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Activity-based selection of a proteolytic species using ribosome display

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ABSTRACT

We have examined the potential of displaying a protease species *in vitro* using ribosome display and demonstrate specific capture on the basis of its catalytic activity. Using a model bacterial cysteine protease, sortase A (SrtA), we show that this enzyme can be functionally expressed *in vitro*. By overlap PCR we constructed ribosome display templates with the SrtA open reading frame fused to a C terminal glycine–serine rich flexible linker and a tether derived from eGFP. Using the broad range cysteine protease irreversible inhibitor E-64 linked to acrylic beads, we show that we can isolate SrtA ribosome display ternary complexes, and recover their encoding mRNA by RT-PCR. This recovery was lost when applied to a SrtA catalytically inactive mutant, or could be alleviated by competition with free inhibitor. This sensitive technique could be further developed to allow the screening of proteases against putative inhibitors and/or the identification of novel proteolytic species.

Proteases represent a broad range of hydrolytic enzymes that are involved in a myriad of crucial cellular processes such as protein turnover, cell signaling, cell cycle control, and apoptosis. It has been estimated that 2% of all genes in the human genome encode proteolytic species [1,2]. The aberrant expression or activity of proteases can underlie a range of pathological conditions including cancer, rheumatoid arthritis, neurodegenerative disorders, and infectious disease [3]. Methodologies that can elucidate and characterize protease species are keenly sought after, particularly in light of their focus as druggable targets for the treatment of these disease states [4,5].

Protein display technologies have become useful tools for the selection, isolation and identification of proteins on the basis of their biological activity. These techniques involve the use of a 'bait' to allow selective capture of 'prey' binders. The approaches for characterizing interactions with bait diversify in the approach used for the presentation of the library of prey, followed by its selection and characterization. Two such techniques, ribosome and mRNA display are performed using *in vitro* transcription–translation systems where the principle is to couple together the nascent proteins or peptides to its individual encoding mRNA molecule. In the case of ribosome display, the mRNA template contains no stop codon and under appropriate conditions, as the ribosome moves along to the end of the template it is unable to dissociate and it stalls, thereby generating a stable ternary complex providing a link between the phenotype (protein or peptide) and its genotype (mRNA molecule). These ternary complexes can then be incubated against an appropriate 'bait' and proteins which bind identified rapidly and sensitively by means of their mRNA by RT-PCR [6,7].

Ribosome display has been used for the evolution of function in proteins, namely the selection and maturation of antibodies based on their affinity with cognate antigen [8–10]. Here we have examined the ability to use this technique in the display of a protease, sortase A (SrtA). SrtA is a cysteine protease–transpeptidase found in the cell envelope of *Staphylococcus aureus* where it is responsible for the cleavage and covalent linkage of surface proteins containing the conserved -Leu-Pro-Xaa-Thr-Gly- sequence motif [11,12]. Many of the proteins anchored to the cell wall by this reaction are involved in interaction with host tissues enabling colonization and infection and therefore SrtA has been proposed as a target for the development of novel anti-microbial agents [13]. In this study we generated ribosome display ternary complexes *in vitro* to display SrtA and examined the capture and retrieval of this enzyme based on its catalytic activity using the general cysteine protease inhibitor E-64 (schematically demonstrated in Fig. 1).

Materials and methods

Generation of in vitro templates. The open reading frame for SrtA, devoid of its N terminal transmembrane domain was amplified from *S. aureus* (ATCC 9144) using SrtAF and SrtAR (see Table 1) which contain a BamHI and SalI restriction site, respectively, and cloned into pQE30 (Qiagen) restricted with BamHI and SalI resulting in the generation of pSRT-WT. An inactive mutant was generated from pSRT-WT by site-directed mutagenesis using primers mutF and mutR (Table 1). This mutation results in a generation of a C184S mutation, thus rendering the active site of the protease inactive. The site-directed mutagenesis was carried out using the QuikChange site-directed mutagenesis kit (Stratagene) as per the manufacturer's instructions, resulting in the generation of pSRT-CΔS. The pSRT-WT and pSRT-CΔS vectors were used as templates for subsequent PCR amplifications.

