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Surface display of *Aggregatibacter actinomycetemcomitans* autotransporter Aae and dispersin B hybrid act as antibiofilm agents

Chandran Ragunath[1], **Kristina DiFranco**[2], **Mayilvahanan Shanmugam**, **Prerna Gopal**, **Vishal Vyas**, **Daniel H. Fine**, **Carla Cugini**, and **Narayanan Ramasubbu**[*]

Department of Oral Biology, Rutgers School of Dental Medicine, 185 South Orange Ave, Newark NJ 07103, USA

Abstract

Among the various proteins expressed by the periodontopathogen *Aggregatibacter actinomycetemcomitans*, two proteins play important roles for survival in the oral cavity. The autotransporter Aae facilitates the attachment of the pathogen to oral epithelial cells, which act as a reservoir, while the biofilm-degrading glycoside hydrolase dispersin B facilitates the movement of daughter cells from the mature biofilm to a new site. The objective of this study was to utilize the potential of these two proteins to control biofilms. To this end, we generated a hybrid construct between the Aae C-terminal translocating domain and dispersin B, and mobilized it into *E. coli* Rosetta (DE3) pLysS cells. Immunofluorescence analysis of the modified *E. coli* cells confirmed the presence of dispersin B on the surface. Further, the membrane localization of the displayed dispersin B was confirmed with western blot analysis. The integrity of the *E. coli* cells displaying the dispersin B was confirmed through FACS analysis. The hydrolytic activity of the surface-displayed dispersin B was confirmed by using 4-methylumbelliferyl-β-D-gluocopyranoside as the substrate. The detachment ability of the dispersin B surface-displaying *E. coli* cells was shown using *Staphylococcus epidermidis* and *Actinobacillus pleuropneumoniae* biofilms in a microtiter assay. We concluded that the Aae β-domain is sufficient to translocate foreign enzymes in the native folded form and that the method of Aae mediated translocation of surface displayed enzymes might be useful for control of biofilms.

Keywords

Autotransporter; surface display; biofilm; dispersin B; oral pathogen

INTRODUCTION

Autotransporters are a superfamily of virulence factors that are large in size and are secreted by Gram-negative bacteria to carry out diverse functions. They consist of a) an N terminal

[*]Corresponding author. Department of Oral Biology, C-634, MSB, RBHS-SDM, 185 South Orange Ave, Newark, NJ 07103 USA. Tel.: (973) 972-0704, Fax: (973) 972-0045, ramasun1@sdm.rutgers.edu.

[1]Present Address: Scientific Chemical Technologies, 6 Pleasant St., Malden, MA 02148

[2]Present Address: Department of Oral Biology, University of Florida at Gainesville.

signal sequence that is responsible for crossing the inner membrane and is eventually cleaved off; b) a mature functional protein designated as the passenger domain; and c) a carboxy terminal β domain, which forms a β barrel that is anchored into the outer membrane (Jose *et al.*, 1995). The C-terminal β barrel domain, necessary for translocating the passenger domain into the extracellular space, folds in the periplasm and inserts into the outer membrane as a barrel (~10 Å pore) via an energetically independent pathway (Henderson *et al.*, 2000). Although some autotransporters require trimerization others are monomeric and possess the ability to translocate passenger domains (Cotter *et al.*, 2005). The simplistic mechanism of autotransport provides a vehicle for exchanging a protein of interest to be secreted in place of the original passenger domain. There have been such reports utilizing the β domain's ability to display proteins on the surface of cells for either delineating the mechanism of translocation (Skillman *et al.*, 2005) or to assess the proper folding of the translocated proteins by testing their enzyme activity (Freeman *et al.*, 1996; Lattemann *et al.*, 2000; Van Gerven *et al.*, 2009). For example, the surface display of various proteins using IgA protease in *Neisseria* and OMP-A and AIDA-I in *Escherichia coli* have already been well characterized as the anchoring protein for surface display (Freeman *et al.*, 1996; Lattemann *et al.*, 2000; Van Gerven *et al.*, 2009).

The display of heterologous proteins on the surface of bacteria offers many practical applications, such as epitope presentation for vaccines, screening of peptide libraries, and whole cell biocatalysis (Klauser *et al.*, 1990; Rutherford & Mourez, 2006). Although initial research was done using bacteriophages to display select foreign proteins, bacteria are now the preferred organisms (Jose & Meyer, 2007). This is due to bacteria's fast replication time, which allows for more recombinant proteins to be made at high levels with little to no toxic effects (Rutherford & Mourez, 2006), as well as their size, which makes them easy to view under a microscope. In spite of these studies using surface display of enzymes, none have been performed to display an enzyme on the bacterial surface for the purpose of degradation and detachment of biofilms.

Biofilms are responsible for a myriad of medical problems including nosocomial infections of catheters and heart valves, as well as diseases like cystic fibrosis pneumonia (Costerton *et al.*, 1995; Costerton *et al.*, 1999). Biofilms are complex communities of bacteria that are encased in a sticky matrix that allows them to adhere to both biotic and abiotic surfaces. The self-synthesized, three-dimensional matrix (extracellular polysaccharide, EPS) participates in holding the bacterial cells together in a mass and firmly attaches the bacterial mass to the underlying surface (Costerton *et al.*, 1999). The EPS matrix also protects the attached bacteria from antibiotics and the host antibodies, thus making them extremely difficult to eradicate. Almost 80% of pathogenic bacteria exist as biofilm communities and utilize a number of matrix substances for attachment and colonization. While the matrix is largely composed of EPS, it is often more complex and may be interacting with other macromolecules such as proteins and extracellular DNA (Izano *et al.*, 2008; Izano *et al.*, 2007). The EPS also facilitates in the aggregation and coaggregation of pathogenic as well as non-pathogenic bacteria in to multispecies biofilms.

