Arch. Biol. Sci., Belgrade, 63 (4), 943-948, 2011 DOI:10.2298/ABS1104943S

ICE NUCLEATION PROTEIN AS A BACTERIAL SURFACE DISPLAY PROTEIN

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Abstract - Surface display technology can be defined as that phenotype (protein or peptide) which is linked to a genotype (DNA or RNA) through an appropriate anchoring motif. A bacterial surface display system is based on expressing recombinant proteins fused to sorting signals (anchoring motifs) that direct their incorporation on the cell surface.

Key words: INP, surface display, anchoring motifs, *Pseudomonas syringae*.

UDC 602.6:579.84

INTRODUCTION

The introduction of recombinant DNA techniques in the late 1970s allowed researchers to successfully express various proteins that play a major role in a number of natural processes like adhesion, colonization of target surfaces, motility, signal transduction and enzymatic degradations in different host organisms including bacteria, yeasts, fungi, mammalian cells and plants (Lang, 2000). One of the most exciting developments in recent years has been the possibility to directly target proteins on the cell surface of different host organisms by fusion of the protein of interest with the anchoring motifs of a naturally occurring bacterial surface protein at the N-terminal or C-terminal, or as a sandwich. This results in the chimeric protein being expressed and displayed on the cell surface of the host cell (Stahl and Uhlen, 1997; Lang, 2000; Lee et al., 2003).

Phage surface display system

Since the development of the first phage expression system (Smith, 1985) in which the protein of interest was expressed as a fusion with the phage coat protein, phage display has been used to express enzymes (Marks et al., 1991), enzyme inhibitors (Roberts et al., 1992), cytokines (Wright et al., 1995) and in many other applications (Benhar, 2001). However, the size of the foreign protein to be displayed on the phage surface was limited (Li, 2000).

Bacterial surface display

To solve the problem of the size of the protein that can be displayed on the surface of the cell, a bacterial display system through genetic fusion with carrier proteins was developed (Freudl et al., 1986). Consequently, various bacterial surface display systems have been developed and investigated to direct target foreign proteins onto the cell surface of different host organisms including bacteria, yeasts, fungi, mammalian cells, and plants (Samuelson et al., 2002).

The ability to display heterologous proteins on the surface of bacteria covers a wide range of biotechnological and industrial applications. The most common application of bacterial surface displays has been the development of live bacterial vaccine delivery systems to expose heterologous epitopes on the cell walls of live attenuated pathogenic bacteria to elicit antigen-specific antibody response (Liljeqvist et

al., 1997; Lee et al., 2000). This system has been used to raise polyclonal antibodies against the expressed protein in animals (Charbit et al., 1988; Martineau et al., 1991; Gunneriusson et al., 1999). It has also been used as biosorbents for the removal of heavy metals (Kotrba et al., 1999; Bae et al., 2000; Bae et al., 2001; Cho et al., 2002), development of biosensors by anchoring enzymes, receptors or other signal-sensitive components for diagnostic, industrial or environmental purposes (Anderson et al., 1981; Dhillon et al., 1999; Ye et al., 2000; Wang et al., 2002) and as whole cell biocatalysts for the detoxification of organic contaminants (Wang et al., 2002).

Gram-negative bacteria have a unique and a complex membrane structure comprising of a typical phospholipid bilayer inner membrane, a thin periplasm layer of peptidoglycan, and an outer cellular membrane. The outer membrane consists of a phospholipid bilayer with the outer lipopolysaccharide molecules arranged within the external phospholipids layer (Lim, 1997). Thus the surface anchoring motifs, fused with the protein to be displayed, have to pass through this complex membrane structure. In order to achieve surface display of a foreign protein on Gram-negative bacteria, a natural outer membrane protein should be used. The foreign protein to be displayed on the surface of the bacteria has to be fused to the anchoring protein of interest at the gene level, and the expressed fusion protein needs to be transported through the inner cellular membrane to the surface and to maintained on the surface of the bacteria (Samuelson et al., 2002).

Many different types of proteins of gram-negative bacteria have been used as a carrier to display foreign peptides on the bacterial cell surface. The outer membrane proteins, Omp, of Gram-negative bacteria were found to be able to serve as carriers of heterologous gene products to be displayed at the outer cell surface. The first examples of heterologous surface display were reported a decade ago, when short gene fragments (35-45 bp) were inserted into the genes for the *E. coli* outer membrane proteins LamB (Charbit et al., 1986) and OmpA (Freudl et al., 1986) and the gene fusion products were found to be accessible to the outer surface of the recombinant bacteria. The upper size limit seems to be 60–70 amino acids (Charbit et al., 1988). The outer membrane display systems, however, were not suitable in most cases for the display of large proteins. Since then, not only outer membrane proteins but also lipoproteins such as TraT, a coat protein fragment of type-1 polyvirus, were used to allow the display of large protein fragments on the surface of *E. coli* (Taylor, 1990). Using subunits of surface appendages such as flagellar proteins, peptides, ranging in size between 48 and 302 amino acids representing the middle region of the collagen-binding YadA adhesin of *Yersinia enterocolitica* were expressed (Westerlund-Wikstrom et al., 1997). Other surface gram negative display systems are reviewed in Samuelson et al., (2002).

One of the problems encountered with such display systems is the limited size of the heterologous insert that can be displayed without affecting the structure and/or function of the carrier protein (Benhar, 2001; Chen and Georgiou, 2002). To overcome this problem a display system based on the ice nucleation protein (INP) from *Pseudomonas syringae* was described (Jung et al., 1998a; Jung et al., 1998b; Kim and Yoo, 1999).