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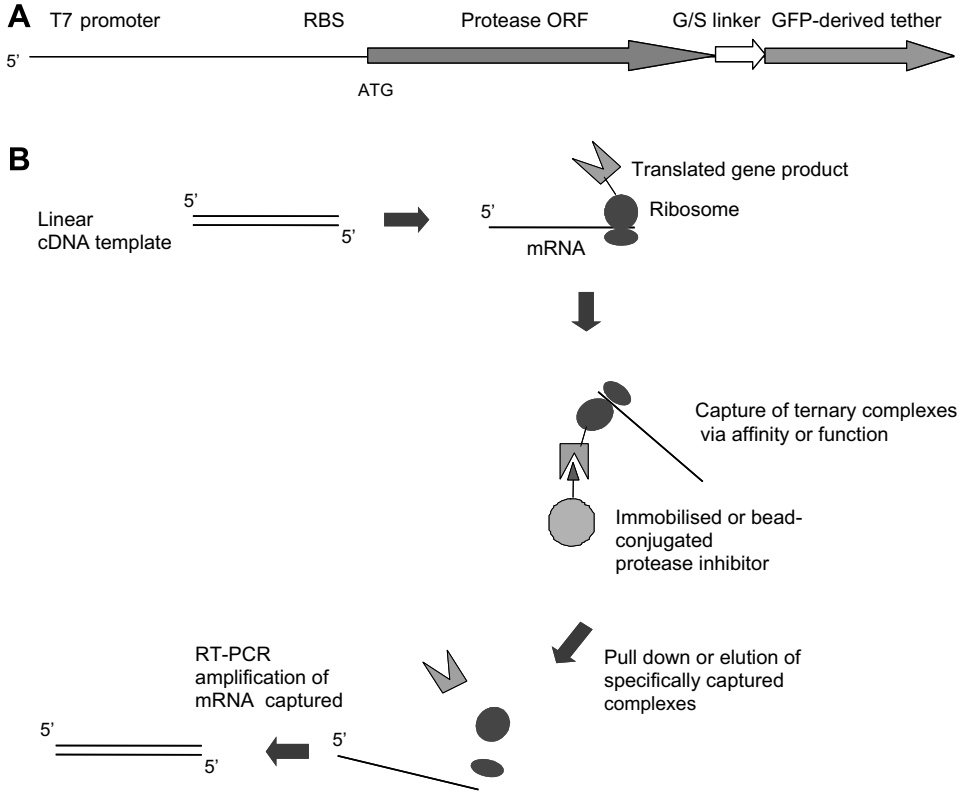

Fig. 1. Diagrammatic overview of selection of proteolytic species using ribosome display. Linear templates (A) are prepared by PCR to include all elements required for ribosome display including a 5′ T7 promoter, a Shine–Dalgarno ribosome binding site (RBS), and translation initiation methionine ATG codon. Crucially, no stop codon is present in this template to allow stalling of the ribosome at the 3′ end of resulting mRNA transcripts. Coupled transcription–translation resulting from this template results in the generation of the protease followed directly by a glycine–serine rich flexible linker (G/S linker) and a ribosome tunnel tether region derived from eGFP as a fusion protein. Upon addition of this template to *E. coli* transcription/translation lysates, ternary complexes are formed whereby the ribosome provides a physical link between the encoding mRNA and resultant nascent protein (B). These complexes are then incubated with immobilised protease inhibitor to isolate complexes on the basis of functionality. Successful capture is then determined by recovery of bound mRNA by RT-PCR.