Among the oral pathogens that are causative agents for periodontal diseases, *Aggregatibacter actinomycetemcomitans* has been shown to be involved in localized

aggressive periodontitis (Slots *et al.*, 1980). This Gram-negative bacterium possesses a secreted, non-cell associated matrix-degrading enzyme called dispersin B (DspB), which is capable of detaching biofilms (Kaplan *et al.*, 2003a; Manuel *et al.*, 2007). DspB belongs to the family 20 glycosyl hydrolase (β-hexosaminidase; EC 3.2.1.52), whose substrate is a linear homopolymer of N-acetyl-D-glucosamine residues in β(1,6)-linkages. The crystal structure of DspB in complex with glycerol and an acetate at the active site suggested that the active site architecture of DspB is similar to the family 20 β-hexosaminidases (Ramasubbu *et al.*, 2005). Dispersin B has been shown to inhibit biofilm formation and/or promote biofilm detachment in different Gram-negative and Gram-positive bacteria: *A. actinomycetemcomitans* (Kaplan *et al.*, 2003a); *Actinobacillus pleuropneumoniae* (Kaplan *et al.*, 2004); *E. coli, Pseudomonas fluorescens* and *Yersinia pestis* (Itoh *et al.*, 2005); *Bordetella bronchiseptica* (Parise *et al.*, 2007); and *Staphylococcus epidermidis* (Kaplan *et al.*, 2004). Recently, we demonstrated that transgenic tobacco plants expressing DspB are resistant to *Pectobacterium carotovorum* infection (Ragunath *et al.*, 2011).

Since enzymes that degrade exopolysaccharide, such as DspB, are capable of causing the detachment of cells from biofilms (Johansen *et al.*, 1997), the matrix-degrading enzymes are attractive anti-biofilm agents. Utilizing such agents against biofilm bacteria renders the bacteria more susceptible to the effect of antibiotics. Also, the targeted bacteria are not under selective pressure to develop resistance to enzyme mediated dispersal because these enzymes do not kill bacteria or inhibit their growth (Kaplan *et al.*, 2003a).

A. actinomycetemcomitans possess a number of autotransporters that can be utilized for surface display as described above. One such protein is Aae (Rose *et al.*, 2003), which is a 90-kDa surface-anchored protein. Aae is a member of the autotransporter family of bacterial proteins that is homologous to an epithelial cell adhesin (Hap) produced by *Haemophilus influenzae* (Hendrixson *et al.*, 1997; Hendrixson & St Geme, 1998). This monomeric autotransporter, which has a truncated passenger domain, is primarily involved in the binding of *A. actinomycetemcomitans* to human buccal epithelial cells (Fine *et al.*, 2005; Rose *et al.*, 2003). In this study, we have used the translocating potential of the β-domain of Aae and the biofilm degrading potential of DspB to form a hybrid protein that is displayed on the surface of *E. coli*. While DspB is a secreted protein, displaying it on the surface of a bacterium is unlikely to enhance its catalytic activity; however, it is expected to retain its catalytic activity when it is exported in the folded conformation. It is noteworthy that autotransporter systems have been used in the surface display of enzymes such as lipase (Becker *et al.*, 2005) or sorbitol dehydrogenase (Jose & von Schwichow, 2004), while retaining their corresponding catalytic activity. Our hypothesis is that Aae β-domain will translocate DspB in a correctly folded form. Our results show that the protein encoded by the hybrid construct was functional and able to detach *S. epidermidis* and *A. pleuropneumoniae* biofilms. Utilization of surface-displayed biofilm degrading enzymes provides a novel method to control biofilm infections independently or in combination with other applications (Jose, 2006).

METHODS

Bioinformatics

The C-terminus of the autotransporters is the region of highest homology among its close homologs (Rose *et al.*, 2003) and presumably adopts a β-barrel used for translocation of the passenger domain. The goal for the hybrid generation was to identify the right size of the C-terminal domain such that only a few N-terminal amino acid(s) will exude out of the outer membrane. To select the appropriate β-domain length of Aae for our study we used *in silico* analysis. First, the amino acid sequence of the Aae protein (deduced from the genome of *A. actinomycetemcomitans* strain IDH781) was analyzed for protein homology detection and structure prediction using the HHpred server (Soding *et al.*, 2005). Homologs of Aae were identified to generate a multiple alignment through multiple iterations of PSI-BLAST searches and the alignment was annotated with predicted secondary structure and confidence values from PSIPRED (Jones, 1999). Based on the predicted secondary structure, a profile hidden Markov model (HMM) was constructed, which was then compared with HMMs from the Protein Data Bank (PDB) using the HHsearch software (Soding, 2005). Using position-specific gap penalties, secondary structure similarity was scored and used to construct local alignments with the Aae sequence and the matching PDB database sequences. The best-matched proteins from the PDB database were selected as templates for comparative modeling with the MODELLER software (Sali & Blundell, 1993). The resulting structure models were viewed with PyMOL version 1.0 (DeLano, 2002). Using this analysis, we predicted that amino acids 623-912 would be of sufficient length to display the passenger domain completely outside the surface.

Bacterial strains, media and growth conditions

E. coli strains DH5α and Rosetta (DE3) pLysS were used for cloning and protein expression, respectively. The *E. coli* strains were grown in LB broth supplemented with kanamycin (DH5α; 30 μg/mL), or kanamycin/chloramphenicol (pLysS; 30 μg/mL each), and induced with 0.1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) when appropriate. *S. epidermidis* (NJ 9709; (Kaplan *et al.*, 2004)) strains were grown in Trypticase soy broth (TSB; Becton Dickinson) supplemented with 6 g of yeast extract and 8 g of glucose per liter (Kaplan *et al.*, 2003a). *A. pleuropneumoniae* (IA5) was grown in TSB with 6 g of yeast extract supplemented with 10% sodium bicarbonate, 20% dextrose and NAD.

Recombinant DNA techniques

First, the N-terminal portion of Aae that encodes the signal sequence (corresponding to bp 1 to 159 in GenBank accession no. AY487820) was amplified by PCR by using genomic DNA isolated from *A. actinomycetemcomitans* strain IDH781 using the primers P1 and P2 (Table 1). The PCR product was digested with NdeI and KpnI and ligated into the NdeI/KpnI sites of the T7 expression vector pET29b (Novagen), resulting in plasmid pKD10. Secondly, to generate a plasmid containing the T7-tag (MASMTGGQQMG) and the Aae β-domain (bp 1869 and 2736), the IDH781 genomic DNA was amplified using the primers P5 (forward with KpnI site) and P4 (reverse with XhoI site). The forward primer, P5, has the DNA sequence corresponding to the T7 tag built into it. The resulting product was double digested with KpnI and XhoI, and ligated into the KpnI/XhoI sites of pKD10. The resulting plasmid,

pKD11, has the following sequence: Aae signal sequence, T7 tag, and the β-domain of Aae. This construct was used to verify whether or not the β-domain (as determined in the Bioinformatics section above) is of sufficient length to display a foreign peptide (T7 tag) outside the cell. Nucleotide sequencing was used to confirm the insert sequence.

