Progress in *Bacillus subtilis* Spore Surface Display Technology towards Environment, Vaccine Development, and Biocatalysis

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Keywords

Bacillus subtilis spores · Bioremediation · Biocatalysis · Surface display · Vaccine development

Abstract

Spore surface display is the most desirable with enhanced effects, low cost, less time consuming and the most promising technology for environmental, medical, and industrial development. Spores have various applications in industry due to their ability to survive in harsh industrial processes including heat resistance, alkaline tolerance, chemical tolerance, easy recovery, and reusability. Yeast and bacteria, including gram-positive and -negative, are the most frequently used organisms for the display of various proteins (eukaryotic and prokaryotic), but unlike spores, they can rupture easily due to nutritive properties, susceptibility to heat, pH, and chemicals. Hence, spores are the best choice to avoid these problems, and they have various applications over nonspore formers due to amenability for laboratory purposes. Various strains of *Clostridium* and *Bacillus* are spore formers, but the most suitable choice for display is *Bacillus subtilis* because, according to the WHO, it is safe to humans and considered as “GRAS” (generally recognized as safe). This review focuses on the application of spore surface display towards industries, vaccine development, the environment, and peptide library construction, with cell surface display for

enhanced protein expression and high enzymatic activity. Different vectors, coat proteins, and statistical analyses can be used for linker selection to obtain greater expression and high activity of the displayed protein. © 2017 S. Karger AG, Basel

Introduction

In extreme conditions *Bacillus subtilis* has the ability to enter the resting phase of sporulation and survive for a long time under harsh environmental conditions. The spores are covered by a thick peptidoglycan layer called the cortex, which is then covered by another multilayered protein coat and an outer coat layer [Henriques et al., 2004]. Thus, spores have the ability to survive in stress conditions such as high temperature, ultraviolet radiation, enzymatic and chemical damage, and desiccation. Display of proteins and peptides on the surface of microbes is becoming a fundamental tool to overcome the problems in bioprocesses, during harsh industrial processes, vaccine development, and environmental protection [Kim and Schumann, 2009]. Enzymes used for therapeutic purposes, such as lipoprotein lipase, have been broadly used to treat cardiovascular and obesity-associat-

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ed complications [Chen et al., 2016b]. Immobilization of such enzymes on the surface of *B. subtilis* spores, can lead them to be used orally for the treatment of disease and disorders. In today's developing world, whole cell biocatalysis plays a key role in industrial bioprocesses, which can be limited by solvent toxicity and low mass transfer to host cells. Surface immobilization can reduce the limitations imposed by cell membranes [Jung et al., 2006]. For successful display, the target protein needs to be fused with an anchor protein [Van Bloois et al., 2011] in order to display translocated-incompatible and multimeric proteins [Kim and Schumann, 2009]. Pan et al. [2014] reported a novel concept of displaying a target protein without a fusion motif by expressing the target protein under mother cell-specific sigma-factors (σ^E σ^K) in the last sporulation phase to absorb on the spore surface normally. This method can broaden the area for displaying those proteins and antigens on the spore surface which are unable or have difficulty to fuse with display motifs. Spores are unique structures coated with 70 different types of proteins [Setlow, 2012]. Commonly used display motifs (spore coat proteins) of *B. subtilis* include CotB, CotC, CotG, and CotZ, and some exosporium proteins like exosporium protease (InhA) and insecticidal protoxin (Cry1Ac) from *Bacillus thuringiensis* [Hinc et al., 2013; Pan et al., 2012]. For successful display, it is necessary to choose an appropriate anchor protein because they contain signal sequences for efficient promotion of transportation, immobilization, and stability to the cell surface [Tanaka et al., 2012]. The carrier protein can be fused N- or C-terminally depending upon the anchoring protein. If the anchoring protein is located on the C-terminal end, then the carrier will be on the N-terminal side, but in cases of anchoring motifs fused N-terminally, then the carrier will be on C-terminal side [Vida et al., 2012]. This review focuses on the applications of spore surface display systems and their importance for industry, the environment, and pharmaceuticals. We also examine their advantages in comparison to nonspore formers for overcoming problems in harsh industrial processes and easy transportation of vaccines in the natural environment due to their ability to survive in harsh conditions.

