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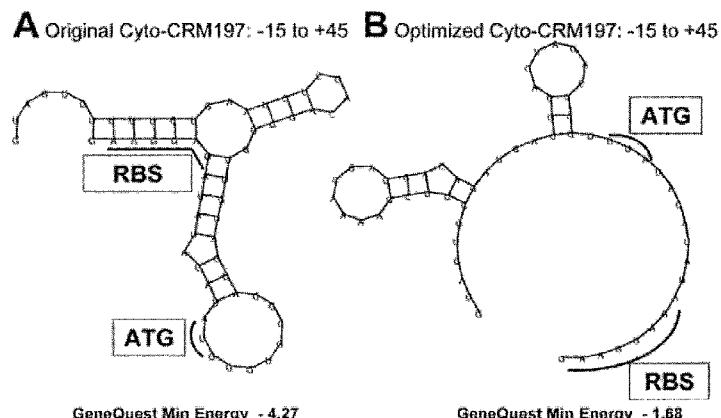
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(54) Title: ENHANCED PRODUCTION OF RECOMBINANT CRM197 IN *E. COLI*

Figure 1



(57) Abstract: Reduced genome or native K12 strain *E. coli* bacteria comprising expression vectors encoding a recombinant CRM197 protein and their use in the production of CRM 197 is provided. The CRM 197 protein may be fused to a signal sequence that directs the expressed CRM197 protein to the periplasm of the *E. coli* host.

ENHANCED PRODUCTION OF RECOMBINANT CRM197 IN *E. COLI***CROSS-REFERENCE TO RELATED APPLICATIONS**

[0001] This application claims the benefit of U.S. Provisional Application No. 61/947,234 filed March 3, 2014, the contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

[0002] The present invention is directed to the production of recombinant CRM197 in *E. coli*, preferably in reduced genome *E. coli* K12 strains.

BACKGROUND OF THE INVENTION

[0003] Diphtheria toxin (DTx) is a two-component exotoxin of *Corynebacterium diphtheriae* synthesized as a single polypeptide chain of 535 amino acids containing an A (active) domain and a B (binding) domain linked together by a disulfide bridge. The toxin binds to a cell receptor (HB-EGF receptor) and enters the cell by endocytosis where the A domain is released from the B domain by proteolytic cleavage. The A domain then exits the endosome through pores made by the B domain and enters the cytoplasm where it inhibits protein synthesis ultimately resulting in cell death.

[0004] CRM197 is a mutated form of Dtx containing a single amino acid substitution of glutamic acid for glycine (G52E) that renders the protein enzymatically inactive and non-toxic. CRM197 has been found to be an ideal carrier for conjugate vaccines against encapsulated bacteria. Conjugate vaccines comprise CRM197 covalently linked to poorly immunogenic and T-cell independent capsular polysaccharides, thus creating conjugate antigens that are highly immunogenic and result in long-lasting immunity against the antigen(s).

[0005] Vaccines containing CRM197 as a carrier protein have been successfully used to immunize millions of children and include Menveo®, a tetravalent conjugate

vaccine against serogroups A-C-W135-Y of *Neisseria meningitidis*, Menjugate® and Meningitec® (against serotype C of *N. meningitidis*), Vaxem-Hib® and HibTITER® (against *Haemophilus influenzae* type B, Hib), and the multivalent pneumococcal conjugate Prevnar™.

[0006] In contrast to tetanus and diphtheria toxins, CRM197 does not require chemical detoxification and can therefore be purified to homogeneity and used directly for conjugation. CRM197 is currently manufactured by the fermentation of either *Corynebacterium diphtheriae* C7, where it is expressed from multiple lysogens of the β phage, or from a plasmid system in *Pseudomonas fluorescens*. The yield of CRM197 (which is released into the media during *C. diphtheriae* fermentation) is low ranging from tens of mg/L to ~200 mg/L and requires biosafety level 2 facilities, resulting in a retail price of about \$500 US per milligram of CRM197. A single dose of vaccine typically contains about 10 and 60 μ g of CRM197 and over 150 million doses are used each year. Current demand for conjugate CRM197 vaccines has outpaced supply and has resulted in delays in initiating vaccination programs in developing countries placing the health of millions of children at risk.

[0007] Moreover, a possible therapeutic use for CRM197 in treating cancers such as ovarian cancer has recently been reported, based on CRM197's ability to bind the soluble form of heparin-binding epidermal growth factor (pro-HB-EGF), which is highly expressed in some cancers. The research and development of this therapeutic potential places even more of a strain on current production methods.

[0008] The single greatest factor contributing to the high price and short supply of CRM197 is the historical inability to generate high amounts of CRM197 in the production workhorse *E. coli*. Although an insoluble form of CRM197 can be fermented in *E. coli* to relatively moderate yields, only a fraction of the insoluble product can be converted to the soluble form (Stefan et al., 2011). Producing high amounts of soluble CRM197 in *E. coli* has been even more challenging. A method for reliably and inexpensively producing high amounts of CRM197 for therapeutic use would constitute a significant advance in the art.

SUMMARY OF THE INVENTION

[0009] The present invention relates to a method for producing a recombinant CRM197 protein in an *E. coli* host cell. In several embodiments, the method comprises incubating a reduced genome *E. coli* comprising an expression vector comprising a nucleic acid sequence encoding a CRM197 protein operably linked to an expression control sequence under conditions suitable for the expression of the recombinant CRM197 protein. A significant increase in yield of CRM197 is achieved in a reduced genome *E. coli* host cell according to the invention compared to production in wild type *E. coli* strains such as BL21. The nucleic acid sequence encoding the CRM197 protein is preferably codon-optimized for expression in an *E. coli* host cell. In a preferred embodiment, the native parent *E. coli* strain is a K12 strain. In another embodiment, the method comprises incubating a native K12 strain *E. coli* comprising an expression vector comprising a nucleic acid sequence encoding a CRM197 protein operably linked to an expression control sequence under conditions suitable for the expression of the recombinant CRM197 protein

[0010] In one aspect, the nucleic acid sequence encoding a CRM197 protein is fused to a nucleic acid sequence encoding a signal sequence that directs transfer of the CRM197 protein to the periplasm of the *E. coli* host cell (preferably a reduced genome *E. coli* host cell), whereby a yield of about 1 gram per liter to about 10 grams per liter of soluble CRM197 is achieved. According to this aspect of the invention, the *E. coli* host (preferably a reduced genome *E. coli* host) comprises an expression vector comprising a nucleic acid sequence comprising a 5' signal sequence portion encoding a polypeptide having an amino acid sequence capable of directing transport of CRM197 to the *E. coli* periplasm and a 3' CRM197 portion encoding the CRM197 protein lacking its native signal sequence. Preferably the expression of CRM197 is inducible and the method comprises the steps of (a) growing the *E. coli* (preferably a reduced genome *E. coli*) and (b) inducing expression of CRM197. Preferably, the method is carried out in a fermentor.

[0011] In related aspects, the (e.g. reduced genome) *E. coli* host cell is transformed with an expression vector comprising an inducible promoter (e.g. a lac derivative

promoter) operatively linked to the protein coding sequence and expression of CRM197 is induced by the addition of a suitable amount of inducer (e.g. Isopropyl β -D-1-thiogalactopyranoside (IPTG)). Preferably, under shake flask conditions, induction occurs at an optical density (OD) at 600 nm (at which wavelength 1 OD unit corresponds to about 0.8×10^9 cells/ml) of about 0.1 to about 1.5 (more preferably about 0.2 to about 0.9, even more preferably about 0.3 to about 0.6). Under fermentation conditions, induction preferably occurs at an OD₆₀₀ of about 100 to 400, more preferably about 150 to 300, most preferably between 200 to 275 (e.g. 230 and 250). In other related aspects, the pH of the culture during growth and/or induction is from about 6.5 to about 7.5, the growth and/or induction temperature is from about 20 °C to about 30 °C (preferably about 25 °C) and the growth media is free of serum, yeast extract and animal-derived by-products. In particularly preferred embodiments, growing the (e.g. reduced genome) *E. coli* comprises a relatively short initial incubation at 37 °C (e.g. 1 to 3 hours) followed by growth at 20 °C to 30 °C (preferably at about 25 °C) prior to and subsequent to induction or comprises continuous growth at 20 °C to 30 °C (preferably at about 25 °C) prior to and subsequent to induction.

[0012] In related embodiments, the yield of soluble CRM197 obtained is at least about 0.5 g/L, at least about .7 g/L, at least about 1.0 g/L, at least about 1.3 g/L, at least about 1.5 g/L, at least about 1.7 g/L, at least about 2.0 g/L, at least about 2.3 g/L, at least about 2.5 g/L, at least about 2.7 g/L, at least about 3.0 g/L, at least about 3.5 g/L, at least about 3.7 g/L, at least about 4.0 g/L, at least about 4.5 g/L, at least about 5 g/L, at least about 5.5 g/L, at least about 6.0 g/L, at least about 7.0 g/L, at least about 8.0 g/L, at least about 9.0 g/L or at least about 10.0 g/L. In other embodiments, the yield of soluble CRM197 obtained is from about 1.0 g/L to about 10.0 g/L, from about 1.0 g/L to about 9.0 g/L, from about 1.0 g/L to about 8.0 g/L, from about 1.0 g/L to about 7.0 g/L, from about 1.0 g/L to about 6.0 g/L, from about 1.0 g/L to about 5.0 g/L, from about 1.0 g/L to about 4.0 g/L, from about 1.0 g/L to about 3.0 g/L or from about 1.0 g/L to about 2.0 g/L. In other embodiments, the yield of soluble CRM197 obtained is from about 2.0 g/L to about 10.0 g/L, from about 2.0 g/L to about 9.0 g/L, from about 2.0 g/L to about 8.0 g/L, from about 2.0 g/L to about 7.0 g/L, from about 2.0 g/L to about 6.0 g/L, from about 2.0 g/L to about 5.0 g/L, from about 2.0 g/L to about 4.0 g/L, from about 2.0 g/L to about 3.0 g/L or from about 2.0 g/L to about 2.0 g/L.

about 4.0 g/L, from about 2.0 g/L to about 4.0 g/L or from about 2.0 g/L to about 3.0 g/L. In other embodiments, the yield of soluble CRM197 obtained is from about 3.0 g/L to about 10.0 g/L, from about 3.0 g/L to about 9.0 g/L, from about 3.0 g/L to about 8.0 g/L, from about 3.0 g/L to about 7.0 g/L, from about 3.0 g/L to about 6.0 g/L, from about 3.0 g/L to about 5.0 g/L, or from about 3.0 g/L to about 4.0 g/L.