To generate a plasmid containing the sequence: Aae signal sequence, DspB, and the β-domain of Aae, the following protocol was used. First, the gene encoding DspB was amplified using primer pairs, P6 and P7, with KpnI and BamHI sites using the construct pRC3 (Kaplan *et al.*, 2003b). The PCR product was digested with KpnI and BamHI, and ligated into the KpnI/BamHI sites of pKD10 resulting in the plasmid pKD2. Secondly, the Aae β-domain (corresponding to bp 1869 and 2736) was amplified by PCR using primer pairs, P3 (forward) and P4 (reverse), introducing BamHI and XhoI sites, respectively by using the genomic DNA isolated from *A. actinomycetemcomitans* strain IDH781. The PCR product was double digested with BamHI and XhoI, and ligated into the plasmid BamHI/XhoI sites of pKD2. This resulting plasmid (pKD3) was sequenced to confirm the insert sequences corresponding to the signal peptide, DspB as well as the β-domain of Aae.

Immunofluorescence microscopy

Recombinant DspB (Kaplan *et al.*, 2003b) was used in the immunization of specific-pathogen-free female New Zealand White rabbits (Pocono Rabbit Farm and Laboratory, Canadensis, PA.) to obtain a rabbit anti-DspB polyclonal antibody. Rosetta (DE3) pLysS containing pKD3 and pKD11 were grown overnight in LB broth containing kanamycin and chloramphenicol at 37°C. The overnight cultures were transferred to fresh medium (1:100) containing kanamycin and the flasks were incubated at 37°C with agitation (200 rpm) until the optical density of the culture at 600 nm reached 0.6 (ca. 3 h). At this time, IPTG was added to a final concentration of 0.1 mM, and the flask was incubated for an additional 2 h with agitation. Cells were spun down for 5 min at 4000 × *g* and washed with PBS buffer (3x). Cells (1×10^7) were fixed in 0.3% glutaraldehyde and incubated overnight at 4°C (Freeman *et al.*, 1996), centrifuged at 4°C for 5 min at 4000 × *g* and washed with PBS (3x). The cells were spread on poly L-lysine coated cover slips and dried at room temperature. Cover slips were incubated with 5% normal goat serum (NGS) followed by primary antibody (rabbit anti-T7 polyclonal antibody raised against the T7 peptide (Convance, CA)) for pKD11 incorporated Rosetta (DE3) pLysS (1:200 dilution) or rabbit anti DspB polyclonal antibody for pKD3 and control (1:500) in PBS with 5% NGS and incubated for 1h. After washing (3x for 5 min in PBS), secondary antibody (1:100 dilution, Alexa Fluor 488-goat anti rabbit IgG (Invitrogen)) in PBS with 5% NGS was added to the cover slips and incubated for 1h in the dark. Cover slips were washed 3x for 5 min in PBS, dried at room temperature, mounted on microscope slides with VECTASHIELD H-1000 (Vector Labs, Burlingame, CA) and examined by fluorescent microscopy using an Olympus BX50WI microscope using anti DspB polyclonal antibody.

Flow-cytometric analysis and FACS

Cultures of *E. coli* strains (Rosetta (DE3) pLysS containing pKD3 and Rosetta (DE3) pLysS containing pET29b) were grown overnight at 37°C and subcultured 1:50 at 37°C (100 mL) until the optical density at 600 nm (OD600) reached 0.6. The cells were further grown for an

additional 1 hr after induction with 0.1 mM IPTG. Cells were pelleted by centrifugation and resuspended in 1 μL of PBS. To 10 μL of cells, 1 μl of the anti-rabbit DspB antibody was added and the cells were incubated for 5 min at room temperature and pelleted. After washing the pellet with 500 μl of PBS, cells were centrifuged and resuspended in 10 μL of PBS containing secondary antibody (1:100 dilution, Alexa Fluor 488-goat anti rabbit IgG (Invitrogen)). After 5 min of incubation at room temperature and addition of 500 μL of PBS, cells were pelleted and resuspended in 100 μL of PBS for flow cytometry analysis (NJMS Flow Cytometry And Immunology Core Laboratory, RBHS). A total of 300,000 events were collected on a BD FACSCalibur. Parameters were set as follows: forward scatter at E02 with a gain of 5.00, side scatter 906 with a gain of 1.00 (both in linear mode) and FL1 (fluorescein isothiocyanate) at a voltage of 777 (LOG mode).

Surface localization of DspB

The Rosetta (DE3) pLysS *E. coli* cells expressing DspB (pKD3) or pET29b (control cells) were fractionated into cytoplasmic and membrane fractions by sonication (Georgiou *et al.*, 1996) and analyzed using SDS-PAGE (10%) by Coomassie staining and immunoblot. Western blotting (Burnette, 1981) was performed by electrotransfer of proteins from SDS-PAGE gels to Immobilon-P membranes (Millipore). After blocking with 5% (w/v) non-fat milk in Tris buffered saline with Tween 20, and washing, the blots were incubated with polyclonal rabbit anti-DspB, washed with PBS, and then incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (Promega). Finally, the blots were developed using the Pierce Supersignal (ThermoScientific).

