Display of functional nucleic acid polymerase on Escherichia coli surface and its application in directed polymerase evolution

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Funding information
European Union’s Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie, Grant/Award Number: 722287

Abstract
We report a first of its kind functional cell surface display of nucleic acid polymerase and its directed evolution to efficiently incorporate 2′-O-methyl nucleotide triphosphates (2′-OMe-NTPs). In the development of polymerase cell surface display, two autotransporter proteins (Escherichia coli adhesin involved in diffuse adherence and Pseudomonas aeruginosa esterase A [EstA]) were employed to transport and anchor the 68-kDa Klenow fragment (KF) of E. coli DNA polymerase I on the surface of E. coli. The localization and function of the displayed KF were verified by analysis of cell outer membrane fractions, immunostaining, and fluorometric detection of synthesized DNA products. The EstA cell surface display system was applied to evolve KF for the incorporation of 2′-OMe-NTPs and a KF variant with a 50.7-fold increased ability to successively incorporate 2′-OMe-NTPs was discovered. Expanding the scope of cell-surface displayable proteins to the realm of polymerases provides a novel screening tool for tailoring polymerases to diverse application demands in a polymerase chain reaction and sequencing-based biotechnological and medical applications. Especially, cell surface display enables novel polymerase screening strategies in which the heat-lysis step is bypassed and thus allows the screening of mesophilic polymerases with broad application potentials ranging from diagnostics and DNA sequencing to replication of synthetic genetic polymers.

KEYWORDS
cell-surface display, directed evolution, enzyme engineering, nucleic acid, polymerase

1 | INTRODUCTION

Microbial cell-surface display (CSD) is a powerful platform to present and immobilize the protein of interest on microbial surfaces. The protein presented on the microbial surface can be freely accessed by supplemented substrates or ligands without the interference from cytosol proteins and metabolites. Yeast and bacteria are the most common hosts for which CSD have been developed (van Bloois, Winter, Kolmar, & Fraaije, 2011; Lee, Choi, & Xu, 2003). Yeast has been frequently employed for displaying enzyme (e.g., glucose oxidase, sortase, and protease) or antibody libraries. The displayed libraries can be screened later on by coupling desired protein property to fluorescent readout and screening with fluorescence-activated cell sorting (FACS; I. Chen, Dorr, & Liu, 2011; Cochran & Cherf, 2015; Mei et al., 2017;...
Ostafe, Prodanovic, Nazor, & Fischer, 2014; Yi et al., 2013. Bacterial hosts have been mainly employed for displaying polypeptides with small molecular weights, such as antigen and antimicrobial peptides (Apitius, Rübsam, Jakesch, Jakob, & Schwaneberg, 2019; van Bloois et al., 2011; Samuelson, Gunneriusson, Nygren, & Ståhl, 2002). Amongst bacterial hosts, Escherichia coli is most frequently used due to the simplicity of gene manipulation and high number of transformants (van Bloois et al., 2011). Numerous peptide libraries were displayed on E. coli surfaces and screened for desired properties such as polymer or zinc oxide binding affinity (Apitius et al., 2019; Kjaergaard, Sørensen, Schembri, & Klemm, 2000; Samuelson et al., 2002). During recent years, a few autotransporter proteins have been reported to transport large enzymes to E. coli surface (van Bloois et al., 2011). The translocator domain of adhesin involved in diffuse adherence (AIDA-E) from Pseudomonas aeruginosa 2787 (O126:H27) was reported to successfully transport a 54-kDa cytochrome P450 (Queh, Schüürmann, Hollender, & Jose, 2017) and a 63-kDa recombinant protein comprising organophosphorus hydrolase and green fluorescent protein (Li et al., 2008). The translocator domain of an outer membrane (OM)-anchored esterase A (EstA) from Pseudomonas aeruginosa was reported to display active lipases with up to 62 kDa (Becker et al., 2005). However, to the best of our knowledge, neither bacteria nor yeast has been reported to successfully display nucleic acid (NA) polymerases. NA polymerases frequently used in biotechnological applications are large in size (generally between 65 and 100 kDa; Aschenbrenner & Marx, 2017) and might be more challenging to be translocated onto the microbial surface. Polymerase evolution campaigns and corresponding screening technologies are of high interest for polymerase chain reaction (PCR) and DNA sequencing-dependent applications, such as diagnostics, nucleic acid therapeutics, forensic DNA analysis, paleogenetics, epigenetics, DNA digital data storage, and the replication of synthetic genetic polymers (Aschenbrenner & Marx, 2017; D’Abbadie et al., 2007; Dien, Holcomb, & Romesberg, 2019; Houllihan, Arangundy-Franklin, & Holliger, 2017). Responding to the growing application scope of polymerases, increasing selection approaches for polymerase mutant libraries have been reported. The approaches include microtiter plate (MTP)-based methods (e.g., primer extension assays and real-time PCR; Huber, Von Watzdorf, Klenow & Henniger, 2011), water–oil emulsion-based methods, phage display (Jestin, Kristensen, & Winter, 1999; Xia et al., 2002), and in vivo strategies such as phage-assisted continuous evolution (Esvelt, Carlson, & Liu, 2011). Plate-based methods generally enable direct isolation of individual improved variants and have a throughput of a few thousand clones per round (Bornscheuer, Hauer, Jaeger, & Schwaneberg, 2019). Phage-display-based strategies have often been used to screen polymerase library for increased thermostability and processivity, and have been employed for displaying thermophilic polymerases, yet due to the requirement of a heat-lysis step in the three methods, they might be less suitable for evolving mesophilic polymerases.