[0013] In a related aspect, the 5' signal sequence portion encodes a signal recognition particle (SRP) dependent signal sequence such as the DsbA, TolB and TorT secretion signals, a Sec-dependent signal sequence such as the OmpF, OmpT, OmpA, PhoA, MalE, LamB, LivK and PelB secretion signals, or a twin arginine translocation (TAT) signal sequence such as the TorA and Sufl secretion signals. In some embodiments, the 5' signal sequence portion encodes a Sec-dependent signal sequence, preferably the OmpA or OmpF secretion signal. In a particularly preferred embodiment, the 5' signal sequence portion encodes the ompF secretion signal.

[0014] In other preferred embodiments, the 5' signal sequence portion encodes a signal sequence selected from an MglB, MalE, OppA, RbsB, Agp, FkpA, YtfQ, HdeA, HdeB, OmpC and GlnH secretion signal. In a particularly preferred embodiment, the 5' signal sequence portion encodes the YtfQ secretion signal.

[0015] In another related aspect, the *E. coli* host cell additionally comprises one or more nucleic acids comprising a sequence encoding one or more proteins for assisting the translocation and/or folding of CRM197 in the periplasm, operably linked to an expression control sequence. The nucleic acid(s) comprising a sequence encoding one or more proteins for assisting the translocation and/or folding of CRM197 in the periplasm may be part of the same expression vector comprising the nucleotide sequence encoding CRM197 or may be located on a different expression vector. Proteins for assisting the translocation and/or folding of CRM197 include, without limitation, chaperones such as Skp, DnaK, DnaJ, CaflM, and CaflA; disulfide bond formation proteins such as DsbA, DsbB, DsbC and DsbD; peptidyl-prolyl cis-trans isomerases such as PpiA, PpiD, FkpA and SurA; soluble partner proteins such as MBP, GST, and thioredoxin; secretion pathway proteins such as YebF, MalE, HlyA,

Hirudin, OmpF, and Spy; protease inhibitors such as YccA; and proteins that relieve export saturation such as PspA.

[0016] In another embodiment, the nucleotide sequence encoding a CRM197 protein is not fused to a signal sequence, whereby a yield of insoluble CRM197 of about 2 grams per liter to about 25 grams per liter is achieved. According to this aspect of the invention, the (e.g. reduced genome) *E. coli* host comprises an expression vector comprising a nucleic acid sequence encoding a CRM197 protein lacking its native signal sequence, whereby the CRM197 protein is expressed in the cytoplasm of the *E. coli* host.

[0017] In several aspects, the present invention relates to a method for producing a recombinant CRM197 protein in a (e.g. reduced genome) *E. coli* host cell, the method comprising: ligating into an expression vector a nucleotide sequence encoding a CRM197 protein fused to a signal sequence that directs transfer of the CRM197 protein to the periplasm; transforming the *E. coli* host cell with the expression vector; and culturing the transformed *E. coli* host cell in a culture media suitable for the expression of the recombinant CRM197 protein; wherein the yield of soluble CRM197 is about 1 to 10 g/L, preferably about 2 to 10 g/L, and further comprising harvesting the *E. coli* cells from the culture and lysing the harvested cells by a mechanical method in the absence of detergent. Optionally, the method further comprises obtaining a soluble fraction of the resulting lysate (e.g. by centrifugation to separate a soluble and insoluble fraction) and subjecting the soluble fraction (comprising soluble CRM197 produced by the *E. coli* host) to one or more purification steps. In one embodiment the soluble CRM197 is subjected to hydrophobic interaction chromatography and/or anion exchange chromatography. In preferred embodiments, the *E. coli* host cell is a reduced genome *E. coli* host cell.

[0018] In other aspects, the invention relates to a (e.g. reduced genome) *E. coli* host cell comprising an expression vector, the expression vector comprising a nucleic acid sequence comprising nucleic acid sequence comprising a 5' signal sequence portion encoding a polypeptide having an amino acid sequence capable of directing transport of CRM197 to the *E. coli* periplasm and a 3' CRM197 portion encoding the

CRM197 protein lacking its native signal sequence operably linked to an expression control sequence. In preferred embodiments, the *E. coli* host cell is a reduced genome *E. coli* host cell that lacks at least the genes deleted from reduced genome *E. coli* strain MDS42 or lacks at least the genes deleted from reduced genome *E. coli* strain MDS69.

[0019] These and other embodiments of the present invention are described in more detail herein below.

DESCRIPTION OF THE DRAWINGS

[0020] Figure 1 depicts the DNA sequence changes that result in a release of hairpin structures in the CRM197 sequence used in experiments aimed at examining the insoluble form of CRM197. The optimized sequence (B) generates a higher minimal energy (-1.68 for the optimized sequence compared to -4.27 for the original sequence) and relaxes secondary structure enhancing recognition of both the start site (ATG) and ribosomal binding site (RBS) relative to the original sequence.

[0021] Figure 2 depicts cytosolic expression of CRM197 in reduced genome *E. coli* strain MDS42 *recA* (MDS42 strain with a *recA* deletion). Shake flask cultures were grown in minimal media to an optical density (OD) of 0.5 and then induced with either 0 or 250 μ M IPTG as indicated. Electrophoresis was with a 4-12% gradient acrylamide Bis-Tris gel followed by protein staining using GelCode stain reagent. 0.04 OD of material was loaded per lane. M = marker lane; T = total cellular protein; S = soluble fraction; I = insoluble fraction. High amounts of cytosolic CRM197 were present in the insoluble fraction (arrows) validating the robust nature of the construct and strain in the production of insoluble CRM197.

[0022] Figure 3 depicts signal sequences examined in relation to periplasmic delivery of CRM197 in reduced genome strain MDS42*recA*. The top panel (A) is an illustration of the heterologous signal sequence fused to the 5' end of the codon optimized CRM197 sequence. The lower left panel (B) illustrates signal sequences representative of the three *E. coli* secretion pathways. The lower right panel (C)

illustrates proteins that were co-expressed with CRM197 to test their ability to assist translocation and/or folding of CRM197 in the periplasm.

[0023] Figure 4 depicts protein gel analysis of CRM197 expression in MDS42*recA* using OmpA and OmpF signal sequences. Early (Panels A and C; inducer added at OD₆₀₀ of 0.01) and late (Panels B and D; inducer added at OD₆₀₀ of about 0.4) induction at 25 °C using either Δaa1-5 YccA (OmpA + CRM197 +/- YccA) or Δaa1-5 YccA (OmpF +CRM197 +/- YccA) as chaperones. Arrows indicate the highest level of CRM197 in each inducer series. Note the endogenous *E. coli* protein that migrates directly below CRM197. The gel method was as described for Figure 2. The periplasmic samples were prepared with the aid of Periplasting Buffer (Epicentre, Madison, WI). A 2 OD sample was harvested by centrifugation at 7,500 x g for 10 minutes in a 1.5 ml Eppendorf tube. The supernatant was removed and the cell pellet gently resuspended in 50 μl of Periplasting Buffer (200 mM Tris-HCl [pH 7.5], 20% sucrose, 1 mM EDTA, and 30 U/μl Ready-Lyse Lysozyme). After 5 minutes at room temperature, 50 μl of ice cold water was rapidly added to the resuspended pellet. The mixture was incubated on ice for 5 minutes prior to fractionating the periplasmic fraction from the spheroplasts by centrifuging at 4,000 x g for 15 minutes. The supernatant representing the periplasmic fraction was prepared for SDS-PAGE analysis. An amount equivalent to 0.12 OD units was loaded.

[0024] Figure 5 compares the effects of using glucose or glycerol as a carbon source on CRM197 expression in MDS42*recA* host cells. Panel A depicts total cellular protein (0.04 OD loaded per lane). Panel A depicts isolated periplasmic proteins (0.12 OD loaded per lane). Note that higher levels of CRM197 were generated in glucose-supplemented media. Lanes labeled “42 only” are control lanes with MDS42*recA* without the expression vector (i.e. not expressing CRM197).

[0025] Figure 6 depicts protein gel analysis of CRM197 expression in eight reduced genome *E. coli* strains carrying an expression vector coding for CRM197 fused to an ompA signal sequence including four strains built onto the MDS42 platform and four strains built onto the MDS69 platform. MDS42 strains tested are: (i) MDS42, (ii) MDS42*recA*, (iii) MDS42 metab (MDS42 strain comprising

corrections of the *rph* and *ilvG* frameshift mutations and deletions of the *iclR* and *arpA* genes) and (iv) MDS42 Blon metab (MDS42 metab further comprising a modification of the Lon protease (b0439) promoter region to mimic the sequence of the lon promoter region of B strain *E. coli*, in which an IS insertion separates the -35 region from the -10 region of the ancestral *E. coli lon* promoter.). MDS69 strains tested are: (i) MDS69 metab (MDS69 strain comprising corrections of the *rph* and *ilvG* frameshift mutations and deletions of the *iclR* and *arpA* genes) (ii) MDS69 Blon metab (MDS69 metab further comprising the Lon protease promoter modification described above) (iii) MDS69 lpp metab (MDS69 metab further comprising a deletion of the *lpp* gene (nucleotides 1755260-1755687 of MG1655) and (iv) MDS69 Blon, lpp metab. Panels A and C depict total cellular protein isolated after induction at the IPTG concentration indicated. Panels B and D indicate periplasmic isolation done in parallel. The gel method was as described in Figure 2.

[0026] Figure 7 depicts protein gel analysis of CRM197 expression in MDS42 and MDS69 protease strains. Panels A and C depict total cellular protein isolated after induction at the IPTG concentration indicated. Panels B and D depict periplasmic isolation done in parallel. The gel method was as described in Figure 2.

[0027] Figure 8 depicts protein gel and Western blot of fermentation samples following fed-batch fermentation of reduced genome *E. coli* strain MDS42 metab carrying an expression vector coding for CRM197 fused to an OmpA signal sequence in defined minimal media at the 10 liter scale. Panel A: total cell protein (TCP) and periplasmic (Peri) preparations were collected at the fermentation ODs indicated. IPTG was added at OD = 200. Panel B: Western blotting with anti-diphtheria toxin antibody was used to definitively identify CRM197. Note that CRM197 was not expressed prior to induction. SFC = shake flask control. 0.04 and 0.12 OD was loaded per lane for TCP and Peri samples, respectively. The gel method was as described in Figure 2.