Enzyme assays

The substrate 4-methylumbelliferyl-β-D-glucosamine (Sigma) was used to determine the enzyme activity (Manuel *et al.*, 2007) of the displayed DspB. The DspB expressing *E. coli* cells (pKD3) were washed (3x), pelleted, weighed, and resuspended in 50 mM sodium phosphate buffer (pH 5.8). In a typical experiment, 10 μL of the cells were used (corresponding to 10^7 cfu). As a negative control, T7-expressing cells (pKD11) were used, while purified recombinant DspB was used as a positive control. Typically, enzyme reactions were carried out in 100 μL suspended mixtures using a substrate concentration (1 to 5 mM) in 50 mM sodium phosphate buffer (pH 5.8). All assays were carried out in 96 well microtiter plates placed in a 37°C incubator. The reactions were terminated at various times by adding 10 μL of 10N NaOH. Additionally, wells having suspended *E. coli* cells without addition of NaOH or without addition of the substrate were also monitored. The increase in fluorescence resulting from the release of 4-methylumbelliferone (excitation at 360 nm and emission at 460 nm) in each well was measured using a fluorescence/luminescence/visible light microplate reader (BIO-TEK Instruments). All assays were performed in triplicate on at least three separate occasions, which exhibited similar results with minimal variations among them.

Biofilm detachment assay

The biofilm of *S. epidermidis* strain NJ 9709 or *A. pleuropneumoniae* strain IA5 was prepared as previously described in a 96 well plate (Kaplan *et al.*, 2003b; Kaplan *et al.*, 2004; Manuel *et al.*, 2007). The *A. pleuropneumoniae* biofilm was grown in a 96-well plate

for 20 hours at 37 °C 10% CO_2 incubator. Rosetta (DE3) pLysS cells containing pKD3 were grown and treated with IPTG as described above for immunofluorescence microscopy. Cells were spun down for 5 minutes at 4000 × *g*, washed 3x with PBS and resuspended in 500 μL PBS. Wells with *S. epidermidis* biofilm or *A. pleuropneumoniae* biofilm were treated with varying amounts of Rosetta (DE3) pLysS cells containing pKD3 (10^5 to 10^8 in 200 μL). Mock treated (PBS) wells (negative control), DspB treated wells (positive control) and bacteria treated wells were incubated for 1h at 37°C, washed and stained with crystal violet for 1 minute. After washing and drying, 33% acetic acid was added to each well and the plates were scanned using a microplate reader (Bio-Rad) at 590 nm. Optical intensities of the residual biofilm representing the biofilm detachment were recorded (Kaplan *et al.*, 2003b; Kaplan *et al.*, 2004; Manuel *et al.*, 2007). This experiment was performed in triplicate on at least three separate occasions with consistent results.

RESULTS

Identification and modeling of Aae β-domain

Since the C-terminal domain of autotransporters are vital to the export of the passenger domain, we sought to determine the correct size of this domain so that only a few N-terminal amino acids will exude out of the pore. If the estimated length of the C-terminus is too short, the foreign protein/peptide will be prevented from being brought outside the barrel completely. Our intent was to keep the hybrid size as small as possible without affecting the transport function. To do this, we used the server HHpred (Soding *et al.*, 2005), which detected and aligned homologous sequences in the Protein Data Bank (PDB). The C-terminal domain of Aae had best matches with a probability of > 90% (hits) to the *E. coli* EspP (Barnard *et al.*, 2007) and to the *Neisseria meningitidis* NalP (Oomen *et al.*, 2004) autotransporters. These proteins form an outer membrane pore composed of antiparallel β-strands with the N-terminal part of the chain threaded through the barrel. The HHpred server modeled Aae based on these hits and produced a barrel structure for Aae (data not shown). From this analysis, we selected residues 623 through 912 (290 residues) to be the appropriate length/size for our purpose to generate a hybrid protein with DspB as the passenger domain (Fig. 1).

Construction of recombinant fusion protein encoding genes

In order to determine whether or not the 290 residues selected for Aae β domain was large enough to be able to extrude the full passenger protein, a T7 tag as the passenger domain was first used (Fig. 1). Since the Aae has three domains, we retained the Aae signal sequence and the selected C-terminal domain in the construct, but used the T7 tag instead of the passenger domain. This was cloned into a pET29b plasmid (pKD11). A second construct (pKD3) was created after confirming the exposure of the T7 tag outside membrane (see below) wherein DspB was used as the passenger domain. The use of Aae signal sequence is apparently important since constructs using the signal sequence of DspB instead of Aae were not successful in displaying either the T7 or DspB (data not shown). The signal sequence of DspB is smaller (20 aa residues) compared to that of Aae (53 aa residues).

Visualization with immunofluorescence microscopy

The display of the T7 tag and DspB with the 290 C-terminal residues of the Aae β-domain was confirmed by immunofluorescence microscopy (Fig. 2). Control cells of Rosetta (DE3) pLysS containing pET29b displayed no fluorescence (Fig. 2a) with or without IPTG (data not shown). Interestingly, the Rosetta (DE3) pLysS mobilized with either pKD11 (T7 tag; Fig. 2b) or pKD3 (DspB; Fig. 2c) were positively stained irrespective of whether or not IPTG was used for induction. The results for the T7-tag hybrid pKD11 cells suggest that the length of the Aae β-domain identified by the bioinformatics analysis is sufficient to bring out even the 11-mer T7 peptide outside of the cell surface.

The cellular localization of the fusion protein was also analyzed by fluorescence-activated cell sorting (FACS), which clearly demonstrated that the DspB could be detected on the surface of pKD3 cells (Fig. 3). The peak fluorescence channel of the pKD3 strain that corresponds to a measure of the number of surface-exposed DspB molecules per cell turned out to be approximately 2208.

Surface localization of DspB

In order to assess whether or not the expressed hybrid protein containing DspB is localized in the membrane fractions, the *E. coli* cells (Rosetta (DE3) pLysS containing pKD3 and Rosetta (DE3) pLysS containing pET29b with or without IPTG induction) were fractionated into cytoplasmic and membrane fractions. These extracts were probed with rabbit anti DspB polyclonal antibody (Fig. 4). The cells with pKD3 showed the presence of DspB in both the cytoplasmic as well as membrane fractions; however, the membrane fractions showed a much higher presence of DspB. The *E. coli* cells with pET29b did not show any bands. These results clearly suggest that pKD3 cells are capable of exporting DspB to the membrane while fused to the Aae β-domain (~70 kD).

Enzyme assays

To assess whether or not the displayed DspB was enzymatically active, we tested the catalytic activity of DspB using a fluorescent substrate (Fig. 5). Only pKD3 cells, which were designed to express DspB on the surface, showed enzyme activity whereas the pKD11 control cells did not. These results suggest that the DspB was enzymatically active on the surface. The number of cells in each of the wells was approximately 1.0×10^7 cells. Interestingly, pKD3 cells that were uninduced with IPTG did not show any activity (data not shown).