2.1 | Materials and Methods

Chemical reagents and solvents with an analytical grade or higher purity were purchased from either Sigma-Aldrich (Hamburg, Germany), AppliChem (Darmstadt, Germany), or Carl Roth (Karlsruhe, Germany). Enzymes were all purchased from New England
Biolabs (Frankfurt, Germany) or Fermentas (St. Leon-Rot, Germany). All oligonucleotides used in this study were purchased from Integrated DNA Technologies (Leuven, Belgium). V/flat-bottom polystyrene (PS) 96-well MTPs and flat-bottom black chimney well 96-well MTPs were purchased from Greiner Bio-One GmbH, (Fricke-nhausen, Germany). Both Plasmid Purification Kit and gel and PCR Purification Kit were purchased from MACHEREY-NAGEL (Düren, Germany). The cell membrane-impermeable DNA intercalating fluorescent dye (EvaGreen™) was purchased from Biotium, Inc. (California). The lysogeny broth (LB) medium used for cloning and precultures consisted of 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl. In the case of LB agar plates, 20 g/L agar was added. The TBMg10 medium used for expressions comprised 12 g/L peptone, 24 g/L yeast extract, 4 g/L glycerol, 2.31 g/L KH2PO4, 12.5 g/L K2HPO4, and 10 mM MgSO4. The phosphate-buffered saline (PBS) comprised 140 mM NaCl, 10 mM KCl, 6.4 mM Na2HPO4, 2 mM KH2PO4. A 1X PolCSD buffer consists of 50 mM Tris–HCl (pH 7.1), 10 mM (NH4)2SO4, 10 mM KCl, 1.5 mM MgCl2, and 20% glycerol. A 10X Pol buffer consists of 500 mM Tris–HCl (pH 7.1), 100 mM (NH4)2SO4, 100 mM KCl, and 15 mM MgCl2.

2.2 | Cloning

2.2.1 | The AIDA-I-E-KF construct

An AIDA-I autotransporter construct (including a leading sequence, an E-epitope, a linker G3SGGS(G3)3, a β1 extracellular domain, and a translocator domain of AIDA-I) was synthesized and cloned to the expression vector pALXtreme1a (Blanusa, Schenk, Sadeghi, Marienhagen, & Schwaneberg, 2010) using XbaI and HindIII restriction sites (Table S5). The gene encoding E. coli KF was amplified from the genome of E. coli DH5α using P_KF1 and P_KF2 primers (Table S1). The PCR was carried out according to the general PCR protocol (Table S4; with a 5 min initial cell lysis and DNA denaturation step) and E. coli DH5α cells were used as a template. KF was then inserted between the leading sequence and the E-epitope of the AIDA-I autotransporter construct using Nhel and BamHI restriction sites to generate pALX1a-AIDA-I-E-KF (see Supporting Information).

2.2.2 | The EstA-E-KF construct

An EstA autotransporter construct comprising a leading sequence, a passenger peptide, a 10-Alanine spacer, an E-epitope, an inactivated EstA extracellular domain, and an EstA translocator domain was kindly provided by Dr. Kristin Rübsam. A PCR using P_EstA1 and P_EstA2 primers (Table S1) was performed on the EstA autotransporter construct to replace the passenger peptide with a flexible linker (G3)3. The modified EstA autotransporter construct was then amplified by PCR using P_EstA3 and P_EstA4 primers and then cloned into the expression vector pALXtreme1a (Blanusa et al., 2010) by Ndel and HindIII restriction sites to generate pALX1a-EstA-E (Table S5). Finally, the gene encoding E. coli KF was amplified from the genome of E. coli DH5α using P_KF1 and P_KF2 primers (Table S1) and then inserted between the leading sequence and the linker of the EstA autotransporter construct using Nhel and KpnI restriction sites to generate pALX1a-EstA-E-KF (see Supporting Information).

2.3 | Expression

pALX1a-AIDA-I-E-KF and pALX1a-EstA-E-KF were transformed into E. coli BL21 DE3 LacIq and plated on LB agar containing 50 µg/ml kanamycin. The resulted single colonies on the plates were used to inoculate 150 µL LBkan in a 96-well MTP (PS, F-bottom) as a pre-culture. This preculture was cultivated at 30 °C for 18–22 hr. Ten microliters of the preculture were then used to inoculate 150 µL TBMg10kan in an MTP (PS, V-bottom) as the main culture. The main culture was cultivated at 37 °C for 90 min, and then induced with 0.2 mM isopropyl β-thiogalactopyranoside (IPTG). Cultivation of the main culture was subsequently continued at 18 °C for 18 hr.