[0028] Figure 9 depicts protein gels of (i) total cell protein (TCP) isolated using a conventional detergent-based buffer (Panel A and left three lanes of Panel B) and (ii) periplasmic protein preparations (right three lanes of Panel B), following expression

of OmpA-CRM197 in MDS42*recA* strain. Panel A: samples of total cell protein (left) were centrifuged for 10 minutes at high speed (21k g) and then reanalyzed (right). CRM197 is not found in the soluble fraction. Panel B: high speed centrifugation of total cell protein (TCP) and periplasmic fractions showing that CRM197 is insoluble in the cytoplasmic fractions exposed to detergent (left) and soluble in the periplasmic fractions (right) that were not exposed to detergent. The gel method was as described in Figure 2. The arrow indicates CRM197 in TCP that does not appear in the soluble fraction.

[0029] Figure 10 depicts protein gels of detergent and mechanical lysis and CRM197 solubility. MDS42*recA* cells carrying an expression vector encoding *ompA* fused to CRM197 were subjected to fed-batch fermentation as described for Figure 8. Cells were lysed using either (A) detergent (Bugbuster® (Novagen), a proprietary mixture of non-ionic detergents that disrupt the cell membrane) or (B) mechanical (sonication) lysis in the presence of solubilization agents. Note that lysis in the absence of detergent resulted in high levels of soluble CRM197. GSH:GSSG = reduced to oxidized ratio of glutathione; M = marker; Sol = soluble fraction; TCP = total cell protein. Inducer (IPTG) = 35 μ M.

[0030] Figure 11 is a graph depicting fermentation of MDS69 metab host cells carrying an expression vector encoding OmpA-CRM197. CRM197 was found to increase in fed-batch fermentation up to the 30 hour time point where a maximum yield of 1.95 g/L was achieved.

[0031] Figure 12 depicts protein gels from the fermentation of Figure 11 showing a high amount of soluble CRM197 in fermentation samples. Strain MDS69 metab was subjected to fed-batch fermentation in minimal media with glucose as the carbon source. Samples collected prior to the addition of inducer (22 hrs) or at either 28 or 29 hrs were homogenized by sonication and different cellular fractions isolated. CRM197 was found exclusively in the soluble (Sol) fraction. TCP = total cellular protein fraction; Insol = insoluble fraction.

[0032] Figure 13 depicts the results of a two column purification of CRM197. Panel A: 50 OD of the 28 hr fermentation sample shown in Fig 12 was subjected to microfluidizer (MF) homogenization. Soluble and insoluble (IS) fractions were isolated and soluble material (25 OD equivalents) was loaded onto a phenyl sepharose column. The three lanes labeled “Soluble” are 0.1, 0.07 and 0.04 OD of pre-loaded soluble samples, respectively. CRM197-containing material was eluted with 10 mM NaCl, 10 mM phosphate buffer, pH 7.5. Five consecutive 2.5 ml samples were collected (left to right) and fractions indicated by a circle were pooled. Note the small amount of unprocessed CRM197 (arrows) that was purified away from the main eluted sample and found following elution using distilled water only. Panel B: Pooled samples from Panel A were loaded onto a DEAE sepharose column and eluted using different salt concentrations (0.1 M NaCl followed by 1 M NaCl and finally, 1.5 M NaCl). CRM197 of highest purity was eluted at 1 M NaCl. Anti-diphtheria toxin antibody (1:1000 dilution) Western blots are shown alongside protein-stained gels run in parallel. F, flow through; W, column wash; MF = total homogenate following microfluidization; IS = insoluble fraction following centrifugation of microfluidization homogenate; S = soluble fraction following centrifugation of microfluidization homogenate.

[0033] Figure 14 protein gel analysis of CRM197 expression in MDS42*recA* compared to wild type B strain BLR(DE3). Cells were transformed with an expression vector encoding an ompA-CRM197 fusion directing the protein to the periplasm and grown at 25 °C for 19 hours in shaken flasks. Expression of CRM197 was induced at OD = 0.3 (late stage) by addition of IPTG at the indicated concentrations. Periplasmic proteins were isolated and analyzed. Note the endogenous *E. coli* protein that migrates directly below CRM197.

[0034] Figure 15 protein gel analysis of the periplasmic fractions generated from pSX2-OmpA CRM197 and pSX2-OmpF CRM197 expression in MDS69 metab and MDS69 metab low mutation rate hosts. The OmpF-CRM197 clone produces more soluble periplasmic CRM197 in the MDS69 meta low mut host (lanes 5-6) than the ompA-CRM197 clone (lanes 8-9) in the same host and using the same induction conditions. Lanes 11-15 represent samples of the medium supernatant after cell

harvest and demonstrate that little to no CRM197 is released to the medium under these induction and growth conditions.

[0035] Figures 16A-16C depict CRM197 DNA and amino acid sequences. Figure 16A depicts the DNA sequence of the CRM197 ORF optimized for expression with a signal sequence to direct expression and processing of the mature CRM197 protein to the periplasmic space. Figure 16B depicts the DNA sequence of the CRM197 ORF optimized for expression in the cytoplasm. Figure 16C depicts the amino acid sequence of the mature CRM197 protein produced after signal sequence processing (Figure 16A) or N-terminal Methionine removal (Figure 16B).

[0036] Figure 17 depicts signal sequences examined in relation to periplasmic delivery of CRM197 in reduced genome strain MDS69 metab, classified according to their relative abundance in *E. coli* K and/or B strains.

[0037] Figure 18 depicts the $\mu\text{g}/\text{OD}$ (top panel) and $\mu\text{g}/\text{ml}$ of periplasmic CRM197 (Caliper analysis) with the following signal sequences: OmpF, MalE, HdeA, OppA, HdeB and GlnH (Induction A) and OmpF, MglB, Agp, OmpC, RbsB, FkpA, and YtfQ (Induction B) at 25 μM inducer (IPTG) added at $\text{OD} \sim 0.3$ in reduced genome *E. coli* strain MDS69 metab.

[0038] Figure 19 depicts the $\mu\text{g}/\text{OD}$ (top panel) and $\mu\text{g}/\text{ml}$ of periplasmic CRM197 (Caliper analysis) with the following signal sequences: OmpF, MalE, HdeA, OppA, HdeB and GlnH (Induction A) and OmpF, MglB, Agp, OmpC, RbsB, FkpA, and YtfQ (Induction B) at 35 μM inducer (IPTG) added at $\text{OD} \sim 0.3$ in reduced genome *E. coli* strain MDS69 metab.

[0039] Figure 20 depicts the $\mu\text{g}/\text{OD}$ (top panel) and $\mu\text{g}/\text{ml}$ of periplasmic CRM197 (Caliper analysis) with the following signal sequences: OmpF, MalE, HdeA, OppA, HdeB and GlnH (Induction A) and OmpF, MglB, Agp, OmpC, RbsB, FkpA, and YtfQ (Induction B) at 50 μM inducer (IPTG) added at $\text{OD} \sim 0.3$ in reduced genome *E. coli* strain MDS69 metab.

[0040] Figure 21 compares the $\mu\text{g}/\text{OD}$ (top panel) and $\mu\text{g}/\text{ml}$ of periplasmic CRM197 (Caliper analysis) obtained with the OmpF signal sequence at 0, 25, 35 and 50 μM inducer and with the YtfQ signal sequence at 0, 25, 35, 50, 75, 100, 150 and 250 μM inducer added at $\text{OD} \sim 0.3$ in reduced genome *E. coli* strain MDS69 metab. The results are the average of two sets of experiments.

[0041] Figure 22 is a protein gel analysis comparing CRM197 yield in periplasm (P) and media (M) of MDS69 metab with OmpF or YtfQ signal sequence at inducer concentration of 50 μM (OmpF) or 50, 75, 100, 150, 250 μM (YtfQ) added at $\text{OD} = 0.3$ (late stage). Samples were collected at the specified OD for analysis.

[0042] Figure 23 compares periplasmic expression of CRM197 in MDS metab cells with OmpF or YtfQ signal sequence and in BL21(DE3) cells with ompF signal sequence at the indicated inducer concentrations. Inducer was added at $\text{OD} \sim 2$ (very late induction) and samples were analyzed by Caliper. The g/L of (soluble) periplasmic CRM197 (extrapolated to $\text{OD}600 = 250$) is indicated at each concentration of inducer.

DETAILED DESCRIPTION OF THE INVENTION

[0043] While the present invention is capable of being embodied in various forms, the description below of several embodiments is made with the understanding that the present disclosure is to be considered as an exemplification of the invention, and is not intended to limit the invention to the specific embodiments illustrated. Headings are provided for convenience only and are not to be construed to limit the invention in any manner. Embodiments illustrated under any heading may be combined with embodiments illustrated under any other heading.

[0044] The use of numerical values in the various ranges specified in this application, unless expressly indicated otherwise, are stated as approximations as though the minimum and maximum values within the stated ranges were both preceded by the word “about.” In this manner, slight variations above and below the stated ranges can be used to achieve substantially the same results as values within the ranges. As used herein, the terms “about” and “approximately” when referring to a

numerical value shall have their plain and ordinary meanings to one skilled in the pertinent art at issue. Also, the disclosure of ranges is intended as a continuous range including every value between the minimum and maximum values recited as well as any ranges that can be formed by such values. This includes ranges that can be formed that do or do not include a finite upper and/or lower boundary. This also includes ratios that are derivable by dividing a given disclosed numeral into another disclosed numeral. Accordingly, the skilled person will appreciate that many such ratios, ranges, and ranges of ratios can be unambiguously derived from the data and numbers presented herein and all represent various embodiments of the present invention.

[0045] A “reduced genome” bacterium as used herein means a bacterium having about 1% to about 75% of its genome (e.g. protein coding genes) deleted, for example about 5%, about 10%, about 20%, about 30% about 40%, about 50% or about 60% of the genome deleted. In one embodiment, the reduced genome bacteria used in the practice of the present invention have a genome that is preferably genetically engineered to be at least two percent (2%) and up to twenty percent (20%) (including any number therebetween) smaller than the genome of a native parent strain. Preferably, the genome is at least five percent (5%) and up to thirty percent (30%) smaller than the genome of a native parent strain. More preferably, the genome is eight percent (8%) to fourteen percent (14%) to twenty percent (20%) (including any number therebetween) or more smaller than the genome of the native parent strain. Alternatively, the genome may be engineered to be less than 20%, less than 30%, less than 40% or less than 50% smaller than the genome of a native parental strain. The term “native parental strain” means a bacterial strain found in a natural or native environment as commonly understood by the scientific community to represent the foundation of a strain line and on whose genome a series of deletions can be made to generate a bacterial strain with a smaller genome. Native parent strain also refers to a strain against which the engineered strain is compared and wherein the engineered strain has less than the full complement of the native parent strain. The percentage by which a genome has become smaller after a series of deletions is calculated by dividing “the total number of base pairs deleted after all of the deletions” by “the total number of base pairs in the genome before all of the deletions” and then multiplying

by 100. Similarly, the percentage by which the genome is smaller than the native parent strain is calculated by dividing the total number of nucleotides in the strain with the smaller genome (regardless of the process by which it was produced) by the total number of nucleotides in a native parent strain and then multiplying by 100.