Table 1
Details of oligonucleotide primers employed in this study

Primer name	Primer sequence
SrtAF	5′TTTTTTGGATCCAAACCACATATCGATAATTATTCACG
SrtAR	5′TTTTTTGTCGACTCATTTGACTTCTGTAGCTACAAATTTTACG
mutF	5′GATAAACAATTAACATTAATTACTTCTGATGATTACAATGAAAAGA CAGGCG
mutR	5′ CGCCTGTCTTTTCATTGTAATCATCAGAAGTAATTAATGTTAATTGT TTATC
SrtRiboF	5′AGACCACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTTAAGAA GGAGATATATCCATGAAACCACATATCGATAATTA
SrtRiboR	5′GCCTCCAGAGCCACCTCCGCTGCCACCTCCTTTGACTTCTGTAGCTAC AAAGATTTTACG
gfpF	5′GGAGGTGGCAGCGGAGGTGGCTCTGGAGGCGAGTACAACTACAAC AGCCACAACGTC
gfpR	5′ CCGCACACCAGTAAGGTGTGCGGTGGCGGTCACGAACTCCAGCAG
ssrA	5′ TTAAGCTGCTAAAGCGTAGTTTTCGTCGTTTGC
Int-srt-F	5′GTATATCCAGGACCAGCAACACC
Int-srt-R	5′CTAGAACTCCTACATCTGTAGGC

In vitro translation and labeling. The plasmids pSRT-WT was used to generate a linear PCR template for the expression of the proteolytic species in *Escherichia coli* lysates using SrtRiboF and SrtAR primers. The forward primer introduces an upstream T7 promoter and Shine–Dalgarno sequence to facilitate efficient transcription and translation in T7 RNA polymerase supplemented RTS 100 *E. coli* HY kit (Roche) *E. coli* extracts. PCR reactions were prepared using 150 nM of each primer and 10 ng vector template in a final reaction volume of 50 μl. After an initial denaturation step at 96 °C for 5 min, 32 cycles of 30 s at 94 °C, 30 s at 55 °C and 45 s at 72 °C were carried out followed by a final extension of 10 min. After amplification, samples were analyzed by agarose gel electrophoresis. Neat, un-purified PCR products (50 ng) were added to *E. coli* extract (100 μl, Roche) and incubated for 30 min at 30 °C. To an aliquot of this translation product (30 μl), the affinity binding probe Biotin-Ahx (aminohexanoyl)-Leu-Pro-Ala-Thr-CHN_2 (Bio-LPAT-DK, 50 μM), previously shown to specifically and irreversibly label SrtA [13], was added and incubated for a further 30 min at 37 °C. Samples (10 μl) were separated by SDS–PAGE analysis (4–12% density gradient gel, Invitrogen), and blotted onto a nitrocellulose membrane (Amersham). The membrane was blocked with 3% BSA (Sigma) and the detection of incorporated biotinylated affinity probe was disclosed with a streptavidin–horseradish peroxidase conjugate at a concentration of 1 in 5000 (Vector Laboratories) by chemolluminence (ECL, GE Healthcare).

Construction of ribosome display overlap linker region and ribosome tether. To generate ribosome display SrtA WT and SrtA CΔS templates it is necessary to have both a linker and tether region fused to the C terminus of the nascent protease species (Fig. 1A). In this investigation the tether chosen consisted of a fragment of enhanced Green Fluorescent Protein (eGFP) comprised of residues (143–227) and an additional upstream flexible glycine–serine rich linker (GGGSGGGSGG). This construct was produced by PCR using the following primers gfpF and gfpR which facilitated incorporation of the linker region in the forward primer as shown in Table 1. Amplification reactions (25 μl) were performed using 1× BioMix Taq polymerase (BioLine), 150 nM of each primer and 1 ng of the pEGFP-N1 plasmid as template (Clontech) amplification conditions were as described above. After amplification, samples were subjected to agarose gel electrophoresis and purification to isolate the desired amplification product (Qiaquick Minielute, Qiagen).

Construction of ribosome display SrtA wt and SrtA mut with overlap linker region. The linear templates for SrtA-WT and CΔS and mutant templates for overlap PCR with the linker and tether region were amplified using SrtRiboF and SrtRiboR (Table 1). SrtRiboR primer amplifies the C terminus of SrtA devoid of its stop codon and introduces the linker region for subsequent overlap PCR with the tether region. Amplification reactions were performed using 1× BioMix Taq polymerase (BioLine), 150 nM of each primer and 1 ng of either pSRT WT or pSRT CΔS plasmids as template in a 25 μl reaction volume. Amplification conditions were the same as described before with the exception that the cycled 72 °C extension step was elongated to 1 min. After amplification samples were subjected to gel electrophoresis and purification to isolate the desired amplification product as before.