Biofilm detachment assay

The activity of the surface-displayed DspB/Aae hybrid was then tested for its ability to detach a *S. epidermidis* biofilm using a 96-well microtiter plate assay (Kaplan *et al.*, 2003b; Kaplan *et al.*, 2004; Manuel *et al.*, 2007). As shown in Fig. 6a, only *E. coli* cells transformed with pKD3 construct exhibited detachment whereas the control cells did not. The absorbance of the residual biofilm was measured after the addition of acetic acid (Fig. 6b). The amount of biofilm detachment by the DspB displaying *E. coli* cells when compared with purified DspB (1 mg/mL to 1 pg/mL) suggests that a minimum of 1.0×10^7 cells/200 μL with plasmid pKD3 are needed for any observable biofilm detachment. The number of

pKD3 *E. coli* cells to reduce the biofilm by 50% was estimated to be about 5×10^7 cells/200 μL. The DspB displaying pKD3 cells were also able to detach biofilms of *A. pleuropneumoniae* (Fig. 7). We have shown earlier that DspB detaches *A. pleuropneumoniae* biofilms (Kaplan *et al.*, 2004). A minimum of 1.0×10^7 pKD3 cells was also required for observable biofilm detachment. As with *S. epidermidis* biofilms, the number of pKD3 *E. coli* cells to detach 50% of *A. pleuropneumoniae* biofilms was estimated to be 10^8 cells (Fig. 7b). Control *E. coli* cells did not detach biofilms.

DISCUSSION

The results from our study support our hypothesis that the β-domain of the autotransporter Aae is able to display foreign proteins that remain functional on the surface of *E. coli*. This is the first time that the autotransporter from the periodontal pathogen *A. actinomycetemcomitans* has been utilized as a translocator unit for surface display. The size of the β-domain selected through *in silico* analysis is sufficient for this purpose. Several notable points emerge from our study. The Aae β-domain we have identified consists of only 291 amino acids and unlike other trimeric autotransporters, is a single chain and thus well suited for such purpose. The display of DspB on the surface was confirmed using immunofluorescence in which we noted that DspB was detected regardless of whether or not IPTG was present (Fig. 2; IPTG absent data not shown). To ensure that the display of DspB is not an artifact, and that the immunofluorescence did not arise due to fixing of the cells with glutaraldehyde, we provide data on the results of two subsequent experiments. Firstly, western blot analysis showed that neither the cytoplasmic nor the membrane fractions of uninduced cells had detectable DspB. As shown in Fig. 4, only IPTG induced cells were positive for the presence of DspB. Thus, the expressed protein is properly shuttled to the membrane where it could be processed for display. Secondly, and more importantly, the FACS analysis (Fig. 3) strongly suggests that the *E. coli* cells decorating the enzyme on their surface are intact.

In the biofilm detachment assay, only those bacteria that were induced with IPTG were capable of detaching the *S. epidermidis* biofilm. The surface mobilized enzyme was able to hydrolyze a substrate (Fig. 5) and detach *S. epidermidis* (Fig. 6) or *A. pleuropneumoniae* biofilms (Fig. 7) suggesting that DspB is catalytically active and is in the correctly folded conformation on the surface. The detachment ability of cells that were not induced was minimal and comparable to the control Rosetta (DE3) pLysS cells. Thus, a possible explanation for the display of immunofluorescence in the uninduced cells could be that DspB is mobilized to the cell surface through leaky expression from T7 promoter.

It is known that the success of mobilizing a functional protein to the outer membrane surface depends on the protein's ability to be folded correctly in the periplasm. Dispersin B apparently does so partly due to the absence of disulfide bonds, which increases the efficiency of transport since their presence could pose complications during the folding stages in the periplasm. For those proteins that do possess disulfide bonds, techniques such as utilizing site directed mutagenesis to remove the cysteines, using an oxidoreducatase negative mutant (*dsbA*), or adding 2-mercaptoethanol have been proposed as a remedy (Rutherford *et al.*, 2006; Rutherford & Mourez, 2006). While we have demonstrated that the

Aae β-domain is capable of exporting a protein with no disulfide bonds, studies to demonstrate the capability of the Aae β-domain to successfully bring out proteins with disulfide bonds to the surface need to be explored. Nevertheless, the use of the Aae C-terminal β-domain for surface display offers many new possibilities in clinical and environmental applications in biofilm degradation, vaccinology, or biocatalysis. The use of pKD3 containing cells to degrade different bacterial biofilms shows great promise in the use of bacterial cells displaying antibiofilm enzyme(s) on their surface for biofilm degradation of pathogenic bacteria. In summary, we have shown that foreign peptides and proteins could be successfully displayed as an Aae hybrid and that use of EPS degrading enzymes could detach biofilms.

Acknowledgments

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Abbreviations

DspB	dispersin B
NGS	normal goat serum
IPTG	Isopropyl β-D-1-thiogalactopyranoside
EPS	extracellular polysaccharide

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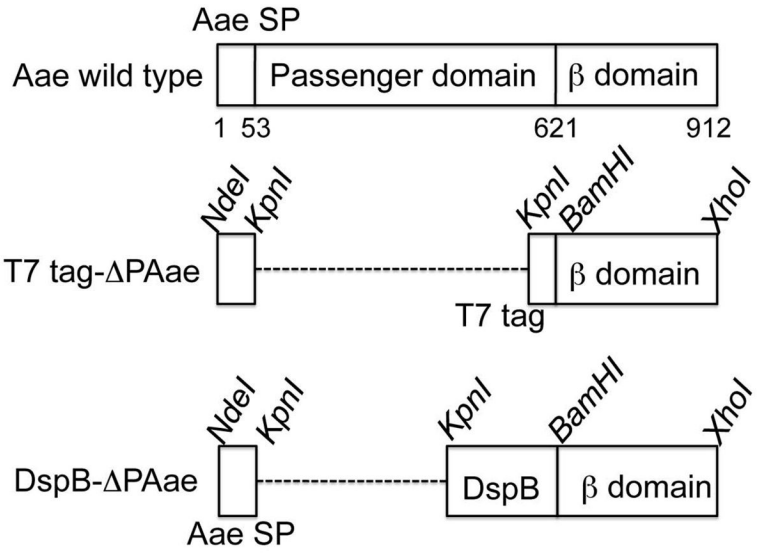

Figure 1.