[0046] In one embodiment, a “reduced genome” bacterium means a bacteria for which removal of the above amounts of genome does not unacceptably affect the ability of the organism to grow on minimal medium. Whether removal of two or more genes “unacceptably affects” the ability of the organism to grow on minimal medium in the present context depends on the specific application. For example, a 30% reduction in proliferation rate may be acceptable for one application but not another. In addition, adverse effect of deleting a DNA sequence from the genome may be reduced by measures such as changing culture conditions. Such measures may turn an otherwise unacceptable adverse effect to an acceptable one. In one embodiment, the proliferation rate is approximately the same as the parental strain. However, proliferation rates ranging from about 5%, 10%, 15%, 20%, 30%, 40% to about 50% lower than that of the parental strain are within the scope of the invention. More particularly, doubling times of bacteria of the present invention may range from about fifteen minutes to about three hours. Non-limiting examples of suitable reduced genome bacteria, as well as methods for deleting DNA from a bacterium such as *E. coli*, are disclosed in U.S. Pat Nos. 6,989,265, 7,303,906, 8,119,365, 8,039,243 and 8,178,339, each of which is hereby incorporated by reference herein.

[0047] The term “b number” used herein refers to the unique ID assigned to each gene of the K-12 MG1655 strain as described in Blattner *et al.*, Science 277:1453-1474 (1997).

[0048] The term “CRM197” used herein refers to cross-reacting material 197 (CRM197), a diphtheria toxin variant having a single G → A transition leading to the substitution of glycine (at position 52 in the wild-type toxin) with glutamic acid in CRM197. This missense mutation is responsible for the loss of ADP-ribosyltransferase activity. See e.g. Giannini *et al.*, Nucleic Acids Res. 12(10):4063-4069 (1984).

[0049] In several embodiments, a method for producing a recombinant CRM197 protein in a reduced genome *E. coli* host cell is provided. It has been found that a surprisingly high yield of recombinant CRM197 can be produced in insoluble or soluble form using reduced genome *E. coli* host strains e.g. compared to wild type *E. coli* host strains. In one aspect, the method leads to increased production of insoluble CRM197 in the cytoplasm of the host cell. In other aspects, the method leads to increased production of soluble CRM197 in the periplasm of the host cell. In preferred embodiments, the native parent *E. coli* strain used to create the reduced genome *E. coli* host cell is a K-12 strain such as K-12 strain MG1655.

[0050] In some embodiments, a native K-12 strain such as K-12 MG1655 is used to produce recombinant CRM197 according to the methods herein described.

[0051] The nucleotide sequence of CRM197 for use according to the present invention may be prepared using recombinant DNA technology. For example, CRM197 can be chemically synthesized or can be prepared by site-directed mutagenesis based on the known nucleotide sequence of the wild type structural gene for diphtheria toxin carried by cornyebacteriophage β (Greenfield et al., Proc Nat Acad Sci, 80:6953-6957 (1993)). Preferably, the nucleotide sequence of CRM197 is optimized for expression in *E. coli*.

[0052] A variety of sequence features of the heterologous nucleic acid can be optimized including, without limitation, modification of translation initiation regions, alteration of mRNA structural elements, and the use of different codon biases. Methods for optimizing nucleic acid sequence to improve expression in *E. coli* host cells are known in the art and described e.g. in U.S. Patent No. 7,561,972, the contents of which are incorporated herein by reference. Preferably, optimization of the nucleotide sequence of CRM197 for expression in *E. coli* comprises at least codon optimization. The presence of codons in the heterologous nucleic acid sequence that are rarely used in *E. coli* can delay translation of the encoded protein and result in a reduced expression in the *E. coli* host cell. Thus, in one aspect, the general codon usage in *E. coli* is used to optimize the expression of CRM197 in *E. coli*. Optimization of CRM197 for expression in *E. coli* also preferably includes

minimization of interfering secondary structure. Interfering secondary structure can result in reduced expression of heterologous proteins in *E. coli* by impeding transcription and translation. For example, mRNA secondary structure at the initiation site has been inversely correlated to translational efficiency. An exemplary CRM197 nucleotide sequence, optimized for expression in the periplasm of *E. coli* when attached to an upstream region encoding a signal sequence is provided as SEQ ID NO: 1 (Figure 16A). An exemplary CRM197 nucleotide sequence, optimized for expression in the cytoplasm of *E. coli* when attached to an upstream ATG start codon is provided as SEQ ID NO: 3 (Figure 16B). It is to be understood that the methods of the present invention are not limited to the CRM197 nucleotide sequence set forth as SEQ ID NO: 1. Additional strategies for optimizing heterologous nucleotide sequences for expression in *E. coli* are known in the art and can be used in addition to or as an alternative to the strategies described above.

[0053] Processes for preparing recombinant heterologous proteins from genetically engineered bacterial host cells such as *E. coli* comprising expression systems are well known to those skilled in the art. Recombinant CRM197 can be expressed in (e.g. reduced genome) *E. coli* host cells by any of these methods. In one aspect, the present methods relate to reduced genome *E. coli* host cells comprising expression systems, the expression systems comprising nucleotide sequence encoding CRM197 operably linked to an inducible promoter such that CRM197 is expressed in the host cells when the promoter is induced. In a preferred aspect, the promoter is induced by addition of a suitable amount of IPTG. Introduction of a polynucleotide into the reduced genome *E. coli* host cell can be accomplished by any of several standard molecular biology techniques such as those described in Davis et al., *Basic Methods in Molecular Biology* (1986) and Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989) including, without limitation, calcium phosphate transfection, microinjection, electroporation, conjugation, infection and the like. Similarly, any system or vector suitable to maintain, propagate or express polynucleotides and/or express a polypeptide in a host may be used to practice the present invention. For example, the appropriate DNA sequence may be inserted into a vector such as a plasmid by standard techniques.

[0054] One aspect of the invention relates to periplasmic expression of CRM197 in a (e.g. reduced genome) *E. coli* host cell. The expression of proteins in the periplasm has been used for industrial use and has been reviewed in Hanahan, *J. Mol. Biol.*, 166:557-580 (1983); Hockney, *Trends Biotechnol.*, 12:456-632 (1994); and Hannig *et al.*, *Trends Biotechnol.*, 16:54-60 (1998), each of which is incorporated herein by reference. Thus, in several embodiments, methods are provided comprising growing a (e.g. reduced genome) *E. coli* comprising an expression vector comprising a nucleic acid sequence encoding a CRM197 protein fused to a signal sequence, operably linked to an expression control sequence under conditions suitable for the expression of the recombinant CRM197 protein, wherein the signal sequence directs transfer of the CRM197 protein to the periplasm of the *E. coli* host. According to these methods, a surprisingly high yield of intact soluble CRM197 is produced and substantially all of the soluble CRM197 can be recovered.

[0055] The presence of a signal sequence on a protein facilitates the transport of the newly translated protein across the inner membrane of *E. coli* into the periplasmic space. The signal sequence is then cleaved; accordingly replacement of the native *C. diphtheriae* signal sequence with a signal sequence that directs transfer of CRM197 to the periplasm of *E. coli* ultimately results in a mature protein having the same amino acid sequence.

[0056] Representative examples of signal sequences capable of directing heterologous proteins to the *E. coli* periplasm are listed below. It is to be understood that signal sequences useful in the methods of the present invention are not limited to those listed below. Preferably, the signal sequence results in direction of at least 70, 80, 90 or 100% of the polypeptide to the periplasm when expressed in *E. coli*.

Signal Sequence	Amino acid sequence
PelB (pectate lyase B)	MKYLLPTAAAGLLLLAAQPAMA
OmpA (outer-membrane protein A)	MKKTAIAIAVALAGFATVAQAA
StII (heat-stable enterotoxin 2)	MKKNIAFLLASMFVFSIATNAYA
Endoxylanase	MFKFKKKFLVGLTAAFMSISMFSATASA
PhoA (alkaline phosphatase)	MKQSTIALALLPLLFTPVTKA
OmpF (outer-membrane protein F)	MMKRNILAVIVPALLVAGTANA
PhoE (outer-membrane pore protein E)	MKKSTLALVVVMGIVASASVQA

Signal Sequence	Amino acid sequence
MalE (maltose-binding protein)	MKIKTGARILALSALTMMFSASALA
OmpC (outer-membrane protein C)	MKVVKVLSLLVPALLVAGAANA
Lpp (murein lipoprotein)	MKATKLVLGAVILGSTLLAG
LamB (λ receptor protein)	MMITLRKLPLAVAVAAGVMSAQAMA
OmpT (protease VII)	MRAKLLGIVLTTPIAISSFA
LTB (heat-labile enterotoxin subunit B)	MNKVKCYVLFTALLSSLYAHG
MglB (methyl galactose transporter)	MNKKVLTLSAVMASMLFGAAAHA
OppA (oligopeptide transporter)	MTNITKRSLSVAAGVLAALMAGNVALA
RbsB (subunit ribose transporter)	MNMKKLATLVS AVALSATVSANAMA
Agp (glucose-1 phosphatase, 3-phytase))	MNKTLIAAAVAGIVLLASNAQA
FkpA (peptidyl-prolyl cis-trans isomerase)	MKSLFKVTLLATTMAVALHAPITFA
YtfQ (galactofuranose binding protein, subunit ABC transporter)	MWKRLLIVSAVSAAMSSMALA
HdeA (stress response induced by acidic conditions)	MKKVLGVILGGLLLLPVVSNA
HdeB (stress response induced by acidic conditions)	MNISSLRKAFIFMGAVAALSLVNAQSALA
GlnH (subunit of glutamine ABC transporter)	MKSVLKVSLAALT LAFAVSSHA

[0057] Additional signal sequences for use according to the invention include, without limitation, CpdB (3'-nucleotidease/2',3'-cyclic nucleotide 2'-phosphodiesterase), YdeN (putative sulfatase), OsmY (induced by hyperosmotic stress), ArtI (subunit Arginine ABC transporter), GltL (glutamate ABC transporter), and CybC (cytochrome b562).

[0058] In preferred embodiments, the signal sequence is selected from the ytfQ, OmpA and OmpF signal sequences. In a particularly preferred embodiment, the signal sequence is the OmpF signal sequence. In another particularly preferred embodiment, the signal sequence is the YtfQ signal sequence.