2.4 | Immunofluorescence staining of E. coli cells displaying KF

The E-epitope in the EstA-E and EstA-E-KF recombinant constructs enables the staining of cells expressing the two recombinant proteins using a fluorescein isothiocyanate (FITC)-conjugated anti-E antibody. A concentration of 83.3 µL of cells (OD600 = 6) was centrifuged at 3,250g, 4 °C, for 2 min and then the resulting supernatant was removed. The resulting pellet was resuspended in 500 µL ice-cold PBS and centrifuged at 3,250g, 4 °C for 2 min. The resulting supernatant was again removed, and the pellet was resuspended in 10 µL of 100-fold-diluted antibody solution (E-epitope antibody conjugated with FITC; Novus Biologicals). The cell-antibody suspension was incubated in the dark at room temperature (RT) for 75 min and then further suspended in 1 mL PBS. The suspension was subsequently centrifuged at 3,250g, 4 °C for 2 min and then the resulting supernatant was removed. The resulting cell pellet was resuspended in 50 µL of PBS and then transferred into a black 96-well MTP (PS, F-bottom, chimney well) for analysis. Tecan Infinite M1000 96-well plate reader (Tecan Austria GmbH, Salzburg) was used for detection at excitation/emission wavelength 490/525 nm. Three biological replicates were performed for each strain.

2.5 | Activity assay for cell-surface-displayed KF

A concentration of 83.3 µL of the cells (OD600 = 6) expressing EstA-E-KF and AIDA-I-E-KF were harvested by centrifugation at 3,220g, for 45 s at 4 °C. The resulting cell pellets were resuspended in 37.5 µL 1X PolCSD buffer with gentle pipetting. Each 75 µL reaction consists of 37.5 µL of KF-displaying cells and 37.5 µL of reagent mix. The reagent mix comprises 100 µM deoxyribonucleotide triphosphates (dNTPs), 12 µL of 20X DNA intercalating fluorescent dye, and 2 µM...
prehybridized primer templates (primed template) in 1X PolCSD buffer. The primed template comprises a 49-nt template DNA (Table S2, Temp_49) and a 10-nt primer DNA complementary to the first 10 nt of the template DNA (Table S2, Pri_10). Each 75 µl reaction is incubated in black 96-well MTP (PS, F-bottom, chimney well) at 37°C. The fluorescence signal was detected with Tecan Infinite M1000 96-well plate reader at excitation/emission wavelengths 500/530 nm. Three biological replicates were performed for each strain.

2.6 | OM fractionation

The cells expressing AIDA-I-E, AIDA-I-E-KF, EstA-E, and EstA-E-KF were resuspended in 20 mM Tris buffer with 100 mM NaCl (pH 8) and then sonicated at 40% amplitude for 5 min with 15 s on and 15 s off intervals. The sonicated cell lysate was subsequently centrifuged at 3,225 g, 4°C for 1 hr, and the resulting supernatant was centrifuged at 100,000 g, 4°C for 30 min. The resulting pellet was resuspended in PBS containing 0.01 mM MgCl₂, 2% Triton X-100, and incubated at RT for 30 min. Finally, the suspension was centrifuged at 100,000 g, 4°C for 30 min to precipitate the OM fraction. The resulting pellet was resuspended in PBS for further analysis (Grimm et al., 2018).

2.7 | Library generation

For each library, two fragments were amplified from pALX1a-EstA-E-KF by PCR (general PCR protocol, Tables S4 and S8) using primers listed in Table S3. The PCR-amplified fragments were then column purified. Fragments Nos. 1 and 2 were assembled to generate the EstA-E-KF I709E E710SSM library using NEBuilder DNA Assembly Kit (New England Biolabs; Table S6, Figure S9). The PCR-amplified Fragments Nos. 3 and 4 were assembled to generate the EstA-E-KF I709SSM E710G library using NEBuilder DNA Assembly Kit (New England Biolabs; Table S6, Figure S9).

2.8 | CSD-based screening system for KF

EstA-E-KF I709SSM E710G and EstA-E-KF I709E E710SSM libraries were transformed into E. coli BL21 DE3 LacF21 and plated on LB agar containing 50 µg/ml kanamycin. The resulted single colonies on the plates were used to inoculate 150 µl LBkan in a 96-well MTP (PS, F-bottom) as a preculture. This preculture was cultivated at 30°C for 18–22 hr. A concentration of 10 µl of the preculture was then used to inoculate 150 µl TBMg10kan in an MTP (PS, V-bottom) as the main culture. The main culture was cultivated at 37°C for 90 min, and then induced with 0.2 mM IPTG. Cultivation of the main culture was subsequently continued at 18°C for 18 hr. The cells expressing EstA-E-KF variants were then harvested by centrifugation at 3,220g for 45 s at 4°C. The resulting cell pellets were resuspended in 37.5 µl 1X PolCSD buffer with gentle pipetting. The remaining procedure is identical as described in Section 2.5.