Domain organization of the autotransporter Aae from *A. actinomycetemcomitans*. Unlike other autotransporters, Aae is lacking a protease like domain at the N-terminus of the extruded passenger domain. The numbers refer to the aa position of the start and end of the signal sequence and the β-domain, respectively. In the middle, the T7-tag containing hybrid with the Aae signal sequence (SP) and associated restriction enzymes sites are shown. When fused together, the hybrid protein will lack the passenger domain of Aae completely and in its place will have the T7 tag. At the bottom, the DspB/Aae hybrid with associated restriction sites are shown. This hybrid will lack the Aae passenger domain but will have DspB as the new passenger domain. In the truncated mutants, the signal sequence and the passenger domains are linked and the dashed line is used to keep the β-domains aligned in the figure.

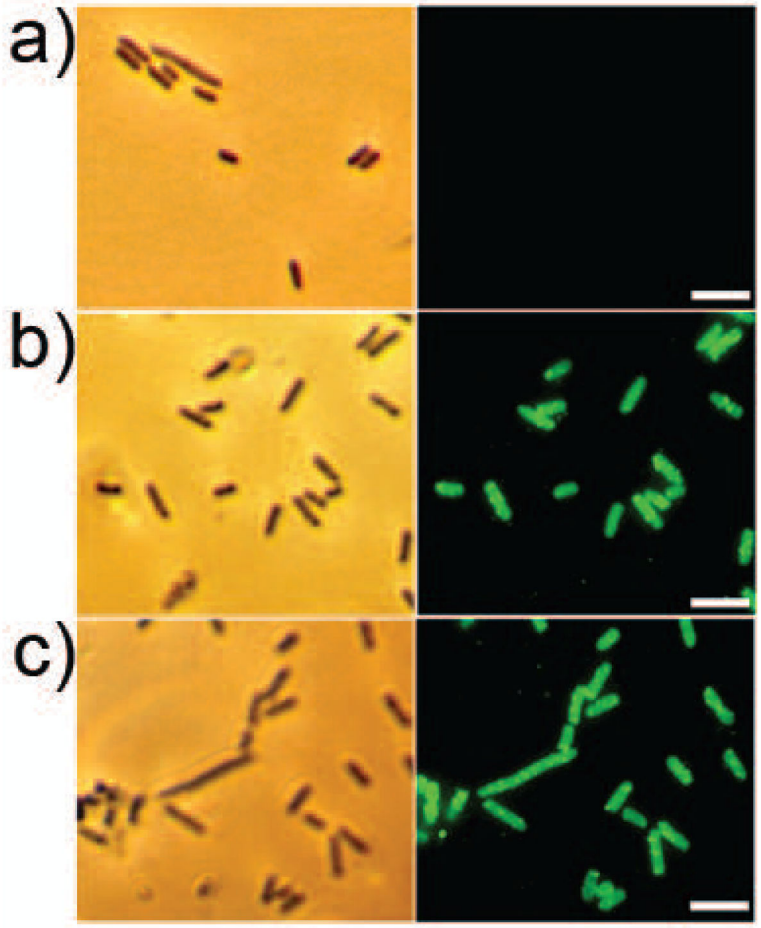

Figure 2.

Immunofluorescence of Rosetta (DE3) pLysS cells. a) Control Rosetta (DE3) pLysS cells transformed with pET29b. b) Rosetta (DE3) pLysS cells mobilized with pKD11 encoding the T7 tag. c) Rosetta cells with pKD3 encoding dispersin B. Shown on the left are the phase contrast images and on the right, immunofluorescence images. The bar indicates 5 μm. Green fluorescence was due to Alexa Fluor 488 labeled secondary antibody.

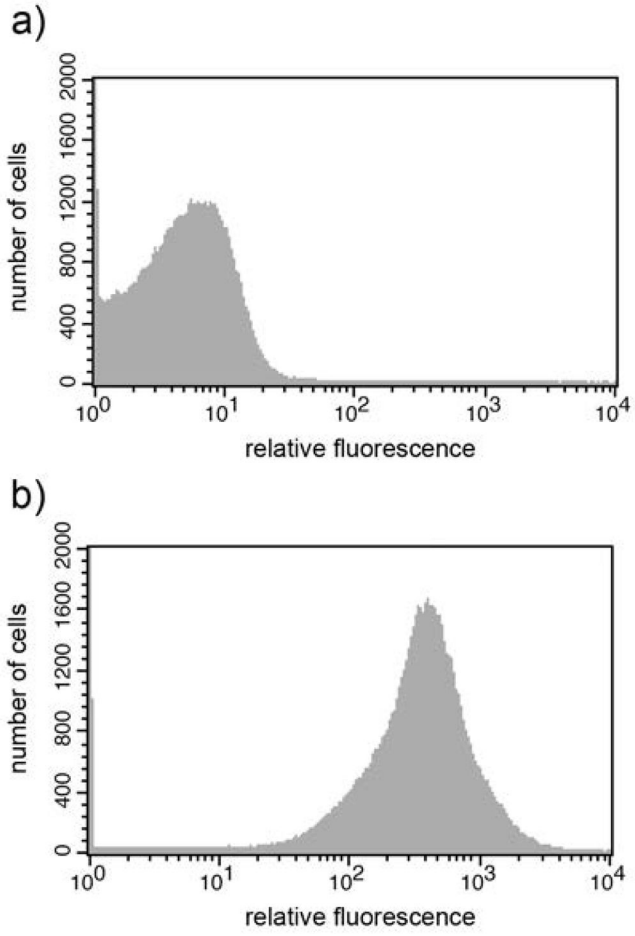

Figure 3.

FACS analysis of cell surface display of DspB as passenger domain. A) FACS histogram of Rosetta cells with pET29b and B) with pKD3. Induced cells were successively incubated with ant-rabbit DspB antibody and Alexa Fluor 488-goat anti rabbit IgG.