Nutritive Cells

Yeast Surface Display

Like other microorganisms (e.g., bacteria and phages), yeast is a promising tool for displaying various enzymes and proteins. Different types of enzymes are displayed

and successfully immobilized on the surface of *Pichia pastoris* and *Saccharomyces cerevisiae* by fusing with the N-terminus of the cell wall [Ren et al., 2007]. Of these, *Yarrowia lipolytica*, *Hansenula polymorpha*, *Kluyveromyces lactis*, and *Schizosaccharomyces pombe* are the most preferred organisms for protein expression and are mostly used in citric acid and isocitric acid production [Kamzlova et al., 2015]. Genetically engineered yeast has many applications in chemical synthesis, biotechnology productions, environmental pollutants adsorptions, biorefineries, and protein evolution [Tanaka et al., 2012]. Displaying costly enzymes on the surface of yeast make them cost-effective and easily available for cultivation and easy recovery with excellent enzymatic characteristics for industrial application, including oil drilling and hydrolysis of coffee [Yang et al., 2009]. However, yeast surface display has certain drawbacks because of its nutritive properties, susceptibility towards heat and other harsh industrial processes, and chances of cell cracking during protein recovery when using different chemicals. The fusion of the target protein either N- or C-terminally is also difficult to decide and has an impact on the function of the target protein. For example, the enzymes with an active site near the C-terminus cannot be fused N-terminally because the target protein must be fused with the GPI at the C-terminus of *S. cerevisiae* [Boder and Wittrup, 1997].

Bacterial Surface Display

A bacterial surface display allowing recombinant proteins and peptides on the bacterial cell surface have made important contributions to the design of whole cell biocatalysis and high-throughput screening [Van Bloois et al., 2011]. Several bacterial species are used for surface display such as *Lactococcus lactis*, *B. subtilis* [Lee et al., 2003], *Streptomyces coelicolor* [Thompson et al., 2010], or *Escherichia coli*, which is the most widely used bacterial host with a variety of display systems among all these bacterial hosts. Surface display is a probable alternative for immobilized enzyme usage because it allows bacterial cell employing for effective cascade reactions for the purpose of multistep conversion, as the catalyzation of immobilized enzymes often requires transformations, which is difficult to attain by traditional chemical ways [Van Bloois et al., 2011]. For successful display and easy recovery, chimeric proteins have to cross the membrane, but some proteins lack the ability to cross the membrane barrier, thus leading to unsuccessful display [DeLisa et al., 2003]. However, using endospores for display of such proteins can lead to the success of surface display and easy recovery [Schuurmann et al., 2014].

Display on Gram-Positive Bacteria

Due to simple and rigid cell wall structures, gram-positive bacteria (*Bacillus* and *Staphylococcus*) strains are the most commonly used and are suitable organisms for the display of proteins on their surface as well as for whole cell biocatalysis and whole cell bioadsorbents [Lee et al., 2000]. The gram-positive bacterial cell wall (*Bacillus thuringiensis*) contain lysine motifs, which offer a promising approach for displaying some antimicrobial proteins and enzymes on the surface of these bacteria, for the production of heat-stable live vaccines [Liu et al., 2008]. Trans-membrane proteins, LPXTG-like proteins, lipoproteins, macromolecular proteins, and cell wall-binding proteins are commonly used surface proteins for surface display [Desvaux et al., 2006]. *Staphylococcus* is another gram-positive bacteria that has numerous approaches for expressing antibody fragments and production of immune libraries [Fleetwood et al., 2013]. Besides all these, PrsA, the molecular chaperone of *B. subtilis* [Kim et al., 2005b] and spores coat proteins are the other examples of surface display development. Most species of gram-positive bacteria are safe to human health and thus it is the most favorable choice for the display and other laboratory purposes.