Generation of ribosome display template by overlap PCR. The previously amplified and gel purified SrtA linear PCR templates with 3′ linker introduced and the tether region (eGFP fragment) were fused together by the complementary linker region using overlap PCR with the following primer pair SrtRiboF and gfpR.

Amplification reactions were performed using 1× BioMix Taq (BioLine), 150 nM of each primer and the previously purified templates for SrtA wild-type/mutant and the tether region 50 ng in a ratio of 1:1. Amplification conditions were the same as described before with the exception that the cycled 72 °C extension step was elongated to 1 min. Fused extended PCR products were analyzed by agarose gel electrophoresis and excised and purified as before.

Activity-based in vitro selection. All *in vitro* coupled transcription–translation reactions for ribosome display were carried out using the RTS *E. coli* HY kit (Roche) as per the manufacturer's instructions with the exception that reactions were supplemented with 1 μM of the primer ssrA (Table 1) which binds to and decommissions the *E. coli* ssrA ribosome rescue system. This system which is responsible for the detection of stalled ribosomes would otherwise cause the termination and destruction of the transcribed templates [7]. Purified fused linear Srt-EGFP templates (50 ng) coding either for WT or CΔS SrtA fused to the eGFP tether were transcribed and translated in *E. coli* extract (100 μl total) for 10–30 min at 30 °C. Reactions were then subjected to a DNase I digest (50 U) to degrade the original DNA template after a 20 min digestion the reactions were then stopped by the addition of 5 volumes of ice-cold selection buffer (PBS, 5 mM $MgCl_2$, Tween 20 0.1% (v/v)). The reactions were then split into three equal aliquots (33 μl) and each was supplemented with either naked acrylamide beads (Calbiochem); E-64 conjugated acrylic beads (Calbiochem) (50 μM final concentration) or an equal volume of RNase/DNase free water was added as a no beads control sample, respectively. Samples were slowly inverted at 4 °C for 30–60 min to allow binding of the E-64 to the active site of the SrtA to occur. After this incubation the samples were centrifuged at 250g for 30 s and the supernatant was removed, the samples were re-suspended in 1 ml of ice-cold selection buffer and the process was repeated a total of four times. After the fifth wash step the samples were re-suspended in RNase/DNase free water (50 μl) and then used immediately as a template for RT-PCR analysis or stored at −70 °C until required. To assess the use of this technique for competition based inhibitor assays, ribosome display was carried out as described above but with an additional pre-incubation step with either free E-64 or the Bio-LPAT-DK affinity probe (both at 50 μM final concentration) before the selection of ternary complexes by E-64 bound to acrylic beads as described above.

Detection of ternary complexes by RT-PCR. Re-suspended acrylic beads samples (1 μl) were used as templates in an RT-PCR reaction. A nested PCR approach was utilized with internal SrtA primer pair srt-int-F and srt-int-R (Table 1) which amplifies a 249 bp fragment of SrtA, convenient to visualize by agarose electrophoresis. RT-PCR reactions were performed using the Qiagen One step RT-PCR kit (Qiagen). Briefly, RT-PCR conditions were carried out using 150 nM of each primer. After an initial reverse transcriptase step at 50 °C for 30 min the reactions were denatured at 96 °C for 15 min, followed by 20–50 cycles of 30 seconds at 94 °C, 30 s at 56 °C and 30 s at 72 °C followed by a final extension step of 10 min. Samples were subjected to agarose gel electrophoresis (2% agarose gel) and successful pull down of ternary complexes was identified by the presence of an amplification product at 249 bp.