3 | RESULTS

Results on the development of the E. coli display system for directed polymerase (KF) evolution are summarized in three parts. In the first part, two autotransporter systems AIDA-I and EstA were assessed for their ability to display KF. CSD of active KF was achieved for the EstA autotransporter system, which was selected to establish a CSD-based screening system for KF-polymerase evolution. In the second part, the stability and applicability of the CSD-based screening system were validated in a semirational protein engineering campaign by screening KF libraries for synthesizing 2'-OMe modified DNA. The conceptual scheme of the screening system is shown in Figure 3. In Section 5, a variant that showed a 50.7-fold increased fluorescence signal for 2'-OMe-NTP incorporation was obtained.

3.1 | CSD of functional KF in E. coli

To display active KF on E. coli surface, we fused the C-terminal of KF to either AIDA-I or EstA autotransporter systems (Figure 1a). The AIDA-I construct consists of a flexible G₄S₅G₃(G₄S)₃ linker, a β₁ extracellular domain, and a translocator domain. The β₁ extracellular domain was reported to assist the folding of the native passenger (Berthiaume, Rutherford, & Mourez, 2007), and a construct including the flexible G₄S₅G₃(G₄S)₃ linker and the β₁ extracellular domain was reported to successfully display a 54-kDa cytochrome P450 (Quehl et al., 2017). EstA consists of a (G₄S)₃A₁₀ linker, an inactivated native passenger, and a translocator domain (Grimm et al., 2018; Rubsam, Weber, Jakob, & Schwaneberg, 2018). An EstA construct including the inactivated native passenger domain was reported to successfully display a 62-kDa lipase (Becker et al., 2005). A (G₄S)₃A₁₀ linker was designed because an A₁₀ spacer was reported to facilitate the spatial separation of the E-epitope to its fusion protein partner (Rubsam et al., 2018). A flexible (G₄S)₃ linker, which was reported to improve the activity of displayed P450 reductase (Quehl et al., 2017), was further added to facilitate a freer movement of the displayed KF.

Subsequently, we examined the expression, the localization, and the activity of displayed KF by each autotransporters. The whole-cell lysate and the OM fraction of cells expressing AIDA-I-E, AIDA-I-E-KF, EstA-E, and EstA-E-KF was analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; Figures S1 and S2). The OM fractions were further analyzed by western blot analysis (Figure S2). Defined bands at the estimated sizes of the four recombinant proteins were observed in the whole-cell lysates and the OM fractions, indicating that all recombinant proteins were present in the OM of the host E. coli. To validate the presence of the passenger domain (E-epitope and KF) outside of the cell, a fluorophore-conjugated anti-E antibody was mixed with the cells expressing AIDA-I-E, AIDA-I-E-KF, EstA-E, or EstA-E-KF. The obtained fluorescence signal after washing directly correlates to the expressed E-epitope numbers. Cells expressing AIDA-I-E, AIDA-I-E-KF, EstA-E, or EstA-E-KF showed a 35.7-, 8-, 8.5-, or 2.3-fold increased fluorescence signal compared to cells harboring only the empty vector...
The results confirmed a successful translocation of the passenger domain on *E. coli* surface by both AIDA-I and EstA autotransporter systems. Subsequently, the activity of the displayed KF in both autotransporter systems was validated using prehybridized primer and template (primed template) in conjunction with dNTPs and a DNA intercalating fluorescent dye (Shoute & Loppnow, 2018). Supposing the displayed KF catalyzes primer extension, more dye intercalates into the extended DNA and results in an increased fluorescence signal (Figure 1b). Although the dye is reported to be impermeable to cell membranes, therefore does not interact with intracellular DNA (Shoute & Loppnow, 2018), separate control measurements consisting of cells, dNTPs, and the dye were performed to determine the fluorescence background (Figures S5a and S5c). The fluorescence signals derived from the DNA products synthesized by displayed KF in the AIDA-I system was, therefore, "negative" throughout the measurement after subtracting its high background and normalizing to the signal of the empty vector (Figure 2b). Furthermore, gradual increases of background fluorescence were detected for cells harboring empty vector and the cells expressing EstA-E and EstA-E-KF throughout the measurement with the cells expressing EstA-E-KF showing slightly higher background than empty vector and EstA-E (Figure S5c). Despite the difference in backgrounds, the fluorescence signal derived from the DNA products synthesized by displayed KF in the EstA system remained generally stable for at least 25 min after reaching its plateau, therefore ensured a stable genotype–phenotype linkage for screening applications (Figure S6d). As this is a catalytic reaction that should continue until all templates have been extended, the DNA synthesis catalyzed by the displayed KF in the EstA system was successfully detected as the increase of fluorescence readout, and a plateau of $\sim 1.3 \times 10^4$ relative fluorescence units (RFU) was reached after 15 min (Figure 2d and Figure S5c,d). The results confirmed that in the EstA system, the 68-kDa KF retained its DNA polymerase activity after being translocated from cytosol to cell surface, and, thus represents the first successful display of an active polymerase on the *E. coli* cell surface. Notably, the entire passenger domain of the EstA-E-KF recombinant protein (native EstA passenger-linker-E-KF) possesses an
estimated size of 105.7 kDa, which is more than three times of the size of the native passenger domain of EstA (31.9 kDa). The OM fractions of the cells expressing EstA-E-KF were further quantified in Figures S3 and S4. An average of 6,851 EstA-E-KF molecules was expressed per cell, with an approximate batch-to-batch variation of 536 molecules.