Display on Gram-Negative Bacteria

Heterologous surface display is the major tool for expressing immunogens on the surface of nonpathogenic bacteria including *E. coli* [Narita et al., 2006] for long-lasting immune responses. For successful display in gram-negative bacteria, the anchoring motif should pass the 3 layers, i.e., the outer membrane, cytoplasmic membrane, and periplasmic spaces. Different types of proteins are used for surface display in gram-negative bacteria with different fusion strategies. Commonly used proteins for gram-negative surface display include Omp, IgA protease, INP, S-layer protein, pilin, PhoA, and flagella. The outer membrane proteins are used as carriers to display the proteins on a gram-negative surface by sandwich fusion, while INP is the most stable and is used for the display of large proteins [Lee et al., 2003]. *E. coli* is the most widely used gram-negative bacteria used in numerous surface displays for different purposes like vaccine development, biocatalysis, bioadsorption, and biodegradation [Lee et al., 2003]. For spore surface display of gram-negative bacteria, the most favorable is *E. coli*, but it needs to be confirmed whether the strain is non-pathogenic. Among all the advantages and disadvantages of both gram-positive and -negative bacteria, gram-positive bacteria are the suitable choice due to safety and spore production of some species, which make them more desirable because of heat and chemical tolerance.

Spore Surface Display (Stress Tolerance: High Temperature, pH, Organic Solvent Resistance, GRAS)

Due to intracellular production, spores have advantages over nonspore producers because heterologously anchored proteins cannot cross any membrane. Moreover, due to the rigid spore coat, the displayed proteins and enzymes become resistant to harsh industrial processes such as high temperature, chemicals, and radiation, as well as the fact that they can be stored for a long time at room temperature [Kim and Schumann, 2009]. Spores have the ability to be reused during bioprocesses; Chen et al. [2016b] reported reusability of recombinant spores in up to 5 reaction cycles. *B. subtilis* spores contain different spore coat proteins, CotA, CotB, CotC, CotD, CotE, CotF, CotG, CotH, CotJA, CotJC, CotM, CotS, CotSA, CotT, CotX, CotY, CotZ, SpoIVA, SpoVID, YabG, and YrbA [Takamatsu and Watabe, 2002], but the most preferred anchored proteins are the outer coat proteins. The most commonly used outer coat proteins are CotB [Duc le et al., 2007; Hinc et al., 2010b], CotG [Hinc et al., 2010b; Kwon et al., 2007], and CotC [Yuan et al., 2013]. In addition, an inner coat protein, OxdD, was also used by Potot et al. [2010] for endogenous phytase exposure. The progressive way for spore surface display is presented in Figure 1.

Vectors for Spore Surface Display

For successful spore surface display, the nature of the proteins and the fusion partner's properties are the basic aspects and therefore a vector with different anchor proteins is needed. The use of different integration loci gives strength and flexibility to design different fusions on the spore surface at the same time. The C-terminal vectors Cot/CgeA genes have multiple cloning sites at the end of the gene, with no stop codon. For N-terminal vectors, however, it is located between the start codon and the remaining gene, but it needs to be verified whether the insert is cloned in the frame of the Cot gene translation and has no stop codon [Bloor and Cranenburgh, 2006]. Successful sporulation and its resistance to chloroform and lysozyme was observed when the two designed vectors (CagA protein from *Helicobacter pylori*, in direct association with the CgeA spore coat protein and in the other heterologous part, was connected through an α -helical linker) were displayed on *B. subtilis* spores [Iwanicki et al., 2014]. The use of different vectors for spore surface display technology in different studies is presented in Table 1.

Applications (Vaccine, Antigen, Antibody, Pharmaceutical Protein)

Bacterial spores have shown some unique properties (heat resistance, pH stability, alkaline tolerance) and can be used for producing oral vaccines by displaying heat-sensitive antigens on bacterial spores to overcome the harsh environment of the stomach [Negri et al., 2013], and they have proven to be useful in such applications. Surface-displayed recombinant spores can be used as an oral vaccine, such as expressing the tetanus toxin fragment C of *Clostridium tetani* or heat labile toxin from *Escherichia coli* with serum IgG titers, which were used in a mouse parental model to induce an immunogenic response and are considered for oral administration [Mauriello et al., 2004]. The efficiency of a vaccine can also be increased to some extent as with VP28, the white spot syndrome virus envelope protein, which is considered to be an effective prophylactic vaccine against white shrimp white spot syndrome and shows a slight decrease in mortality rate up to 31% after being fed VP28-displayed spore-mixed tablets [Nguyen et al., 2014]. Recombinant *B. subtilis* spores has the ability to induce system-specific and mucosal humoral immunity, and Zhou et al. [2015] concluded higher amounts of IgG and IgA antibody titers against *H. pylori* antigen in the mice serum immunized with recombinant spores with urease. *B. subtilis* spores are able to tolerate harsh environments and regrow after inactivation in most conditions. The recombinant spore in active and formaldehyde inactive forms decreased the load of TB antigen in mice in an experiment conducted by Sibley et al. [2014], and was considered to be safe for humans, easily transportable, and heat-stable mucosal vaccines. The currently available vaccine, which is either a live-attenuated or inactivated vaccine, needs to be preserved at low temperature and be used through injecting or instilling, which makes their use difficult to some extent, but by using a spore surface-displayed vaccine, all these problems can be alleviated. The spore surface-displayed vaccine can be stored at room temperature and can be mixed with animal chow for oral use in animals.