Results and discussion

To date ribosome display has been primarily employed for the selection and maturation of antibodies to antigen targets [14], and selection of enzymatically active proteins [15,16]. In this current investigation, we wished to assess the applicability of utilizing ribosome display for the display of a model proteolytic species. The model protease chosen for this study was SrtA. SrtA is a protease from *S. aureus* which cleaves sorting signals at an LPXT$^{\downarrow}$G motif on surface proteins of this and other Gram-positive bacteria [12]. Previously we and others have shown that it is possible to express an active form of SrtA in *E. coli*, demonstrating its activity via utilization of the affinity binding probe Bio-LPAT-DK which binds irreversibly to the active site cysteinyl residue and facilitates visualization through detection of the biotin moiety using streptavidin reagents [13]. Moreover, its ease of expression in *E. coli* lends itself to production in *E. coli* cell-free lysates as ribosome display ternary complexes.

As an initial step we wished to ensure that we could successfully express SrtA *in vitro* in cell-free conditions. Linear transcription/translation templates were prepared by the amplification of SrtA WT using primers that added an upstream T7 promoter and Shine–Dalgarno ribosome binding site. These motifs facilitate the transcription and translation of the SrtA products in *E. coli* lysates supplemented with T7 RNA polymerase. Demonstration that SrtA was active was provided by labeling of the protease species with the affinity binding probe Bio-LPAT-DK (Fig. 2), previously employed by ourselves to demonstrate the activity of SrtA over-expressed in *E. coli* cultures. The labeling of the recombinant protease at the correct size was visualized in the presence of the probe, indicating that the expression of functional SrtA could be performed from a linear template in *E. coli* coupled transcription/translation lysates.

We then examined if ternary ribosome display complexes presenting SrtA in *E. coli* coupled transcription/translation lysates could be prepared. Key to successful folding of nascent peptides as they emerge from the ribosome tunnel is the presence of a C terminal tether which resides in the ribosome, holding the ternary complex together, yet not interfering with the protein being displayed (Fig. 1). Previously, fragments of the bacterial protein TolA [17] or phage TIII protein [7] has been employed for purpose. In this application we decided to employ a two part linker/tether consisting of firstly an upstream flexible linker region used routinely in single chain antibody design to fuse heavy and light domains (GGGSGGGSGG) to ensure that folding of the nascent protein was not prevented by any steric or conformational hindrances. Secondly, the C terminal tether chosen for this study was a fragment of eGFP (residues 143–227). The recombinant expression of eGFP has been well documented in a wide range of expression hosts, and therefore has the potential to be used in all *in vitro* expression systems without toxicity or misfolding issues. Furthermore, in our hands we found that overlap PCR reactions using the GS rich-eGFP linker/tether proved to be remarkably robust even when the complementary overlap PCR template partner varied (results not shown). Individual templates for SrtA WT, a catalytically inactive mutant (SrtA CΔS), and the GS rich-eGFP linker/tether were produced by PCR amplification (Fig. 3A). Overlap PCR was facilitated using the common overlap region on the 3′ of the SrtA PCR products and the 5′ of the GS rich-eGFP linker/tether (Fig. 3B). These fused templates were then applied to the *E. coli* coupled transcription/translation lysates to allow production of the ternary ribosome complexes. The ternary complexes formed in the transcription/translation lysates were then incubated with E-64 coupled acrylic beads. E-64 is a classical epoxide-derived irreversible inhibitor of cysteine proteases previously demonstrated to potently inhibit sortase proteases [18]. After this incubation, the beads were removed from suspension by centrifugation and washed exhaustively prior to detection of specifically bound species by RT-PCR which picks up and amplifies mRNA species present. Using nested gene-specific RT-PCR primers for SrtA, which facilitates detection of mRNA without the need for mRNA elution, we clearly observed the amplification of a DNA band at 249 bp as anticipated (Fig. 4A). Furthermore, this band was not present in

