bacteria that undergo lysis upon entry into mammalian cells (because of impaired cell-wall synthesis due to *dap* auxotrophy) can deliver plasmid DNA to their hosts.

In order to design a genetically better defined system and to expand the host-cell range, we have cloned the 3.2 kb *inv* locus encoding the invasin of *Yersinia pseudotuberculosis* alone or with the 1.5 kb *hly* gene coding for the listeriolysin O from *L. monocytogenes* in the stable *dap* auxotroph *E. coli* BM2710

Figure 2



Bacterial invasion of HeLa cells after three hours. GFP and bacteria inside cell (left). Identical cell labelled with antihuman LAMP1 monoclonal antibody and Texas Red goat antimouse antibody (centre). Superimposition of (a) and (b) showing the co-localisation of bacteria and the LAMP1 antigen (right).

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Table 1

Major studies on bacteria to eukaryote gene transfer.

Bacterial species	Transferred plasmid(s)	Transfer		References
		<i>in vitro</i> : cell lines transfected	<i>in vivo</i> : route of administration	
<i>Shigella flexneri</i> <i>dapA</i> ⁻	pCMV β	BHK P815	Intranasal Intracorneal	[4,5]
Invasive <i>E. coli</i> <i>dapA</i> ⁻	β -gal replicative and integrative eukaryotic vectors pEGFP-C1	HeLa COS-1 CHO A549 J774	ND	[11,12]
<i>Salmonella</i> <i>typhimurium</i> <i>aroA</i> ⁻	pCMV β pCMV <i>actA-hly</i>	Primary mouse macrophage	per os	[7,8]
<i>Listeria</i> <i>monocytogenes</i> Δ <i>mpl actA plcB</i>	pCMVgfp pCMVcat pCMVova	J774 P388D	ND	[10]

cat, chloramphenicol acetyl transjerase; ND, not done; ova, ovalbumine.

[12]. We also introduced by transformation plasmid pEGFP-C1, which directs synthesis of GFP in mammalian cells but not in bacteria. In HeLa, Chinese hamster ovary, and COS-1 cells, 5–20% GFP⁺ cells were observed two days after incubation with invasive bacteria. Transfer efficiency was enhanced by co-expression of the gene for listeriolysin. Expression of the acquired genes occurred both in dividing and in quiescent cells, albeit at a lower efficiency in the latter. Functional transfer of plasmid DNA could be observed in J774 macrophages but not in a mouse dendritic cell line. Expression of acquired DNA was very stable, as tested after two months of culture. The number of viable intracellular bacteria decreased rapidly with no survivors after 24–72 hours, and chromosomal DNA of the donor could not be detected after 23–48 days. Plasmid transfer was obtained at a lower efficiency from *E. coli* BM2711, the *dap* non-requiring counterpart of BM2710, indicating that bacteria may transfer functional genetic information to nonprofessional phagocytes if they can induce their own internalisation. Confocal immunofluorescence microscopy showed that within 1–3 hours and up to 24–48 hours, all bacteria were localised in phagolysosomes of HeLa cells (Figure 2).

Discussion

Direct introduction of DNA from bacteria to mammalian cells has been reported in very few cases: Schaffner *et al.* [13] reported that plasmids carrying tandem copies of the SV40 virus genome could be transferred from *E. coli* to cultured mammalian cells by exposing the cell culture to a bacterial suspension; however, transfer was found to occur at a very low frequency. Similar results were obtained with *E. coli* harbouring plasmids carrying polio virus 1 cDNA, and transfer took place in the presence of high concentrations of DNase

that digest free DNA in the culture medium [14]. In these cases, the vectors were human pathogens and could not be employed outside an experimental setting.

Attenuated intracellular bacteria, such as *Shigella*, *Salmonella*, *Listeria* and invasive *E. coli*, have been found to act as good gene delivery vectors in both phagocytic and non-phagocytic mammalian cells (Table 1). Observations from *in vivo* and *in vitro* experiments are consistent with the hypothesis that, after internalisation into a primary vacuole, bacteria or their plasmid component have to escape into the cytosol to gain access to the nucleus.