Environment

Industry plays a key role in today's developing world where gases, polluted water, and various types of hazardous chemicals and heavy metals like nickel, arsenic, chromium, and lead released from these industries have toxic

effects on the health of mankind, animals, birds, and marine life. Several technologies have been developed, including precipitation-dissolution and complexation [He et al., 2005], to overcome these problems; however, they have not achieved wide attention due to major drawbacks such as high energy requirements and generation of hazardous sludge [Ahalya et al., 2003]. Enzymatic degradation is the best way to get rid of these problems. Nitriles used in chemical industries as intermediates are toxic and carcinogenic in nature [Gong et al., 2012], which can pollute water. To counter this, nitrilases are required to get rid of these toxic chemicals and to convert them to useful nontoxic substances such as carboxylic acid in grass. However, these grasses contain some substances (cellulose, hemicellulose, phytate) that are difficult to digest, especially for nonruminant animals such as poultry and pigs, and xylanase, hemicellulase, cellulase, protease, glycanase, or phytase which are required for digestion [Rao, 2009]. Continued monitoring is needed to overcome the problem and improve the activity of these enzymes. Immobilization is an easy and safe way to enhance and stabilize these enzymes [Chen et al., 2016a] because immobilized enzymes are capable of breaking down or disassembling pollutants repeatedly, as *B. subtilis* spores have the ability to regeminate and constantly display with active enzymes [Cartman et al., 2008; Nguyen, 2006]. Recombinant bacterial spores are also a bioremediation tool for heavy metals as they can absorb these and recover them from the ecosystem, like absorption and recovery of nickel ions using *B. subtilis* spores [Hinc et al., 2010a]. Due to absorptive properties of recombinant spores, surface display of different enzymes on *B. subtilis* spores is becoming a fundamental tool for bioremediation processes as it has a major advantage over conventional methods including a revival of absorbed material, good efficacy, and low cost.

Enzyme Immobilization/Biocatalysis (Industrial Enzymes under a Harsh or Bad Environment)

Displaying different proteins on *B. subtilis* spores has great potential for applications in protein immobilization. Display of proteins on spore surfaces may result in changes in protein structure and surface charges leading to increase in relative activity, thermostability, pH stability, and reusability [Chen et al., 2016a]. For example, an increase of 18% in the retained activity [Chen et al., 2015b], 30–40% in thermostability and pH stability [Chen et al., 2015a], and 83% in reusability [Chen et al., 2016a],

Fig. 1. Construction of fusion protein N- or C-terminal and process of spore displaying target protein on their surface.

Table 1. List of fusion and target proteins, used vectors, and application of *Bacillus subtilis* spore surface-displayed proteins

