Gene Transfer to Fish Cells by Attenuated Invasive Escherichia coli

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Abstract: Genetic immunization has proved effective in a number of applications including vaccination of rainbow trout (Oncorhynchus mykiss) against the fish pathogen infectious hematopoietic necrosis virus. However, injection vaccines, especially in aquaculture, are not as desirable as oral or immersion dosing schemes. In this report we present evidence that attenuated invasive Escherichia coli can infect and deliver plasmid DNA to salmonid fish cells.

Keywords: gene transfer, fish cells, Escherichia coli, DNA vaccine, Yersinia ruckeri, IHNV

INTRODUCTION

Gene transfer to eukaryotic cells has many exciting applications, including gene therapy, production of transgenic animals, and genetic immunization. Plasmid DNA based vaccines have been developed and tested for a variety of diseases, including fish viral diseases (Anderson et al., 1996; Lorenzen et al., 1998; Haynes, 1999; Gurunathan et al., 2000; Heppell and Davis, 2000). Using this technology, our laboratory developed a highly effective vaccine against infectious hematopoietic necrosis virus (IHNV) (Anderson et al., 1996). Injection with this DNA vaccine was shown to induce protection in 80% to 98% of rainbow trout fry (Oncorhynchus mykiss) against a normally lethal dose of IHNV. However, injection of vaccines in fish is labor intensive and its cost can be prohibitive. An easier means of delivering the DNA vaccine to fish reared intensively was needed.

Recent developments in mammalian vaccinology have exploited the use of attenuated bacteria to deliver plasmid DNA to mammalian cells both in vitro and in vivo (Courvalin et al., 1995; Sizemore et al., 1995; Darji et al., 1997; Dietrich et al., 1998). In addition, Dietrich et al. (2001) recently reported the ability of Listeria monocytogenes to invade cell lines of warm-water fish. We report here the first use of attenuated invasive bacteria to transfer DNA into salmonid fish cells.

In this study we used an attenuated invasive Escherichia coli system that was developed by Grillot-Courvalin et al. (1998). An E. coli K12 diaminopimelate auxotroph (BM2710), expressing the invasin gene from Yersinia pseudotuberculosis and the listeriolysin gene from L. monocytogenes, was shown to transfer the DNA reporter plasmid containing the gene for green fluorescent protein (GFP) to HeLa, COS-7, and CHO cell lines with varying efficiency (Grillot-Courvalin et al., 1998). The invasin and listeriolysin genes were carried on a low copy number plasmid,
Lennox agar plates or broth supplemented with 25 μg/ml meso-diaminopimelic acid (DAP), 50 μg/ml kanamycin, and 25 μg/ml streptomycin. Descriptions of the plasmids are given in Table 1.

Infection of CHSE-214 Cells with Invasive E. coli

Chinook salmon embryo (CHSE-214) cells were cultured as previously described (Engelking and Leong, 1981). Twenty-four hours before infection, CHSE-214 cells were seeded onto glass coverslips in 6-well plates at 10^5 cells per well. Bacterial suspensions of BM2710 pEGFP-C1, also carrying one of the invasion plasmids (pGB2, pGB2Ωinv, or pGB2Ωinv-hly), were prepared in cell culture medium plus 25 μg/ml DAP. The monolayered cells were infected at a multiplicity of infection of 100 bacteria per tissue culture cell. After 2 hours of incubation at room temperature, the monolayers were rinsed and the medium was supplemented with 100 μg/ml gentamicin. At 24 and 48 hours after infection, monolayers were fixed with 10% buffered formalin and mounted with Vectashield (Vector Laboratories, Burlingame, Calif.) and viewed by epifluorescence microscopy to detect GFP expression. The dye 4,6-diamidino-2-phenylindole (DAPI) was applied as a counterstain.

Flow Cytometry

CHSE-214 cells were seeded into 25-cm² tissue culture flasks or 6-well plates and infected as described above. After infection and rinsing the medium was replaced with CO₂-independent medium (Invitrogen, Carlsbad, Calif.) plus 10% fetal bovine serum and 200 μg/ml gentamicin to kill extracellular bacteria. The monolayers were incubated at 20°C. Five days after infection, the cells were trypsinized and fixed with 2% methanol-free formaldehde in preparation for flow cytometry. The cell suspensions were analyzed on a Becton-Dickinson (Franklin Lakes, N.J.) FACScalibur. For each sample, 100,000 to 250,000 events were counted.

Macrophage Isolation

Head kidney leukocytes were isolated as described by Crippen et al. (2001). Briefly, individual rainbow trout were killed by an overdose of MS-222. The fish werebled from the caudal vein, then the head kidney was removed to a sterile stomacher bag. The tissue was mixed with 5 ml of a 1:1 mixture of Hank’s balanced salt solution and Alsever’s solution (HBSS/Alsever’s) and homogenized briefly (15–20

**Materials and Methods**

### Bacterial Strains and Plasmids

Strains and plasmids used in this study are listed in Table 1. Escherichia coli strain BM2710 harbouring the reporter plasmid pEGFP-C1 and the invasion plasmids pGB2, pGB2Ωinv, and pGB2Ωinv-hly were generously provided by Dr. Sylvie Goussard of L’Institut Pasteur, Paris, France. E. coli strain BM2710 was routinely cultured at 37°C on LB

![Figure 1. Model of gene transfer by attenuated invasive E. coli. 1: Bacteria invade the cell by inducing phagocytosis. 2: The nutritional deficiency (i.e.) diaminopimelate auxotrophy results in death of the bacteria. Bacterial contents, including plasmid DNA, are released into the phagosome. 3: Phagosomal contents (and plasmid DNA) are released into the cytosol owing to phagosomal lysis or leakage. 4: Plasmid DNA enters the nucleus. 5: The reporter gene is expressed by the host cell.](image)