Studies of microbial pathogenesis have expanded our understanding of the mechanisms employed by bacteria to achieve entry into host cells and to gain access to intracellular compartments. There are two major strategies for bacteria to gain access to host cells [15]. For certain genera, such as *Salmonella* or *Shigella*, contact between the bacteria and the host cells results in the secretion, by the bacteria, of a set of invasion proteins that triggers intracellular signalling events, leading to cytoskeletal rearrangement, membrane ruffling and bacterial uptake by micropinocytose. For other genera, such as *Yersinia* or *Listeria*, binding of a single bacterial protein to a particular ligand on the host cell-surface is necessary and sufficient to trigger entry into phagocytic and non-phagocytic cells by a zipper-like mechanism. Binding of *Yersinia* invasin to β 1 integrins results in entry into cells expressing this integrin at the surface; binding of internalin with E-cadherin mediates entry of *Listeria* into certain cell types. Similarly, introduction in *E. coli* of the virulence plasmid (pWR100) of *S. flexneri* or of the *inv* gene of *Y. pseudotuberculosis* confers to this otherwise extracellular bacteria the ability to invade non-phagocytic cells.

Access to the cell cytosol depends on the mechanisms by which bacteria survive inside the cells [16]. *Listeria* and *Shigella* escape rapidly from the vacuole after lysis of its membrane, by production of listeriolysin in the case of *Listeria*, and replicate in the cytoplasm of the cell. Other bacteria, such as *Salmonella*, remain in the phagosomal vacuole and replicate within this compartment. The successful delivery of genes by *Salmonella* is therefore surprising, as *Salmonella*-containing vacuoles have a unique trafficking pathway, uncoupled from the normal endocytic degradation pathway. Interestingly, infection by *Salmonella* resulted in functional DNA delivery into primary macrophages but not in macrophage cell lines, suggesting that the transport pathway operates efficiently only in primary cells [4,7].

DNA entering the host cell cytoplasm by phagocytosis is less efficiently routed to the nucleus than DNA introduced directly into the cytoplasm (e.g. by gene gun). Consistent with this finding are the observations that more efficient transfer was observed when *Listeria* was destroyed in the cytoplasm of the cell [10] or if the invasive *E. coli* produced listeriolysin that triggers pore formation in the vacuolar membrane [12]. It cannot be excluded, however, that DNA may gain access to the mammalian cell cytoplasm via leakage from host-cell phagosomes, as has been shown for exogenous antigens in macrophages [17].

The property of bacteria to act as a gene delivery system has been mainly exploited for DNA vaccination [18]. Attenuated auxotroph mutants of *Salmonella* are already in use as live vaccines in man and in animals [19], and effective gene transfer to dendritic cells after oral administration of mice with these bacteria has been documented [8]. In these *in vivo* studies, the number of transfected cells varies greatly depending on the type of bacterial vector or cell type. Dendritic cells, however, are highly efficient antigen-presenting cells and a small number of transfected dendritic cells has been shown to be sufficient to stimulate both primary and secondary T- and B-cell responses and to process and present antigens efficiently [20]. Furthermore, per os administration of bacterial vectors could lead to transfection of gut-associated lymphoid cells and stimulation of mucosal immunity [7,8].

The potential use of bacterial vectors for *in vivo* or *ex vivo* gene therapy has not been assayed yet. Genetically engineered *S. typhimurium* have been selected that are able to target multiple tumours from a distant inoculation site [21]. These attenuated hyper-invasive mutants were shown to selectively replicate in the tumours leading to tumour growth retardation and prolonged average survival.

Despite the high numbers of bacteria and plasmids internalised by the host cells, transgene expression remains low and comparable to the levels obtained with other non-viral delivery systems, such as polycation-DNA complexes. Following bacterial internalisation, plasmid

DNA has to be released into the cytosol before nuclear entry can occur. The turnover of plasmid DNA delivered by microinjection in the cytosol has been shown to be rapid, with an apparent half-life of 50–90 min in HeLa and COS-1 cells [22]. Direct delivery into the cytosol of native plasmids by intracellular bacteria may constitute a means of protecting this DNA from degradation by cytosolic nucleases.

Until now, most studies have reported gene transfer to antigen-presenting cells. Future work should concentrate on the ability of these vectors to deliver functional DNA into epithelial cell types and other differentiated tissues. In addition, bacterial gene delivery systems should be improved for better cell type targeting.

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