Bacterial strain	Fusion protein	Target protein	Used vector	Applications	Reference
<i>B. subtilis</i> PY79 (Spo+)	CotC	TTFC and LTB	pRH22 and pIM51	<i>Clostridium tetani</i> and <i>E. coli</i> vaccine	Mauriello et al., 2004
<i>B. subtilis</i> 168	CotB, CotC, CotG, and CotZ	FliD	pDL	<i>C. difficile</i> oral vaccines	Negri et al., 2013
<i>B. subtilis</i> WB600	CotC	urease B	pUS186	Oral vaccine for <i>H. pylori</i>	Zhou et al., 2015
<i>B. subtilis</i> HU58	CotB	MPT64	pcotVac	Vaccine against tuberculosis	Sibley et al., 2014
<i>B. subtilis</i> PY79	CotB	RSM2e3	pDG1664	Influenza vaccine	Zhao et al., 2014
<i>B. subtilis</i>	CotC	Pep23	pDG364	CD4 T-cell-based vaccine	D'Apice et al., 2007
<i>B. subtilis</i> PY79	CotB and CotC	PA	pDG364	Vaccine against anthrax	Duc le et al., 2007
<i>B. subtilis</i> 168 trpC2	CotB	CTB-OVA epi	pHY300	Cholera vaccine	Han and Enomoto, 2010
<i>B. subtilis</i> PY79	CotB	GST-Cpa247-370	pDL243	Vaccine against necrotic enteritis	Hoang et al., 2008
<i>B. subtilis</i> WB600	CotC	CsTP22.3	pUS186	Vaccine against <i>Clonorchis sinensis</i>	Zhou et al., 2008
<i>B. subtilis</i> DB104	CotG	Streptavidin	pHPS9	Diagnosis	Kim et al., 2005a
<i>B. subtilis</i> DB104	CotG	GFPUV	pCSK1	Diagnosis	Kim et al., 2006
<i>B. subtilis</i> 168 (<i>trp</i> −)	CotC	HSA	pJS700	Increased serum albumin	Mao et al., 2012
<i>B. subtilis</i> PY79	CotB	VP28	pDG364	Vaccine for shrimps	Nguyen et al., 2014
<i>B. subtilis</i> PY79 and RH201	CotB	Histidine	pDHAFB	Bioremediation	Hinc et al., 2010a
<i>B. subtilis</i> DB104	CotG	ω-Transaminase	pCSK1	Industrial biocatalysis	Hwang et al., 2011
<i>B. subtilis</i> DB403	CotB	DSM	pHS	Industrial biocatalysis	Chen et al., 2015b
<i>B. subtilis</i> DB104	prsA	celA	pUB18-P43-SP	Industrial biocatalysis	Kim et al., 2005b
<i>B. subtilis</i> PY79	OxdD and CotG	phytase	pDG364	Industrial biocatalysis and animal probiosis	Potot et al., 2010
<i>B. subtilis</i> DB403	CotB	Tm1350	pHS	Industrial biocatalysis	Chen et al., 2015a
<i>B. subtilis</i> PY79	CotB, CotC, and CotG	ureA	pGEM and pDL	Industrial biocatalysis	Hinc et al., 2010b
<i>B. subtilis</i> 168 (<i>trp</i> −)	CotC	<i>adhA</i>	pJS700	Industrial biocatalysis	Yuan et al., 2013
<i>B. subtilis</i> DB403	CotG	nit	pHS	Industrial biocatalysis	Chen et al., 2016a
<i>B. subtilis</i> WB600	CotG	Neu5Ac aldolase	pEB03	Industrial biocatalysis	Gao et al., 2011
<i>B. subtilis</i> 168 (<i>trp</i> −)	CotC	BmADH	pJS700	Industrial biocatalysis	Wang et al., 2011
<i>B. subtilis</i> DB104	CotG	β-Gal	pDG1728	Industrial biocatalysis	Kwon et al., 2007

respectively, was noted in immobilized surface-displayed esterases other than nonimmobilized ones. Biocatalysis, a powerful technique used in biochemical industries, is a one-step process that results in low-cost productivity [Schoemaker et al., 2003]. During biocatalytic reactions, enzymes are inactivated and they need to immobilize and enhance thermostability and tolerance to organic chemicals and pH [Hailes et al., 2007]. Displaying environment-susceptible enzymes and other biocatalysts on the spore surface enhances productivity rate, pH stability, thermostability, and survival in harsh conditions by forming robust spores [Gao et al., 2011; Wang et al., 2011]. Thus, the spore surface-displayed enzymes and biocatalysis might be a way to produce cost-effective chemoenzymatic compounds. Feasible enzymes are not appropriate for industrial purposes because of the increase and decrease of temperature and pH during industrial processes which can affect the activity and stability of feasible enzymes, hence the need to use strong enzymes and spores to tolerate harsh industrial environments.