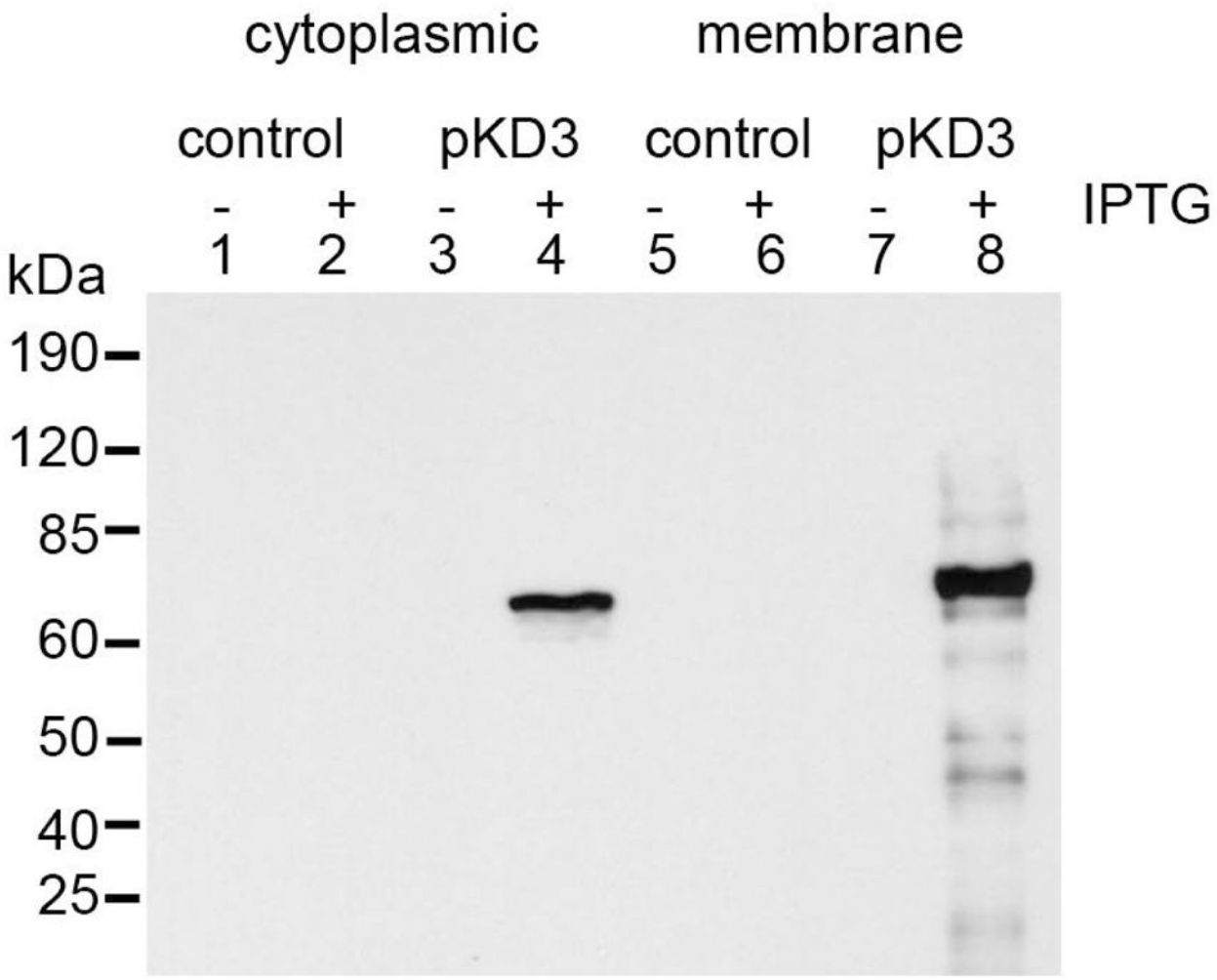

Figure 4.

Western blot analysis of DspB surface localization. The cytoplasmic as well as membrane fractions were boiled in sample buffer. The samples were run on 10% SDS-PAGE, transferred to a membrane and probed using polyclonal anti-DspB antibody. Lanes 1, 2, 5 and 6 were from control Rosetta (DE3) pLysS cells. Lanes 3, 4, 7 and 8, are from the pKD3 cells. Odd numbered lanes were not induced with IPTG (0.1 mM) whereas even numbered lanes were induced with IPTG.

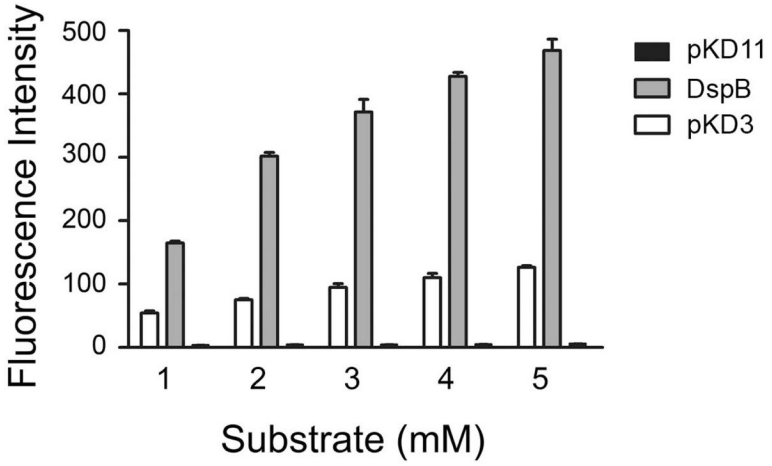

Figure 5.

Hydrolytic activity of surface displayed DspB. The hydrolytic activity of DspB was measured using 4-methylumbeliferyl-β-D-glucopyranoside as substrate in 50 mM phosphate buffer using 10 μL of cells (10^7 cfu) from a wet weight of 0.2 g/mL at pH 5.8. The control cells (pKD11) did not have any activity. The activity of purified recombinant DspB (8 μg per well) is shown for comparison. The DspB reactions were carried out for 15 min.