[0059] Any reduced genome *E. coli* strain may be used as a host cell according to the methods described herein. In one aspect, the reduced genome *E. coli* has a genome that is genetically engineered to be at least two percent (2%) and up to forty percent (40%) (including any number therebetween), such as between 5% and 30% or

between 5% and 20%, smaller than the genome of its native parent strain. The percentage by which a genome has become smaller after a series of deletions is calculated by dividing “the total number of base pairs deleted after all of the deletions” by “the total number of base pairs in the genome of the parental strain before all of the deletions” and then multiplying by 100. In another aspect, the reduced genome bacterium has a genome that is between 4.41 Mb and 3.71 Mb, between 4.41 Mb and 3.25 Mb or between 4.41 Mb and 2.78 Mb. The reduced genome *E. coli* strain for use according to the methods described herein may be produced by cumulative genomic deletions of a parent *E. coli* strain by the methods described in International Patent Publication No. WO 2003/070880.

[0060] The parental *E. coli* strain may be any *E. coli* strain but is preferably a K-12 strain (e.g. MG1655 (ATCC No. 47076) or W3110 (ATCC No. 27325)) or B strain. A particularly preferred parental *E. coli* strain is K-12 strain MG1655 (annotated version m56, NCBI accession no. U000961) with a genome having 4,639,674 base pairs.

[0061] In one aspect, the parental *E. coli* strain is a K-12 strain lacking one or more of the genes listed at Tables 2-20 of U.S. Patent No. 8,178,339, incorporated herein by reference. In a preferred embodiment, the reduced genome *E. coli* K-12 strain lacks at least the following genes (identified by “b” numbers based on the designations set out in Blattner *et al.*, *Science*, 277:1453-74 and in GenBank Accession No. 400096): b0245-b0301, b0303-b0310, b1336-b1411, b4426-b4427, b2441-b2450, b2622-b2654, b2657-b2660, b4462, b1994-b2008, b4435, b3322-b3338, b2349-b2363, b1539-b1579, b4269-b4320, b2968-b2972, b2975-b2977, b2979-b2987, b4466-b4468, b1137-b1172, b0537-b0565, b0016-b0022, b4412-b4413, b0577-b0582, b4415, b2389-b2390, b2392-b2395, b0358-b0368, b0370-b0380, b2856-b2863, b3042-b3048, b0656, b1325-b1333, b2030-b2062, b2190-b2192, b3215-b3219, b3504-b3505, b1070-b1083, b1878-b1894, b1917-b1950, b4324-b4342, b4345-b4358, b4486, b0497-b0502, b0700-b0706, b1456-b1462, b3481-b3484, b3592-b3596, b0981-b0988, b1021-b1029, b2080-b2096, b4438, b3440-b3445, b4451, b3556-b3558, and b4455, which are the genes deleted from *E. coli* K-12 MG1655 to create reduced genome (or multiple deletion) strain MDS39. In another preferred

embodiment, the reduced genome *E. coli* K-12 strain further lacks the following gene: b1786, which is the gene deleted from MDS39 to create reduced genome strain MDS40. In another preferred embodiment, the reduced genome *E. coli* K-12 strain further lacks the following genes: b0150-b01530, which are the genes deleted from MDS40 to create MDS41. In yet another preferred embodiment, the reduced genome *E. coli* K-12 strain further lacks the following gene: b2945 (*endA*) which is the gene deleted from MDS41 to create reduced genome strain MDS42. In still another embodiment, the reduced genome *E. coli* K-12 strain further lacks any of the following genes: b0315-b0331, b0333-b0341 and b0346-b0354, which are the genes deleted from MDS42 to create reduced genome strain MDS43. In yet another embodiment, the reduced genome *E. coli* K-12 strain further lacks any of the following genes : b2481-b2492, b2219-b2230, b4500, b3707-b3723, b0644-b0650, b4079-4090, b4487, b4092-b4106, b0730-b0732, b3572-b3587, b1653, b2735-b2740, b2405-b2407, b3896-b3900, b1202, b4263-b4268, b0611, b2364-b2366, b0839, b0488-b0500, b0502, which are the genes deleted from MDS43 to create MDS60. In yet another preferred embodiment, the reduced genome *E. coli* K-12 strain further lacks any of the following genes: b0566-b0575, b2209, b0160-b0161, b1431-b1444, b3643, b1037-b1043, b0383, b0226-b0234, b2115-b2132, which are the genes deleted from MDS60 to create MDS69. In certain embodiments, the reduced genome *E. coli* K-12 strain for use in the methods described herein is MDS41, MDS42, MDS60 or MDS69.

[0062] *E. coli* host cells for use in the present invention preferably comprise a functional *recA* gene (b2699), although *E. coli* lacking a functional *recA* gene (b2699) can also be used as a host cell for producing CRM197. For example, a reduced genome *E. coli* strain such as e.g. strain MDS40, MDS41, MDS42 or MDS69 can be modified by inactivation of b2699 by complete or partial deletion of the gene from the modified *E. coli* K-12 strain. In one embodiment, CRM197 fused to an OmpA signal sequence is expressed in a reduced genome *E. coli* host lacking a functional *recA* gene.

[0063] In another aspect, the reduced genome *E. coli* comprises one or more non-functional genes selected from the group consisting of the genes encoding Pol II, Pol

IV and Pol V, as described in WIPO Publication No. 2013/059595, the contents of which are incorporated herein by reference. In one embodiment, the reduced genome *E. coli* has non-functional PolB (encoded by b0060, coordinates 63429-65780 on the *E. coli* K12 MG1655 genome) and DinB (encoded by b0231, coordinates 250898-251953 on the MG1655 genome) genes. In another embodiment, the reduced genome *E. coli* has non-functional PolB, DinB and UmuDC (encoded by b1183-b1184, coordinates 1229990-1231667 on the MG1655 genome) genes. Preferably, the gene(s) are rendered inactive by complete or partial deletion. For example, the *polB*, *dinB* and *umuDC* genes may be rendered nonfunctional in a reduced genome *E. coli* strain such as strain MDS40, MDS41, MDS42 or MDS69.

[0064] In another aspect, the reduced genome *E. coli* (e.g. strain MDS40, MDS41, MDS42 or MDS69) has been genetically modified so as to (a) enhance orotate phosphoribosyltransferase activity (b) produce active acetohydroxy acid synthase II and (c) reduce expression of the *iclR* and *arpA* gene products.

[0065] *E. coli* orotate phosphoribosyltransferase, an enzyme that catalyzes synthesis of pyrimidine nucleotides, is encoded by the *pyrE* gene, b-number b3642. The *pyrE* gene is present in an operon with the upstream *rph* gene (b3643). The *pyrE* gene is expressed at sub-optimal levels in *E. coli* K-12 strains such as MG1655 and W3310 due to a -1 frame shift mutation in the coding region of the *rph* gene. Orotate phosphoribosyltransferase activity can be enhanced by a deletion that entirely removes the *rph* coding sequence to bring the promoter of the *rph-pyrE* operon closer to the translation initiation site of *pyrE*. Alternatively, any of the methods described in U.S. Patent No. 8,293,505, the contents of which are incorporated by reference, can be used to enhance orotate phosphoribosyltransferase activity.

[0066] *E. coli* acetohydroxy acid synthase II normally consists of a large subunit, encoded by the *ilvG* gene and a small subunit, encoded by the *ilvM* gene (b3769). The *ilvG* sequence of *E. coli* K-12 strain MG1655 is corrupted and is actually a pseudo gene (b-number b4488), as set forth in GenBank Accession No. AAC77488.1. The *ilvG* pseudo gene is comprised of two separate coding sequences, *ilvG_1* (b3767) and *ilvG_2* (b3768). The *ilvG* pseudo gene sequence in K-12 strains such as MG1655

comprises a deletion of nucleotides GT at positions 983 and 984 relative to the intact *ilvG* genes found in other *E. coli* strains (e.g. B strain, O strain, etc.). The deletion of these nucleotides results in a frameshift mutation and nucleotides TGA at positions 982-984 of the K-12 *ilvG* pseudo gene sequence serve as a premature termination codon resulting in a truncated form of *ilvG* corresponding to *ilvG_1*. Thus, the normal gene product of *ilvG* is not expressed and acetohydroxy acid synthase II is not present in *E. coli* K-12 strains. The reduced genome *E. coli* can be modified to produce active acetohydroxy acid synthase II by the introduction of a mutation which complements a native -2 frameshift mutation in the *ilvG* gene. Alternatively, the reduced genome *E. coli* can be modified to produce active acetohydroxy acid synthase II by any of the methods of U.S. Patent No. 7,300,776, the entire contents of which are incorporated herein by reference.

[0067] The *iclR* and *arpA* genes of *E. coli* K strain are adjacent genes encoding regulatory proteins that modulate expression of the glyoxylate shunt enzymes and of acetyl-CoA synthetase, respectively. The *iclR* (isocitrate lyase regulator) gene, b-number b4018, is described at NCBI Entrez GeneID No. 948524. The *arpA* (ankyrin-like regulator protein) gene, b-number b4017, is described at NCBI Entrez GeneID No. 944933. The *arpA* gene was found to be partially deleted in the genome sequence of B strains such as BL21DE3 and REL606 relative to the K-12 strain sequence. The *iclR* and *arpA* genes can be inactivated (i.e. rendered non-functional) in the reduced genome *E. coli* by deletion of all or part of the *iclR* and *arpA* gene sequences for example by the “scarless” deletion methods described at column 8, line 45 to column 14, line 41 of U.S. Patent No. 6,989,265.

[0068] In other embodiments, the reduced genome *E. coli* comprises a *relA* gene containing any of the mutations described in U.S. Patent No. 8,367,380, the contents of which are incorporated herein by reference. For example, a reduced genome *E. coli* strain such as strain MDS40, MDS41, MDS42 or MDS69 may be modified to incorporate any of these mutations.

[0069] Reduced genome *E. coli* for use according to the invention may comprise any combination of the modifications described above. In some preferred

embodiments, a reduced genome *E. coli* comprising at least the deletions of MDS42 or comprises at least the deletions of MDS69 and has been genetically modified so as to (a) enhance orotate phosphoribosyltransferase activity (b) produce active acetohydroxy acid synthase II and (c) reduce expression of the *iclR* and *arpA* gene products is employed as a host for periplasmic production of CRM197. The reduced genome *E. coli* preferably comprises a functional *recA* gene.