Peptide Libraries

The fusion of a carrier protein with an anchoring protein has a direct impact on successful surface display as well as on the activity and stability of the displayed protein. It is difficult to predict whether the peptide sequence (linkers) is helpful for increasing the stability and activity of proteins, but our unpublished data suggest numerous effects on enzyme activity and stability using different peptide linkers than direct linking with an anchor protein. The UreA subunit from *H. acinonychis* showed improved expression when the peptide linker GGGEAAK-GGG was used rather than EAAAK, but failed to express when GGGGS was used [Hinc et al., 2013]. When using peptide linkers with different amino acid sequences, orientation and length is the best way to enhance the expression of proteins with different properties such as increased activity, stability, alkaline tolerance, and heat resistance. By using different suitable linkers with various properties, which can be done using statistical analysis

like the response surface methodology, peptide libraries can be made for certain proteins with the best expression properties.

Limitations

Spore surface display has numerous applications in industries and environmental and medical science, but there are certain limitations which make it difficult to get the desired results. The success of display on the surface of spores depends on the fusion of anchored and target proteins, so it becomes harder to choose the correct fusion and anchor partner. Hinc et al. [2010b] reported expression failure of UreA from *H. acinonychis* when displayed with CotB and CotC as the carrier, but succeeded when displayed using CotG. Similarly, Iwanicki et al. [2014] reported the failure of surface display when using a flexible linker GGGGS between CotZ and UreA, but achieved promising results when a rigid linker (EAAAK) was used. So it is always difficult to select the right vector and anchor protein for the specific gene, and more effort is needed to get the right choice. Moreover, the use of the right or no linker is also difficult because in most cases the use of a linker makes the target protein more stable than not using one.

Conclusion and Future Perspectives

Due to low cost and auspicious applications, display of proteins and peptides on microorganisms is becoming a leading technology. Microorganisms (bacteria, yeast, and phages) are used for the display of these proteins. Spore surface display is a multimeric strategy for the production of vaccines, as well as certain applications to industrial biocatalysis and environment protection. Using different proteins and antigens on the spore surface can enhance their activity and stability for biodegradation of hazardous compounds, and can have relevance for vaccine approaches. For a successful display, chimeric proteins have to cross at least one membrane; however, some proteins do not have the ability to cross because of fast folding and hydrophobic patches. To avoid all these problems, the best way is to use endospore-forming bacteria like *B. subtilis* because the proteins displayed on the spore surface do not need to cross the membrane barrier. For the extraction of required proteins, nutritive cells need special chemical treatment or ultrasonic waves to disrupt the cell to obtain the required protein, while the

proteins linked to the spore surface can be separated easily and the product by centrifugation. Nutritive cells including bacteria are susceptible to environmental stress and can be disrupted easily, hence there is a chance of wasting desired proteins while spores have the ability to survive in unfavorable conditions. Displaying enzymes on the surface of yeast is more time consuming, about 96 h for reaching the highest activity, but the time is limited for the spores and reach the maximum activity level at less than 48 h.

To enhance the enzymatic activity, we need to study the molecular dynamics of the desired protein and design a suitable linker for fusion protein construction. A relative flexible linker between the carrier and target protein is the better choice for relative free movement of the C- or N-terminal to get a more optimized structural domain. A rigid linker can maintain a certain distance in the two domains so as to avoid interference. Therefore, it is very important to find a suitable linker for surface display on the spores. A linker library should be built up using statistical methods to analyze the effect on folding of the enzyme space structure by the interaction of single factor or more factors concerning different length, flexibility, constituent, hydrophobicity, charge property, sequence of amino acid residues, secondary structure, and to gain a better understanding of the influence of enzyme activity and stress resistance ability. The preliminary spore display model should be set up for linker design and the guiding principle of efficiency and inexpensive immobilization of the industrial enzyme. A multienzyme complex is a large molecular structure formed by noncovalent bonds of enzymes. Displaying enzymes on the spore surface able to form the multienzyme complex is a new way to solve the expression and immobilization of the multienzyme complex. An experimental strategy is to link different enzymes of the multienzyme complex with different coat proteins, and codisplay them on the surface of the spores at the same time. For the large expression of fusion proteins, different expression vectors can be used, but it is better to select the strain for the desired enzyme which has a close relationship to *B. subtilis*, which is a gram-positive bacterium. Spore coat protein CotB is a low abundance protein. Sometimes it is unable to express the desired proteins to improve the enzyme expression level, so another anchor protein or varieties of spore coat proteins such as (CotG, CotC, CotX) would be selected to immobilize the enzyme.

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Disclosure Statement

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