### 3.2 A CSD-based screening system for evolving KF for efficient incorporation of 2′-OMe-NTPs

Using the CSD of the polymerase, we established a screening system for mesophilic KF and applied it for the directed evolution of KF to efficiently incorporate 2′-OMe-NTPs in DNA synthesis (Figure 3). The cells expressing EstA-E-KF recombinant protein were supplemented with the DNA intercalating fluorescent dye, primed template, dNTPs, or 2′-OMe-NTPs before the incubation at 37°C and the measurement of the fluorescence intensity in 96-well MTP formats. The screening system reveals two characteristics of polymerase-catalyzed primer extension reactions: the increase of fluorescence intensity per unit time (slope) and the fluorescence intensity after the reaction reaches a plateau. The slope indicates the intercalating rate of the fluorescent dye into the newly synthesized DNA, and, therefore, correlates to the rate of nucleotide incorporation. The measured fluorescence intensity after the reaction reaches plateau indicates the completeness of primer extension or the polymerase’s ability to incorporate nucleotide successively. Preliminary
experiments suggested that 2′-OMe-NTPs caused KF to perform incomplete primer extensions and resulted in significantly reduced fluorescence readout after the reaction reached a plateau. Therefore, the ability to incorporate nucleotides successively despite incorporating 2′-OMe-NTPs was selected as the screening criteria for KF variants, and the fluorescence intensity after the reaction reaches plateau was measured in each well of the MTP. Furthermore, a mutant EstA-E-KF (KF D705A E710A) with abolished polymerase activity was generated as the negative control for the screening system (Shinkai, Patel, & Loeb, 2001). Before screening mutant libraries, the coefficient of variation of CSD-based screening system in a whole 96-well MTP was determined. The apparent coefficient of variation of the measured fluorescence intensity was 9.0%. After subtraction of the negative control, a true coefficient of variation <15% is routinely employed in successful directed evolution campaigns (Cheng, Schwaneberg, Cheng, & Zhu, 2015; Wong, Wu, Roccatano, Zacharias, & Schwaneberg, 2005), the screening system was, therefore, considered suitable for the directed KF-polymerase evolution.

Two site-saturation mutagenesis (SSM) libraries of KF at positions 709 and 710, respectively, were screened for improved synthesis of 2′-OMe modified DNA to validate the CSD-based screening system. Position I709 was reported to support sugar discrimination (Shinkai et al., 2001) and position E710 was reported to be involved in the exclusion of ribonucleotide 5′-triphosphate (rNTP) via steric hindrance (Astatke, Ng, Grindley, & Joyce, 2002). Furthermore, sequence and structural alignment revealed that positions 709 and 710 are in consensus with the Taq positions 614 and 615 (Beese, Derbyshire, & Steitz, 1993; Li, Korolev, & Waksman, 1998). Taq positions 614 and 615 were reported to facilitate 2′-OMe incorporation when mutated to glutamate and glycine, respectively (T. Chen et al., 2016; Fa et al., 2004).
In the first library, we simultaneously mutated position I709 to glutamate and saturated position 710 with all 20 canonical amino acids using degenerate nucleotide codon “NNK” (N = A or T or C or G; K = G or T). In the second library, we simultaneously mutated position E710 to glycine and saturated position 709 with all 20 canonical amino acids using degenerate nucleotide codon “NNK.” To ensure that all 20 possible amino acid variants are present in randomly picked and screened clones, 168 clones per library were screened employing the CSD-based screening system (theoretical diversity coverage >94%; Firth & Patrick, 2008). In the first library, 44 clones showed a fluorescence readout of >150% of wild-type and 10 clones had a 190–250% improvement. After sequencing of 10 clones, glycine was found in the eight best clones (215–250% of wild-type); aspartate (191% of wild-type), as well as glutamate (203% of wild-type), were found once. In the second library, only six clones showed a fluorescent readout of >150% of wild-type KF. Sequencing of the three best clones (>170% of wild-type KF) revealed a preferred substitution from isoleucine to glutamate (>200% of wild-type KF) and a substitution from isoleucine to aspartate (170% of wild-type KF; Table 1) at position 709. In summary, KF I709E E710G was shown to be the most preferred variant found by CSD-based screening system in both libraries and the variant KF I709E E710G was subjected to further characterization.