[0070] Various protein coding genes can be deleted to form reduced genome bacteria. In *E. coli* and other bacteria, a type of DNA sequence that can be deleted includes those that in general will adversely affect the stability of the organism or of the gene products of that organism. Such elements that give rise to instability include without limitation transposable elements, insertion sequences, and other “selfish DNA” elements which may play a role in genome instability. For example, insertion sequence (IS) elements and their associated transposes are often found in bacterial genomes, and thus are targets for deletion. IS sequences are common in *E. coli*, and all of them may be deleted. For purposes of clarity in this document, we use the term IS element and transposable element generically to refer to DNA elements, whether intact or defective, that can move from one point to another in the genome. An example of the detrimental effects of IS elements in science and technology is the fact that they can hop from the genome of the host *E. coli* into a plasmid during propagation for sequencing. This artifact can be prevented by deletion from the host cells of all IS elements. For a specific application, other specific genes associated with genomic instability, such as active and inactive prophages may also be deleted. In particularly preferred embodiments, the reduced genome *E. coli* host according to the invention has deleted therefrom all insertion sequences (i.e. does not comprise insertion sequences). In a related aspect, the reduced genome *E. coli* host lacks all IS1, IS2, IS3, IS5, IS150 and IS186 insertion sequences.

[0071] Reduced genome bacteria of the invention may also be engineered to lack, for example, without limitation, certain genes unnecessary for growth and metabolism of the bacteria, pseudo genes, prophage, undesirable endogenous restriction-modification genes, pathogenicity genes, toxin genes, fimbrial genes, periplasmic protein genes, invasin genes, lipopolysaccharide genes, class III secretion systems,

phage virulence determinants, phage receptors, pathogenicity islands, RHS elements, sequences of unknown function and sequences not found in common between two strains of the same native parental species of bacterium. Other DNA sequences that are not required for cell survival can also be deleted or omitted.

[0072] In a particularly preferred embodiment, a reduced genome *E. coli* is provided having a genome between five percent (5%) and thirty percent (30%) smaller than the genome of a native parent *E. coli* K strain and lacking all insertion sequence (IS) elements. Positions of the IS elements on a genome map of *E. coli* MG1655 are shown in Fig. 1 and Table 2 of U.S. Patent No. 8,178,339, the contents of which are incorporated herein by reference. Insertion sequence elements which commonly occur in *E. coli* and which may be removed, include without limitation, IS1, IS2, IS3, IS4, IS5, IS30, IS150, IS186, IS600, IS911 and IS10. Preferably, the native parent *E. coli* strain is *E. coli* K-12 strain MG1655.

[0073] In another particularly preferred embodiment, the reduced genome *E. coli* comprises deletion(s) of one or more periplasmic protein genes, including without limitation, the following genes alone or in any combination: b0018, b0150, b0152-b0153, b0161, b0227, b0250, b0291-b0293, b0297, b0316, b0329, b0365, b0371, b0376, b0383-b0384, b0494, b0497-b0498, b0545, b0553, b0559, b0562, b0565, b0567, b0569, b0572-b0574, b0611, b0700, b0704, b0839, b0983-b0986, b1023-b1024, b1072, b1079-b1080, b1083, b1038-b1039, b1041-b1043, b1329, b1357, b1369, b1377, b1383, b1386, b1435-b1436, b1440, b1562, b1878, b1889, b1920, b1995, b2000, b2062, b2123, b2126, b2131-b2132, b2190, b2209, b2487, b2637, b2647, b2945, b3043, b3046-b3048, b3215-b3216, b3219, b3325, b3329, b3338, b3482, b3579, b3584, b3586, b3593, b3596, b4080, b4088, b4105, b4280, b4290-b4292, b4309-b4311, b4314, b4316-b4320, b4412, b4415, b4455, and b4487.

[0074] In another aspect of the invention, a native K-12 strain such as K-12 MG1655 is used to produce recombinant CRM197 according to the methods herein described.

[0075] The recombinant protein may be co-expressed with chaperones/disulfide-bond forming enzymes, which may provide proper folding of the recombinant protein, including but not limited to Skp, DnaK, DnaJ, CaflM, CaflA, DsbA, DsbB, DsbC, DsbD, PpiA, PpiD, FkpA, SurA, MBP, GST, YebF, MalE, HlyA, Hirudin, OmpF, Spy, YccA; and PspA. Nucleic acid sequences of such proteins useful for periplasmic expression of recombinant protein include, without limitation, those described in U.S. Pat. Nos. 5,747,662; 5,578,464 and 6,022,952, each of which is incorporated herein by reference.

[0076] *E. coli* host cells (reduced genome or native K12 strain) transformed with an expression vector encoding CRM197 can be cultured in any fermentation format. For example, shake flask cultures, batch, fed-batch, semi-continuous and continuous fermentation modes may be used herein. As used herein “fermentation” includes both embodiments in which literal fermentation is employed and embodiments in which other non-fermentative culture modes are employed. Further, any scale of fermentation may be employed including 1 liter scale and larger fermentation volumes. In one embodiment, the fermentation volume is or is at least 1 Liter. In other embodiments, the fermentation volume is or is at least 5 Liters, 10 Liters, 15 Liters, 20 Liters, 25 Liters, 50 Liters, 75 Liters, 100 Liters, 200 Liters, 500 Liters, 1,000 Liters, 5,000 Liters, 10,000 Liters, 50,000 Liters, or more.

[0077] In various embodiments, fermentation medium may be selected from among rich media, minimal media and mineral salts media. In preferred embodiments, a minimal medium or mineral salts medium is selected. The media is preferably free or substantially free of serum and animal-derived products. A mineral salts medium typically consists of mineral salts and a carbon source (e.g. glucose, sucrose, or glycerol). The mineral salts used to make mineral salts media include those selected from among, e.g., potassium phosphates, ammonium sulfate or chloride, magnesium sulfate or chloride, and trace minerals such as calcium chloride, borate, and sulfates of iron, copper, manganese, and zinc. No organic nitrogen source, such as peptone, tryptone, amino acids, or a yeast extract, is included in a mineral salts medium. Instead, an inorganic nitrogen source is used and this may be selected from among, e.g., ammonium salts, aqueous ammonia, and gaseous ammonia. A preferred

mineral salts medium will contain glucose as the carbon source. In comparison to mineral salts media, minimal media can also contain mineral salts and a carbon source, but can be supplemented with, e.g., low levels of amino acids, vitamins, peptones, or other ingredients, though these are added at very minimal levels.

[0078] In embodiments, a target culture cell density is reached at which time an inducer, preferably IPTG, is added to initiate protein production. It is understood that the cell density at induction, the concentration of inducer, pH and temperature can be varied to determine optimal conditions for expression

[0079] In preferred embodiments, the pH of the culture is from about 6.5 to 7.5.

[0080] Growth, culturing and/or fermentation of the transformed reduced genome *E. coli* is performed within a temperature range permitting survival but is preferably from about 20 °C to about 30 °C, more preferably is about 25 °C. In another preferred embodiment, the culturing comprises a relatively short initial incubation at 37 °C (e.g. 1 to 3 hours) and is followed by growth at about 20 °C to about 30 °C, preferably about 25 °C prior to and subsequent to induction. In other embodiments, culturing comprises growth at about 25 °C prior to and subsequent to induction.

[0081] In embodiments, under shake flask conditions, inducer is added at an optical density (OD) at 600 nm of about 0.1 to about 1.5, more preferably about 0.2 to about 0.9, even more preferably about 0.3 to about 0.6) at an incubation temperature of 20-30 °C, preferably 25 °C. At 600 nm, 1 OD unit corresponds to about 0.8×10^9 cells/ml. In other embodiments, under fermentation conditions, inducer is added at an OD600 of about 100 to 400, more preferably about 150 to 300, most preferably between 230 and 250.

[0082] The present methods provide for an increase in the level of properly processed CRM197 in comparison with conventional expression systems, such as in wild type *E. coli* B strains. In certain embodiments, the methods provide for an increase in soluble CRM197. In this context, the term “soluble” means that the protein is not precipitated at centrifugation between approximately 5,000 and 20,000 x

gravity when spun for 10-30 minutes in a buffer under physiological conditions. Conversely, “insoluble” means that the protein can be precipitated by centrifugation at between 5,000 and 20,000 x gravity when spun for 10-30 minutes in a buffer under physiological conditions.

[0083] The methods of the present invention can comprise recovery of recombinant CRM197 produced from the (e.g. reduced genome) *E. coli* host cells. When produced in the periplasm as a soluble protein, the recovery of recombinant CRM197 in soluble form is preferably accomplished by mechanically lysing the *E. coli* host cells in the absence of detergents and solubilizers. Mechanical disruption typically involves sonication (Neppiras and Hughes, *Biotechnology and Bioengineering*, 6:247-270 (1964)), microfluidization (Sauer et al., *Biotechnology and Bioengineering*, 33:1330-1342 (1989)), or bead milling (Limon-Lason et al., *Biotechnology and Bioengineering*, 21(5):745-774 (1979)). Other mechanical methods known in the art may also be employed.

[0084] Recombinant CRM197 may be purified by standard techniques known in the art including, but not limited to, ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxyapatite chromatography, immunopurification methods and the like. In a preferred embodiment, purification of recombinant CRM197 comprises hydrophobic interaction chromatography and/or anion exchange chromatography.

[0085] The yield of CRM197 can be determined by methods known to those skilled in the art such as capillary gel electrophoresis and Western blot analysis. Activity assays can also provide information regarding protein yield. Useful measures of protein yield include the amount of recombinant protein per culture volume (e.g. grams of protein /liter of culture), percent or fraction of active protein (e.g. amount of active protein/amount of protein used in the assay), percent or fraction of total cell protein, amount of protein/cell and percent or proportion of dry biomass.

[0086] Activity assays for evaluating CRM197 are known in the art and described in the literature and may include immunological assays, e.g. Western Blot analysis and ELISA, as well as receptor binding assays, e.g. Diphtheria toxin receptor (proHB-EGF) binding. In one embodiment, activity is represented by the % active recombinant CRM197 protein in the extract supernatant as compared with the total amount assayed (i.e. based on the amount of CRM197 determined to be active by the assay relative to the total amount of CRM197 used in the assay). In another embodiment, activity is represented by the % active recombinant CRM197 protein in the extract supernatant compared to a standard e.g. native protein (i.e. based on the amount of active CRM197 protein in the supernatant extract sample relative to the amount of active protein in a standard sample where the same amount of protein from each sample is used in the assay). In embodiments, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 95% to about 100%, or about 99% to 100% of the recombinant CRM197 protein is determined to be active.

[0087] Means of confirming the identity of CRM197 are also known in the art, e.g. a protein can be analyzed by peptide mass fingerprint using MALDI-TOF mass spectrometry, N-terminal sequencing analysis or peptide mapping.