Many of the described gram-negative display systems have been mainly assessed in *E. coli* for surface display of a variety of epitopes, and then applied to *Salmonella* spp. The use of attenuated strains of *Salmonella*, such as the aroA strain of *S. typhimurium*, to deliver cloned antiphagocytic virulence determinants of unrelated bacteria have been investigated. Mice immunized with this recombinant *S. typhimurium* produced serum and salivary IgA, IgG, and IgM antibodies specific against the protein (Poirier et al., 1988). Similar studies by Newton et al., (1989) and Wu et al., (1989) demonstrated the utility of attenuated *Salmonella* as a general vaccine vehicle.

Ice-nucleation protein (INP)

The INP encoding gene known generally as Inak, is present in ice nucleation active bacteria such as

Pseudomonas syringae, Pseudomonas fluorescens and *Erwinia herbicola*. The Inak gene encodes a unique outer membrane associated protein, the INP, which is able to accelerate ice crystal formation in supercooled water at temperatures of -2 to -10^{0}C (Wolber et al., 1986; Turner et al., 1991). Ice nucleation active bacteria are distributed worldwide. They are found in the soil and on plant leaf surfaces. Not all strains within the species described above are ice nucleation active. In fact, the ice nucleation activity has been used as one of several traits to identify strains of *Pseudomonas syringae.* Among strains that are active in ice nucleation, not every cell is active at a given time and temperature (Edwards et al., 1994; Hirano and Upper, 2000). Orser and his colleagues (1985) were the first to show that the ice nucleation phenotype could be transferred to *E. coli* by expression of DNA clones from the genome of *P. syringae* and *E. herbicola*.

Many studies have demonstrated the potential applications of ice nucleation active bacteria in the food industry including freeze concentration and freeze texturing of food, improving the process of freezing of various foods and preparation of frozen emulsified foods such as ice cream to improve the quality of the product (Wolber, 1993; Tegos et al., 2000). The uses of such bacteria in applications have also been suggested, such as in artificial snow making and in the creation of artificial ice islands.

INP is a monomeric protein composed of more than 1,200 amino acid residues with a deduced molecular mass of 118 kDa (von Heijne, 1984) and three distinct domains: (i) an N-terminal domain with 175 amino acids, which is hydrophobic and function as the membrane anchor; (ii) a central cylindrical repeating domain (CRD), 48-residue long, which is not essential for membrane anchoring but can be used as a modular spacer to control the length between a heterologous protein and the cell surface and has a catalytic role in the formation of ice crystals, and (iii) a C-terminal domain of 49 amino acids that are hydrophilic and extracellular (Kozloff et al., 1991; Nemecek-Marshall et al., 1993; Wolber, 1993; Kawahara, 2002).

INP itself has the necessary secretion and anchoring signals. INP is not processed during transport and has no typical signal peptide-type sequences at the N-terminus as other known secreted proteins (Wolber et al., 1986). Studies by Michigami et al., (1995), suggest that INP is synthesized at the inner membrane, it then enters a vesicle and is transferred via the vesicles to the outer membrane.

The N-terminal domain is anchored to the glycosylphosphatidylinositol (GPI) outer membrane lipid moiety. However, both the C and N-termini of INP are free and exposed on the cell surface, so foreign proteins fused to the C- or the N-terminus of INP can be localized to the cell surface (Jung et al., 1998a; Jung et al., 1998b; Kim and Yoo, 1998; Kwak et al., 1999; Lee et al., 2000; Shimazu et al., 2001). INP has the ability to maintain its ice nucleation activity after fusion to a foreign protein, which allows the detection of the recombinant proteins on the cell surface by ice nucleation activity assay.

In other display systems, expression problems have emerged when the displayed protein is longer than 60 amino acids in length (Agterberg et al., 1987; Charbit et al., 1988; Benhar, 2001; Chen and Georgiou, 2002). However, INP appears to have no such limitations since large functional enzymes have been successfully displayed using the INP system (Jung et al., 1998a; Jung et al., 1998b).

The INP protein was used for the first time to display the *Zymomonas mobilis* levansucrase on the *E. coli* surface to produce an immobilized enzyme (Jung et al., 1998a). This was followed by expression of the *Bacillus subtilis* carboxymethylcellulose (CMCase) on the surface of *E. coli* (Jung et al., 1998b), the viral protein of the human immunodeficiency virus type1 (Kwak et al., 1999), and the mutated CMCase gene library generated by gene shuffling (Kim et al., 2000). A recombinant oral vaccine was developed to display the hepatitis C core protein on the surface of *S. typhi* Ty21a (Lee et al., 2000). Using the N- and

C-termini of INP, Jeong et al. (2001) were able to express salmobin, a thrombin-like enzyme on the surface of *E. coli*. More recently an organophosphorous hydrolase was displayed on the surface of *E. coli* using a truncated INP (Cho et al., 2002).

The N-terminal domain of the ice nucleation protein, an outer membrane protein of *Pseudomonas syringae*, was used as an anchor motif for surface display and expression of heterologous antigens of the *Edwardsiella tarda ghosts* and *Ed. tarda cadaver* based combined vaccines (Choi et al., 2010). The ice nucleation protein was used as a whole cell biocatalyst to display a heme- and diflavin-containing oxidoreductase (Yim et al., 2010).

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