3.3 | Characterization of KF I709E E710G

To characterize the polymerase’s ability to successively incorporate 2′-OMe-NTPs, genes encoding KF* (mutant KF with abolished polymerase activity), wild-type KF, and KF I709E E710G were amplified from their CSD construct, cloned into an expression vector, expressed, and purified. A concentration of 0.35 µM of purified KF*, wild-type KF, or KF I709E E710G were supplied in a primer extension reaction comprising the DNA intercalating fluorescent dye, primed template, dNTPs, or 2′-OMe-NTPs at 37°C. Consistent with the criteria in the screening section, we defined the polymerase’s ability to incorporate nucleotide successively as the measured fluorescence readout after the reaction reaches a plateau. When dNTPs were supplied in a primer extension reaction, purified wild-type KF and KF I709E E710G reached comparable fluorescence readout of (1.47 ± 0.03) × 10⁴ RFU and (1.52 ± 0.02) × 10⁴ RFU, respectively, after normalizing to the signal of KF* (Figure 5b). Comparable fluorescence readout suggested that wild-type KF and KF I709E E710G achieved similar completeness of primer extension, thus have similar ability to incorporate nucleotide successively when their natural substrates were supplied. When dTTP, dATP, dCTP, and 2′-OMe-GTP were supplied, wild-type KF and KF I709E E710G reached fluorescence readout of (8.76 ± 1.49) × 10² and (8.68 ± 0.19) × 10³ RFU, respectively, after normalizing to the signal of KF* (Figure 5c). A 9.9-fold increase of fluorescence readout was achieved by KF I709E E710G compared to wild-type KF, suggesting that KF I709E E710G not only incorporated 2′-OMe-GTP but also continued the primer extension more efficiently than wild-type KF. Despite the improvement, complete primer extension was not achieved by KF I709E E710G. The fluorescence readout detected when supplying dTTP, dATP, dCTP, and 2′-OMe-GTP was 43% less than supplying natural substrates (dNTPs). In addition, we analyzed the ability of purified KF I709E E710G to incorporate all four 2′-OMe-NTPs (Figure 5d). Direct comparisons are biased because complete 2′-OMe modification of one DNA strand reduces the measured fluorescence intensity by ~25% (Figure S7). Consequently, the measured fluorescence values were normalized to the signal of KF* and then multiplied by 1.33 before comparison. The fluorescence readout achieved by wild-type KF and KF I709E E710G after incorporating 2′-OMe-NTPs were (1.49 ± 0.56) × 10² RFU and (7.56 ± 0.36) × 10³ RFU, respectively. Compared to wild-type KF, KF I709E E710G achieved a 50.7-fold increase of fluorescence readout when all four 2′-OMe-NTPs were used in primer extension experiments exclusively. The latter suggested that KF I709E E710G is not only able to incorporate 2′-OMe-GTP but also the other three 2′-OMe-dNTPs with much greater efficiency than wild-type KF. To directly evaluate the variant’s ability to incorporate nucleotide successively, we repeated the primer extension reaction with fluorophore-conjugated primer and analyzed the product by denaturing polyacrylamide gel electrophoresis (urea PAGE; Figure 6). Lanes 1–4 show the primer extension with dNTPs and KF* (Lane 2), wild-type KF (Lane 3), KF I709E E710G (Lane 4), or without polymerase (Lane 1). Lanes 5–8 show the primer extension reaction with dATP, dTTP, dCTP, 2′-OMe-GTP, and KF* (Lane 6), wild-type KF (Lane 7) KF I709E E710G (Lane 8), or without polymerase (Lane 5). Lanes 9–12 show the primer extension reaction with 2′-OMe-NTPs and KF* (Lane 10), wild-type KF (Lane 11),

### Table 1

| Library No. | Description | Mutation (DNA codon) | Substitution (amino acid) |
|-------------|-------------|----------------------|---------------------------|
| 1           | SDM I709E + SSM E710 | GAA/GGG | Glu710Gly (E710G) |
|             |             | GAA/GGG | Glu710Gly (E710G) |
|             |             | GAA/GGG | Glu710Gly (E710G) |
|             |             | GAA/GGC | Glu710Gly (E710G) |
|             |             | GAA/GGT | Glu710Gly (E710G) |
|             |             | GAA/GGT | Glu710Gly (E710G) |
|             |             | GAA/GGG | Glu710Gly (E710G) |
|             |             | GAA/GGT | Glu710Gly (E710G) |
|             |             | GAA/GAG | Glu710Glu (E710E) |
|             |             | GAA/GAT | Glu710Asp (E710D) |
| 2           | SDM E710G + SSM I709 | ATT/GAA | Ile709Glu (I709E) |
|             |             | ATT/GAA | Ile709Glu (I709E) |
|             |             | ATT/GAT | Ile709Asp (I709D) |