[0088] The following are among preferred embodiments of the invention

[0089] A method for producing a recombinant CRM197 in a reduced genome *E. coli* K12 strain host comprising incubating a reduced genome *E. coli* K12 strain comprising an expression vector comprising a nucleotide sequence encoding a CRM197 protein fused to a nucleotide sequence encoding OmpF or YtfQ signal sequence that directs transfer of the CRM197 protein to the periplasm of the reduced genome *E. coli* host operably linked to an expression control sequence, under conditions suitable for the expression of the recombinant CRM197 protein, whereby a yield of at least 1 gram, preferably at least 2 grams per liter of soluble CRM197 is obtained and wherein the incubation conditions comprise culturing the *E. coli* host cell in a minimal medium free of animal serum or other animal by-products.

[0090] A method for producing a recombinant CRM197 in a reduced genome *E. coli* K12 strain host comprising incubating a reduced genome *E. coli* K12 strain comprising an expression vector comprising a nucleotide sequence encoding a CRM197 protein fused to a nucleotide sequence encoding OmpF or YtfQ signal sequence that directs transfer of the CRM197 protein to the periplasm of the reduced genome *E. coli* host, operably linked to an expression control sequence under conditions suitable for the expression of the recombinant CRM197 protein, whereby a yield of at least 1 gram, preferably at least 2 grams per liter of soluble CRM197 is obtained, wherein the reduced genome *E. coli* K12 strain has deleted therefrom at least the following DNA segments: b0245-b0301, b0303-b0310, b1336-b1411, b4426-b4427, b2441-b2450, b2622-b2654, b2657-b2660, b4462, b1994-b2008, b4435, b3322-b3338, b2349-b2363, b1539-b1579, b4269-b4320, b2968-b2972, b2975-b2977, b2979-b2987, b4466-4468, b1137-b1172, b0537-b0565, b0016-b0022, b4412-b4413, b0577-b0582, b4415, b2389-b2390, b2392-b2395, b0358-b0368, b0370-b0380, b2856-b2863, b3042-b3048, b0656, b1325-b1333, b2030-b2062, b2190-b2192, b3215-b3219, b3504-b3505, b1070-b1083, b1878-b1894, b1917-b1950, b4324-b4342, b4345-b4358, b4486, b0497-b0502, b0700-b0706, b1456-b1462, b3481-b3484, b3592-b3596, b0981-b0988, b1021-b1029, b2080-b2096, b4438, b3440-b3445, b4451, b3556-b3558, b4455, b1786, b0150-b0153 and b2945 of the *E. coli* K-12 strain MG1655 and optionally has the following additional modifications: (i) deletion of b4017, b4018 and b3643 and (ii) insertion of an AT dinucleotide at position 982 of b4488 and wherein the incubation conditions comprise culturing the *E. coli* host cell in a minimal medium free of animal serum or other animal by-products.

[0091] A method for producing a recombinant CRM197 in a reduced genome *E. coli* K12 strain host comprising incubating a reduced genome *E. coli* K12 strain comprising an expression vector comprising a nucleotide sequence encoding a CRM197 protein fused to a nucleotide sequence encoding OmpF or YtfQ signal sequence that directs transfer of the CRM197 protein to the periplasm of the reduced genome *E. coli* host, operably linked to an expression control sequence under conditions suitable for the expression of the recombinant CRM197 protein, whereby a yield of at least 1 gram, preferably at least 2 grams per liter of soluble CRM197 is

obtained, wherein the reduced genome *E. coli* K12 strain has deleted therefrom at least the following DNA segments: b0245-b0301, b0303-b0310, b1336-b1411, b4426-b4427, b2441-b2450, b2622-b2654, b2657-b2660, b4462, b1994-b2008, b4435, b3322-b3338, b2349-b2363, b1539-b1579, b4269-b4320, b2968-b2972, b2975-b2977, b2979-b2987, b4466-4468, b1137-b1172, b0537-b0565, b0016-b0022, b4412-b4413, b0577-b0582, b4415, b2389-b2390, b2392-b2395, b0358-b0368, b0370-b0380, b2856-b2863, b3042-b3048, b0656, b1325-b1333, b2030-b2062, b2190-b2192, b3215-b3219, b3504-b3505, b1070-b1083, b1878-b1894, b1917-b1950, b4324-b4342, b4345-b4358, b4486, b0497-b0502, b0700-b0706, b1456-b1462, b3481-b3484, b3592-b3596, b0981-b0988, b1021-b1029, b2080-b2096, b4438, b3440-b3445, b4451, b3556-b3558, b4455, b1786, b0150-b0153, b2945, b0315-b0331, b0333-b0341, b0346-b0354, b2481-b2492, b2219-b2230, b4500, b3707-b3723, b0644-b0650, b4079-4090, b4487, b4092-b4106, b0730-b0732, b3572-b3587, b1653, b2735-b2740, b2405-b2407, b3896-b3900, b1202, b4263-b4268, b0611, b2364-b2366, b0839, b0488-b0500, and b0502 of the *E. coli* K-12 strain MG1655 and optionally has the following additional modifications: (i) deletion of b4017, b4018 and b3643 and (ii) insertion of an AT dinucleotide at position 982 of b4488 and wherein the incubation conditions comprise culturing the *E. coli* host cell in a minimal medium free of animal serum or other animal by-products.

Example 1

Cytoplasmic Expression of Insoluble CRM197 in Reduced Genome *E. coli* Hosts

[0092] CRM197 is currently manufactured by fermentation of *Corynebacterium diphtheriae* C7, where it is expressed from multiple lysogens of the β phage, or from a recombinant plasmid system in *Pseudomonas fluorescens*. The yield of CRM197 in *C. diphtheriae* is low (at most ~200 mg/L) and requires biosafety level 2 (BSL2) facilities. Production in *P. fluorescens* results in a higher yield (about 2 g/L); however, both hosts retain numerous mobile elements, cryptic prophages and gene remnants with pathogenic functions. In bacterial fermentations, mobility of insertion sequence (IS) elements can lead to insertions that inactivate the gene of interest. The end result can be fermentation failure or the unwanted expression of a truncated product, both of which are economically problematic and potentially dangerous. In addition, reversion

of CRM197 into its toxic parent could have disastrous consequences. Reversion of CRM197 may have contributed to toxic activity that was detected in tissue culture cells (Qiao et al., 2008). Thus, expressions systems with reduced mutation rates may provide the highest reliability and productivity coupled with the lowest level of risk for reversion.

[0093] The single greatest factor contributing to the high price and short supply of CRM197 is the historical inability to generate high amounts of CRM197 in the production workhorse *E. coli*. CRM197 is insoluble when expressed in the cytoplasm of bacteria and requires re-folding prior to use when made in standard commercial *E. coli* strains. Since relatively low amounts of CRM197 are produced in conventional strains, a reduced genome *E. coli* strain, MDS42, was tested as a production host for insoluble CRM197 in shake flask culture.

[0094] Reduced genome strain MDS42 was produced by the methods described in International Patent Publication No. WO 2003/070880, which is incorporated herein by reference. Briefly, a series of reduced genome strains (MDS01-MDS39), were produced by making a series of 39 cumulative deletions (approximately 14.1% of the genome) of nucleic acid sequences from the parental strain *E. coli* MG1655. Hybridization to genome scanning chips (NimbleGen Systems, Madison, WI) containing the K-12 sequence and all sequences in the IS database revealed that MDS39, the first strain designed to lack all IS elements, unexpectedly contained additional copies of an IS element that had translocated to new locations during its production. These IS elements were deleted to produce MDS40. The *fhuACDB* (the *tonA* locus) was deleted from MDS40 to produce MDS41. The location and function of each cumulative deletion made to produce MDS01-MDS41 can be found at Table 2 of U.S. Patent No. 8,178,339, the entire content of which is incorporated herein by reference. The *endA* gene was then deleted from MDS41 to produce MDS42. Twenty-seven additional nucleic acid deletions were made in MDS42 to create MDS69. MDS42 and all strains based on MDS42 (MDS43, MDS44...MDS69 etc.) are free of insertion sequences.

[0095] For production of insoluble CRM197, a modified CRM197 sequence was employed comprising DNA sequence changes that result in a release of hairpin structures in the CRM197 sequence. The optimized CRM197 sequence removes secondary structure that inhibits translation initiation and enhances recognition of both the start site (ATG) and ribosomal binding site (RBS). See Figure 1.

[0096] The native CRM197 signal sequence was removed and the optimized CRM197 sequence (cyto-CRM197, SEQ ID NO: 3) amplified by PCR and subcloned into expression vector pSX2, which contains a Kanamycin resistance cassette and uses a lactose-inducible promoter to drive expression of the cloned sequences. Plasmid pSX2 containing CRM197 (lacking its native signal sequence) was transformed into reduced genome *E. coli* strain MDS42 and examined in shake flask culture. Briefly, 3 ml cultures were grown to saturation in Korz minimal medium (Korz DJ *et al.*, *J. Biotechnol.*, 39(1):59-65 (1995)) supplemented with 0.2% glucose and 50 µg/ml Kanamycin and used to inoculate 20 ml cultures to an initial OD₆₀₀ = 0.075. The growth temperature and inducer (IPTG) concentration that produced optimal levels of insoluble cytoplasmic CRM197 were then determined (in minimal media supplemented with the plasmid-selectable antibiotic kanamycin) using shake flasks. The optimal IPTG concentration was determined to be 250 µM. Figure 2 is an example of a protein gel analyzing total cell protein (T) and the soluble fraction (S) and insoluble fraction (I) following high speed centrifugation of total cell protein from three separate cultures grown to an OD₆₀₀ of 0.5 (late induction) prior to the addition of IPTG. Surprisingly high amounts of cyto-CRM197 were present in the insoluble fractions (see Figure 2, arrows). When quantified against protein standards, the shake flask results predict 10 to 12 g/L of cyto-CRM197 in a modest fermentation of OD₆₀₀ of 200. Production of insoluble CRM197 in the reduced genome *E. coli* host cell was 10 times higher than in conventional *E. coli* strains.

Example 2

Periplasmic Expression of Soluble CRM197 in Reduced Genome *E. coli* Hosts

[0097] Next, production of soluble CRM197 in reduced genome *E. coli* strains was tested by directing expression of CRM197 to the periplasmic space. CRM197 has

proved notoriously difficult to produce in a soluble form in *E. coli*. Export of highly expressed proteins to the periplasmic space aids stability by providing an optimal non-reducing environment for correct protein folding and formation of disulfide bridges. To this end, six signal sequences, in combination with a number of co-expressed chaperone proteins were examined to identify the signal sequence and chaperone protein that conferred the highest levels of periplasmic delivery of CRM197. Figure 3 illustrates the signal sequences examined and the co-expressed chaperone proteins. The signal sequences examined included representative signal sequences from each of the three *E. coli* secretion pathways

[0098] The CRM197 open reading frame (ORF), codon-optimized for *E. coli* (SEQ ID NO: 1), was ordered from DNA 2.0 (Menlo Park, CA). The CRM197 ORF was preceded by a sequence encoding a PelB signal sequence. The *pelB* and CRM197 ORF were flanked by sequences designed to facilitate cloning into the pSX2 expression vector. The nucleotide sequence of the 5' flanking sequence-PelB signal sequence-CRM197 ORF (including stop codon)-3' flanking sequence is provided at Table 1 below, with the flanking sequences underlined, the nucleotide sequence encoding the PelB signal sequence in bold, and the CRM197 ORF in plain text.