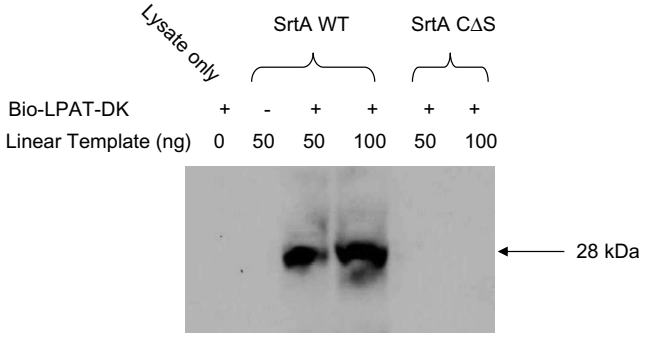

Fig. 2. Detection of functional SrtA expressed from *in vitro* coupled transcription/translation lysate. Lysates were incubated at 30 °C for 1 h with linear PCR templates (50 or 100 ng) to express SrtA WT or CΔS catalytically inactive mutant. Detection of functional SrtA was performed by incubation of lysates with affinity binding probe Bio-LPAT-DK (50 μM) prior to Western blotting analysis were visualization was afforded using streptavidin–horse radish peroxidase.

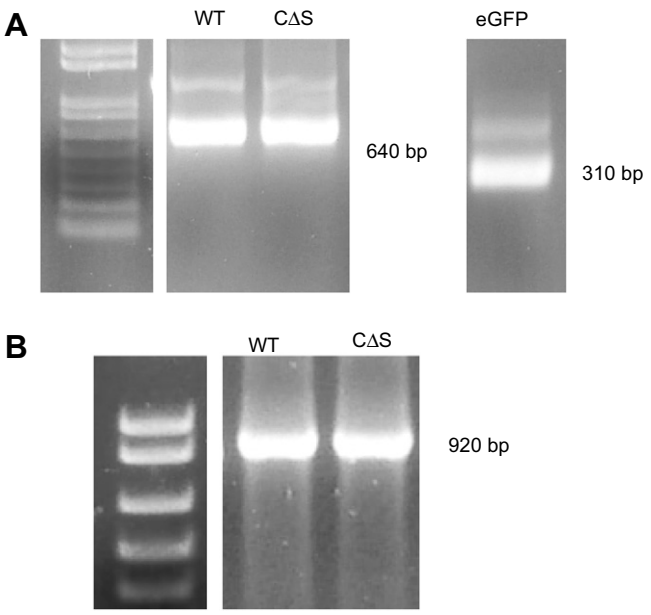

Fig. 3. Construction of ribosome display constructs by overlap PCR. (A) Amplification of SrtA WT and SrtACΔS (650 bp) was performed from plasmids containing wild-type and mutant ORF, respectively. The eGFP tether region with an upstream G/S rich flexible linker was amplified from pEGFP-N1 as described in Materials and methods. (B) Overlap PCR resulted in the generation of a PCR product of the anticipated size for both SrtA WT and CΔS mutant species.

an acrylamide beads-only control demonstrating that this selection was specific to the presence of the E-64 inhibitor. Moreover, no band was detected when the catalytically inactive SrtA CΔS mutant was used as a template in ribosome display, even after 50 rounds of RT-PCR cycles demonstrating the sensitivity of this selection (data not shown). The specificity of this interaction was further demonstrated in another control using acrylic beads displaying the serine protease inhibitor AEBSF, were as expected no SrtA mRNA was detectable (results not shown).

After demonstrating the successful use of ribosome display to select the SrtA protease on the basis of its catalytic activity we asked if the capture of the protease could be competitive. Therefore, the preparation of the ribosome complexes were undertaken as before, but with a pre-incubation step with either free E-64 or the Bio-LPAT-DK affinity binding probe. In these competitive analyses it was observed that this clearly diminished the levels of SrtA mRNA detectable by RT-PCR (Fig. 4B). These RT-PCR results demonstrate that binding of E-64 beads to the active site of wild-type SrtA can be blocked by the prior addition of either free E-64 or the SrtA specific Bio-LPAT-DK affinity binding probe.

Finally, we have also examined the application of the Bio-LPAT-DK affinity binding probe in place of E-64, but we have been unable at this time to demonstrate selectivity for active SrtA enzyme, due to non-specific binding of the ribosome complexes to various streptavidin reagents used to capture the biotin moiety on the probe regardless of conditions used (data not shown). To circumvent these issues we are currently examining the range of alterative capture moieties to replace biotin and the coupling of the LPAT-DK probe to other matrices.