Abbreviation: 2′-OMe-NTP, 2′-O-methyl nucleotide triphosphate.
KF I709E E710G (Lané 12), or without polymerase (Lané 9). When KF* was present, the primers were partially digested by the enzyme’s remaining 3′–5′ exonuclease activity. When dNTPs were supplied, both wild-type KF and KF I709E E710G achieved “full-length synthesis.” When dATP, dTTP, dCTP, and 2′-OMe-GTP was incorporated (see the sequence of primed template, Figure 6a) whereas KF I709E E710G continued to extend the primer after 2′-OMe-GTP incorporation. When 2′-OMe-NTPs were supplied, the exonuclease activity of wild-type KF outperformed its primer-extension efficiency, and as a result, partially digested primers were obtained. KF I709E E710G extended the primers for at least two nucleotides without any observable primer degradation (Figure 6 and Figure S10). The urea PAGE analysis served a direct confirmation of KF I709E E710G’s ability to incorporate 2′-OMe-NTPs and achieve an efficient primer extension. The results also verified that the activity measured by the CSD-based screening system is transferable to purified enzymes.

4 | DISCUSSION

Expanding polymerase functions to efficiently read, copy, and write a broad variety of NA analogs are of high interest for a wide scope of biotechnological and medical applications. For instance, 2′-OMe-modified nucleotides are commonly incorporated in nuclease-resistant NA aptamers. Aptamers are used as therapeutics and in diagnostics with an estimated market size between US $0.7 and $9.1 billion by 2025 or 2026 according to different market analyses (AnalystView Market Insights, 2017; Data Bridge Market Research, 2018; Transparency Market Research, 2018). Being able to use the CSD system for directed polymerase evolution not only extends the scope of cell-surface displayable enzymes to the realm of polymerases but also expands the repertoire of polymerases (e.g., mesophilic polymerase) that we can evolve under conditions (e.g., pH and buffer) close to targeted applications. Being close to application conditions is a prerequisite for successful directed evolution campaigns (Ruff et al., 2013). By genetic fusions to EstA autotransporter,
E. coli can display the 68-kDa KF on the cell surface. The localization of KF was verified by SDS-PAGE and western blot analysis of the host cell's OM fraction and immunostaining of E-epitope. The activity of displayed KF was verified in primer extension reactions with a fluorescence readout (Figures 2b and 2d). A true coefficient of variation of 14.3% was obtained for the CSD-based polymerase screening system in 96-well plate formats (Figure 4), which proved to be sufficient for a successful directed KF evolution campaign (Cheng et al., 2015; Wong et al., 2005). Finally, a double KF variant (I709E E710G) was obtained. The double variant showed for 2′-OMe-GTP incorporation a 9.9-fold fluorescence readout in comparison to wild-type KF. In case that all four 2′-OMe-NTPs were exclusively used instead of dNTPs, the double variant showed in comparison to wild-type KF a 50.7-fold improved fluorescence readout, which directly correlates to the polymerase's ability to incorporate nucleotide successively.

In western blot analysis of the OM fraction of cells expressing AIDA-I-E-KF and EstA-E-KF, some truncated proteins of different sizes were detected (Figure S2b, Lanes 2, 3, and 4). If these truncations occurred between the translocator domain and KF, this could lead to an overestimation of KF surface display efficiency using anti-E antibody. However, considering the incomplete transfer of the full-length proteins due to their large size (Figure S2c, Lanes 2 and 4), the amount full-length proteins were significantly abundant compared to the amount of truncated proteins (Figure S2b, Lanes 2 and 4). Therefore, despite the presence of truncated proteins that could lead to a slight overestimation of KF surface display efficiency, most KF were correctly displayed and could be verified qualitatively by the anti-E antibody. For a quantitative study, an epitope tag at the end of KF can be used in the future.

The first part of the result showed that cells expressing AIDA-I-E-KF exhibited a significant increase of permeability to the DNA intercalating fluorescent dye comparing to AIDA-I-E and empty vector, suggesting a compromised cell membrane integrity due to the expression of AIDA-I-E-KF (Figure S5a). The result resonates with the study of Van Gerven, Sleutel, Deboeck, De Greve, and Hernals-teens (2009) who reported that the surface display of a 30-kDa receptor-binding domain of the F17a-G fimbrial adhesin by AIDA-I autotransporter permeabilized E. coli cells. In contrast to the former, no membrane permeabilization was reported when other larger proteins (34, 36, 54, and 63 kDa) were displayed by AIDA-I autotransporter system, suggesting a general lack of correlation between the host membrane permeabilization and the size of the passenger protein (Andersson, Persson, Ståhl, & Löfblom, 2019; Li et al., 2008; Quehl et al., 2017). Van Gerven et al. (2009) also reported that the sole expression of the AIDA-I translocator domain did not alter the...
viability of the cells. In this study, the cells expressing AIDA-I-E were increasingly permeable to DNA, the intercalating fluorescent dye comparing to cells harboring empty vector throughout the measurement (Figure S5a). Since the 13-residue E-epitope is rather short and has not been reported to hinder membrane integrity, the observed membrane permeabilization in cells expressing AIDA-I-E was likely caused by the AIDA-I translocator domain.