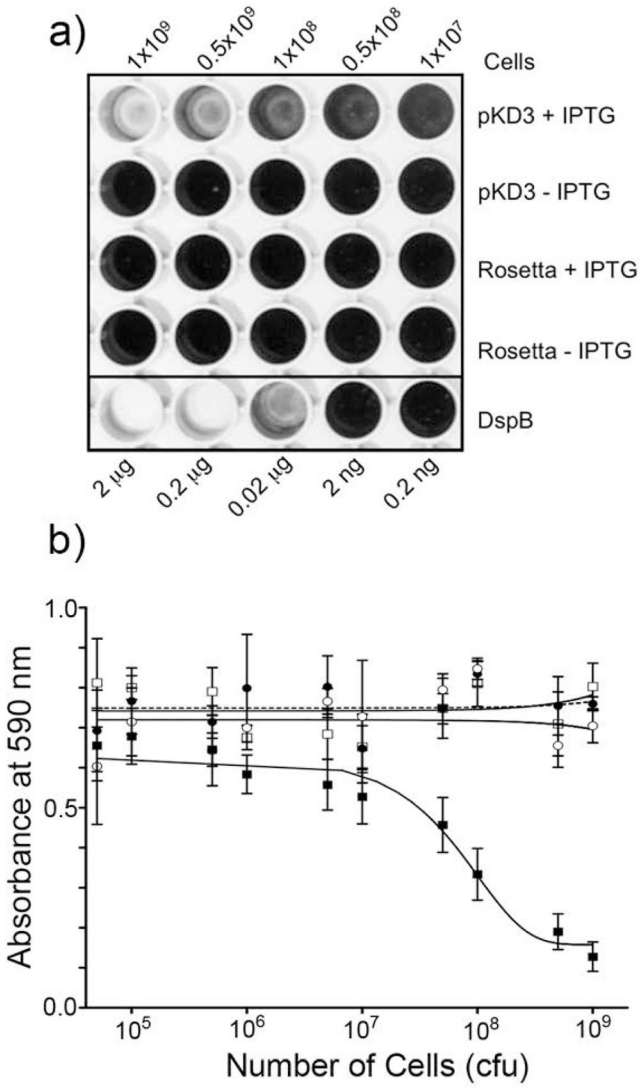

Figure 6.

a) *S. epidermidis* biofilm detachment by Rosetta (DE3) pLysS cells displaying DspB on their surface. The wells containing *E. coli* cells had a range of CFUs from 1.0×10^7 to 1.0×10^9. The bottom wells contained purified DspB. The wells were then rinsed with water and stained with crystal violet. b) OD of the rows of wells in a) corresponding to pKD3+IPTG (filled squares), pKD3-IPTG (open squares), Rosetta+IPTG (filled circles), and Rosetta-IPTG (open circles). The number of cells required for 50% detachment was estimated at 5×10^7 cells.

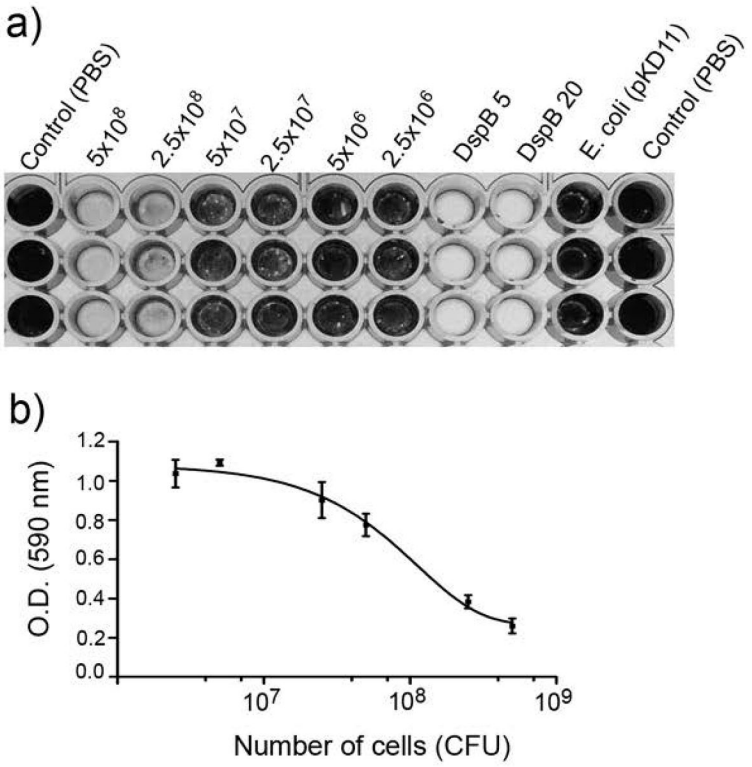

Figure 7.

a) *A. pleuropneumoniae* biofilm detachment by Rosetta (DE3) pLysS cells (pKD3) displaying DspB on their surface shown in triplicate wells. Note that the biofilm is completely detached with 5×10^8 cells after 1 hr at 37°C. Control *E. coli* cells (pKD11) did not detach biofilm while 5 μg of DspB completely detached the biofilm. b) Dose response curve using the absorbance of residual biofilm after treatment with acetic acid. The number of cells required for 50% biofilm detachment is estimated to be 10^8 cells.

Table 1

Aae hybrid-specific PCR Primers used in the study.

Name	Sequence (5′-3′)	Orientation	Restriction site*
P1	GGAATTCCATATGTGTTCTTTTTCAAAA AAGTACTGC	Forward	Nde1
P2	CGGGGTACCCGCAAATGCCGGTTGATTAAA	Reverse	Kpn1
P3	CGCGGATCCAAATTAGGCAGAACAAGTTTC	Forward	BamH1
P4	CCGCTCGAGTTACCAGTAGTAATTCAGTTTTAC	Reverse	Xho1
P5	GCGCGCGGTACCATGGCGAGCATGACCGGCGGC CAGCAGATGGGCATATATAACCCAAATGAAATT	Forward	Kpn1
P6	GCGCGCGGTACCAATTGTTGCGTAAAAGGCAATTCC	Forward	Kpn1
P7	CGCGGATCCCTCATCCCCATTCGTCTTATG	Reverse	BamH1

*Corresponds to the underlined sequence in the primers