Conclusions

We have demonstrated the production of a functional cysteine protease using ribosome display and demonstrated its specific capture using the broad range specificity cysteine protease inhibitor E-64 bound to acrylic beads. This report represents the first example of the selection of a proteolytic species using ribosome display. Previous examples of ribosome display using an inhibitor/ligand to identify ternary complexes require that each enzyme requires its own unique ligand. We however demonstrate the selection of a cysteine protease using a broad range cysteine protease inhibitor (E-64). We also demonstrated that the broad range serine protease

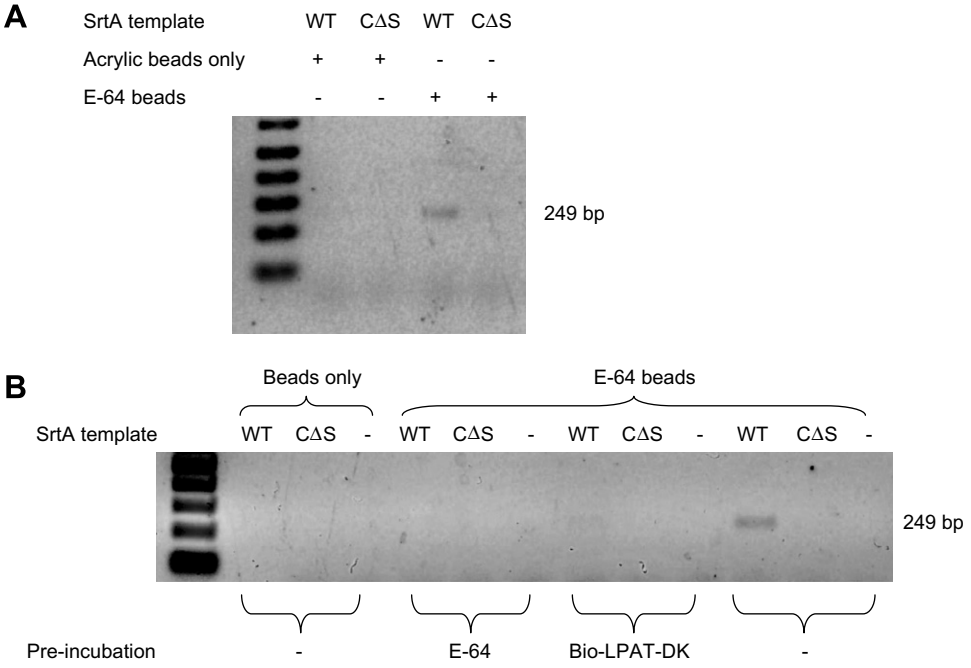

Fig. 4. Ribosome display capture of SrtA on the basis of its catalytic activity using E-64 bound to acrylic beads. (A) Ribosome display was carried out using either SrtA WT or SrtA CΔS inactive mutant as templates (100 ng); after washing 1 ml of re-suspended beads was used as template to detect selected mRNA SrtA templates using nested primers by RT-PCR. (B) Competition selection for SrtA presented during ribosome display was performed in the presence of either free E-64 (50 μM) or Bio-LPAT-DK (50 μM) as described.

inhibitor AEBSF failed to select for the cysteine protease SrtA under the same conditions. Taken together these results suggest that it may be possible to identify classes of proteases irrespective of their substrate specificity with ribosome display using broad range clan-specific inhibitors.

This demonstration that protease species can be displayed, selected and binding competed for *in vitro* will allow further investigation and identification of proteolytic species in the future with ribosome display. Ribosome display and other cell-free protein display technologies offer much promise in the screening of libraries for functional proteins, or whole proteome libraries. Our studies here illustrate that it will be possible to pick out protease species on the basis of their enzymatic activity, and may allow identification of novel species over currently known domains and families. Finally, this technique may prove useful in the design of new inhibitors, whereby screening of a library of mutants of a given protease (generated rapidly by error prone PCR) could provide insight into key residues involved in the inhibitor binding or mechanisms by which the protease could evolve to evade capture by a putative drug molecule.

Acknowledgments

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