The second and third parts of the results confirmed the important role of positions 709 and 710 of KF in accepting incoming nucleotides with 2'-modifications. Positions 709 and 710 lie in a highly conserved region (DYSQELR) of motif A in prokaryotic family A polymerases (Patel, Suzuki et al., 2001; Sattar et al., 2004). The corresponding residue of position 709 in Taq has been reported to be involved in forming a hydrophobic pocket that binds to the base and the sugar part of the incorporating nucleotide (Patel, Kawate, Adman, Ashbach, & Loeb, 2001). The bulky side chains of position 710 have been identified to act as a “steric gate” for sugar discrimination of incoming nucleotides (Astatke et al., 2002; Brown & Suo, 2011). The substitution from glutamate to glycine at position 710 likely reduced the “steric gate” and enlarged the active site for the insertion of 2'-OMe nucleotides (Astatke et al., 2002; Fa et al., 2004). Interestingly and in contrast to literature data, already I709E E710D and I709E alone enabled a significant degree of 2'-OMe-NTPs acceptance (186% and 182% of wt in the screening system), E710D alone has a negligible effect on rNTP incorporation (Shinkai et al., 2001) which suggests that I709E alone facilitated the 2'-OMe-NTPs acceptance without altering the E710 steric gate in KF.

Polymerase CSD provides a novel tool for designing advanced polymerase screening strategies and enables efficient polymerase evolutions close to application conditions such as pH and buffer composition. Furthermore, the polymerase CSD system can potentially be linked to FACS readout by encapsulating single cells displaying polymerase variants in droplets together with its substrates using the water-in-oil-in-water double emulsion method reported by Larsen et al. (2016). In most emulsion-based polymerase screening or enrichment strategies, the cells expressing polymerase variants intracellularly are disrupted by heat lysis to allow the interaction between the polymerases and the substrates and the enrichment of the signal. Combining polymerase CSD with emulsion-based technologies circumvents the need for heat lysis, thus potentially allows ultrahigh throughput enrichment of mesophilic polymerases by FACS. Furthermore, polymerase CSD enables the recovery of the enriched variants easily by culturing sorted cells, circumventing the PCR amplification, and the cloning steps between two enrichment rounds. In the future, a build-in reporter for the surface expression level of individual cells might be interesting when incorporating the polymerase CSD to the emulsion-based methods and screening with FACS. This could be established by inserting a reporter protein (e.g., mCherry fluorescent protein) between the EstA translocator domain and the KF, which might, however, decrease the overall surface expression efficiency due to the increased size of the passenger protein. In this case, removing the native extracellular domain in the surface expression construct could reduce the size of the passenger protein and might rescue the display efficiency (Figure S14).

5 | CONCLUSION

A CSD platform based on the EstA autotransporter was found to be suitable to display a 68-kDa KF and can likely be applied to other polymerases. The applicability and stability of the platform were validated in a directed evolution campaign to significantly improve the incorporation of 2'-OMe modified nucleotides. 2'-OMe modified nucleotides are broadly applied in the design of nuclease resistant aptamers which are used as therapeutics and in diagnostics. In essence, this study provides a technology platform that expanded the scope of cell-surface displayable enzymes to the realm of (mesophilic) polymerases. The extracellular localization of target polymerases circumvents the otherwise inevitable heat-lysis step in numerous polymerases screening strategies, allowing a more efficient screening close to application conditions, especially for mesophilic polymerases.

ACKNOWLEDGMENTS

This study was supported by the European Union’s Horizon 2020 Research and Innovation Programme under the Marie Skłodowska-Curie Action Predictive and Accelerated Metabolic Engineering (Grant Agreement No. 722287). The EstA autotransporter construct comprising a leading sequence, a passenger peptide, an E-epitope, and EstA was kindly provided by Dr. Kristin Rübsam. The authors would like to thank Dr. Zhi Zou for providing helpful advice on experiment design, manuscript writing, and submission. Open access funding enabled and organized by Projekt DEAL.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Conceptualization: M. E. C. and U. S. Methodology: M. E. C., U. S., and A. K. Formal analysis and investigation: M. E. C. Writing—Original draft preparation: M. E. C. Writing—Review and editing: M. E. C., K. G., D. S., and U. S. Funding acquisition: D. S. Supervision: K. G., D. S., and U. S.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Chung M-E, Goroncy K, Kolesnikova A, Schönauer D, Schwaneberg U. Display of functional nucleic acid polymerase on *Escherichia coli* surface and its application in directed polymerase evolution. *Biotechnology and Bioengineering*. 2020;117:3699–3711. https://doi.org/10.1002/bit.27542