[0099] Table 1: *pelB* (bold)-CRM197 nucleotide sequence (plain text) + flanking sequences (underlined):

[00100] CCTCTAGAAATAATTTGTTAAC**TTTGAAGGAGAT**ATACATAT
GAAATA**ACTTGCTGCCAACCGCCGCCGGCCTGCTGCTGCTCGCAG**
CACAGCCGGCTATGGCAGGTGCGGATGATGTTGTGGACAGCTCTAAGTC
TTTGATGGAAAACTTAGCTCGTACCACGGTACGAAGCCAGGTTATGT
CGACAGCATTCAAAAGGTATCCAGAAACCGAAGTCCGGCACGCAGGGT
AACTACGACGACGATTGGAAAGAGTTCTACAGCACCGACAACAAGTATGA
CGCAGCGGGTTACAGCGTTACATGAGAATCCGTTGAGCGGCAAAGCGG
GTGGTGTGTCAAAGTGACGTATCCGGGTCTGACCAAGGTCTGGCGTTG
AAAGTTGATAACGCGGAAACCATTAAGAAAGAGCTGGGTCTGAGCCTGAC
CGAGCCGTTGATGGAGCAAGTTGGTACCGAAGAGTTATCAAACGTTCG
GCGATGGTGCAGCCGCGTTGTCCTGCCTTCGCGGAGGGCAGCT

CCAGCGTTGAGTATATCAATAACTGGGAGCAAGCAAAAGCGCTGTCCGTC
GAACTGGAAATCAATTGAAACGCGCGTAAACGTGGTCAAGATGCAAT
GTACGAGTATATGGCCCAGGCCTGCGCTGGTAATCGTGGTCAAGATGCAAT
TTGGTAGCAGCTTGTCTTGTATCAACCTGGATTGGATGTGATCCGTGATA
AGACCAAGACTAAGATCGAGAGCCTGAAAGAACATGGCCCATTAAAGAA
CAAGATGTCGGAGAGCCGAATAAGACCGTGAGCGAAGAAAAGGCCAAG
CAGTATCTGGAAGAGTTCCACCAAACGGCTCTGGAGCATCCGGAGCTGAG
CGAGCTGAAAACGGTTACGGCACCAACCCGGTGGCAGGTGCGAATT
ACGCGCGTGGCAGTGAATGTGGCGCAGGTGACTCGACTCCGAAACGGCG
GACAATTGGAGAAAACCACCGCAGCGCTGAGCATTCTGCCGGCATCGG
CAGCGTTATGGCATTGCAGATGGTGTGCACCATAACACTGAAGAAA
TCGTAGCGCAAAGCATTGCCCTGTCTAGCTGATGGTGGCGCAGGCTATT
CGCTGGTCGGCGAACTGGTTGATATCGGCTTGCTGCCTACAACTCGTTG
AAAGCATCATTAAACCTGTTCAAGGTGGTCCACAACAGCTATAATGCCCA
GCGTACAGCCCCGGTCACAAGACCCAACCGTCCGCACGATGGCTATGC
GGTGTCTTGGAACACGGCGAAGATAGCATCGTACCGGTTCCAGG
GCGAGAGCGGCCATGACATCAAGATTACTGCAGAAAATACCCGCTGCCG
ATCGCAGGTGTCCTGCTGCCTACGATTCCGGTAAGCTGGACGTTAACAA
AAGCAAAACCCACATTCTGTGAACGGTCGTAAGATTGCATGCGTTGTC
GTGCGATTGACGGCGACGTACCTTCTGCCGTCCGAAGAGGCCGGTCTAC
GTTGGTAATGGTGTGCACGCAACCTGCACGTGGCGTTCACCGCAGCAG
CTCGGAGAAAATCCATAGCAATGAGATTCTAGCGACAGCATTGGCGTTC
TGGGTTACCAAAAGACGGTTGACCATACCAAGTCAATTCCAAACTGAGC
CTGTTCTTGAGATCAAAAGCTAACTCGAGCCCCAAGGGCGACACCCCT

[00101] The nucleotide sequence encoding the PelB-CRM197 ORF was PCR amplified from the DNA 2.0 clone with a sense primer (GGAGATATACATATGAAATACTTGCTGCCAACC) and antisense primer (CTTGTTAGCAGCCGATTAGCTTTGATCTCAAAGAAC) to generate the flanking regions needed for cloning into the pSX2 vector.

[00102] Alternative signal sequences were fused to the CRM197 ORF using a 2 or 3 step PCR process. In the first step, a sense primer covering both the C-terminal coding

region of the signal sequence and the N-terminal coding region of CRM197 was used together with an anti-sense primer covering the C-terminal coding region of CRM197. In the second step, a primer completing the ORF of the signal sequence was used with the same primer covering the C-terminal coding region of CRM197. In the case of the OmpA-CRM197 construct, a third step was used that included a shorter primer covering the N-terminal region of the signal sequence and the same primer covering the C-terminal coding region of CRM197. Primers used to fuse the *E. coli* *ompA* and *OmpF* signal sequence to the CRM197 ORF are described below.

[00103] The following primers used to fuse the *E. coli* *ompA* encoded signal sequence to the CRM197 ORF. For Step 1, the sense primer = 5'-GCTACCGTAGCGCAGGCCGGTGCAGGATGATGTTGTGGA-3' and the antisense primer = 5'-CTTGTTAGCAGCCGATTAGCTTTGATCTCAAAGAAC-3'. For Step 2, the sense primer = 5'-GGAGATATACATATGAAAAAGACAGCTATCGCGATTGCAGTGGCAC TGGCTGGTTTCGCTACCGTAGCGCAGGCC-3' and the antisense primer = 5'-CTTGTTAGCAGCCGATTAGCTTTGATCTCAAAGAAC-3'. For step 3, the sense primer = 5'-GGAGATATACATATGAAAAAGACAGCTATCG-3' and the antisense primer = 5'-CTTGTTAGCAGCCGATTAGCTTTGATCTCAAAGAAC-3'.

[00104] The following primers were used to fuse the *ompF* encoded signal sequence to the CRM197 ORF. For Step 1, the sense primer = 5'-GTTAGTAGCAGGTACTGCAAACGCTGGTGCAGGATGATGTTGTGGA-3' and the antisense primer = 5'-CTTGTTAGCAGCCGATTAGCTTTGATCTCAAAGAAC-3'. For Step 2, the sense primer = 5'-GGAGATATACATATGATGAAGCGCAATATTCTGGCAGTGATCGTCCCTGC TCTGTTAGTAGCAGGTACTGCAAACGCT-3' and the antisense primer = 5'-CTTGTTAGCAGCCGATTAGCTTTGATCTCAAAGAAC-3'.

[00105] Completed signal sequence-CRM197 PCR products were cloned into the pSX2 expression vector. The termini of the signal sequence-CRM197 PCR products

possessed 15 bp of sequence that overlaps the sequence of the pSX2 vector. The pSX2 vector was linearized with the restriction enzymes Kpn I and Sac I to facilitate the cloning reaction. Cloning reactions were transformed into MDS42, MDS42*recA* or MDS42*recA* with a further deletion of IS609, to generate recombinant pSX2 expression vectors. The signal sequence-CRM197 region and flanking vector sequences were verified by sequence analysis.

[00106] Plasmid pSX2 containing the combinations of signal sequence and CRM197 sequence (lacking its native signal sequence) illustrated at Figure 3 (B) were transformed into reduced genome *E. coli* strain MDS42 or MDS42*recA* (MDS42 strain with a deletion of the *recA* gene (the *recA1819* allele)) and examined in shake flask culture. In addition to signal sequence and chaperone protein, culture variables examined included temperature, inducer (IPTG) concentration and time point at which the inducer was added (either early [OD_{600nm} of 0.01] or late [OD_{600nm} of about 0.4]). The following conditions were determined to be optimal for periplasmic secretion of CRM197 and these conditions were used in subsequent experiments: (i) a growth temperature of about 25 °C preceded by a brief 37 °C incubation (e.g. 2 hours) (ii) late induction (addition of IPTG at an OD₆₀₀ of about 0.4) and (iii) an inducer (IPTG) concentration between 15 and 35 μM (about 1/10 that required for optimal expression of cyto-CRM197).

[00107] Briefly, 3 ml cultures were grown to saturation in Korz minimal medium supplemented with 0.2% glucose and 50 μg/ml Kanamycin and used to inoculate 20 ml cultures to an initial OD₆₀₀ = 0.075. The 20 ml cultures (in 125 ml baffled Erlenmeyer flasks) were placed into a 37 °C shaking incubator (250 rpm) for 2 hours. The cultures were then shifted to a 25 °C shaking incubator and monitored until OD₆₀₀ was between 0.3-0.4. At that time, IPTG was added at the indicated concentrations. The induced cultures were incubated overnight in the 25 °C shaking incubator. Total induction time was between 18-22 hours. After induction, the OD₆₀₀ of the cultures was determined. Aliquots of the culture representing 2 OD units were processed to create periplasmic samples. The periplasmic samples were prepared with the aid of Periplasting Buffer (Epicentre, Madison, WI). The 2 OD sample was harvested by centrifugation at 7500xg for 10 minutes in a 1.5 ml Eppendorf tube. The supernatant

was removed and the cell pellet gently resuspended in 50 μ l of Periplasting Buffer (200 mM Tris-HCl [pH 7.5], 20% sucrose, 1 mM EDTA, and 30 U/ μ l Ready-Lyse Lysozyme). After 5 minutes at room temperature, 50 μ l of ice cold water was rapidly added to the resuspended pellet. The mixture was incubated on ice for 5 minutes prior to fractionating the periplasmic fraction from the spheroplasts by centrifuging at 4000xg for 15 minutes. The supernatant representing the periplasmic fraction was prepared for SDS-PAGE analysis. An amount equivalent to 0.12 OD units was loaded per lane.

[00108] The most successful signal sequences and induction characteristics that resulted in the highest secretion of CRM197 into the periplasm are shown in Figure 4. The periplasmic signals OmpA and OmpF were found to facilitate the greatest movement of CRM197 into the periplasm. None of the three co-expressed chaperone proteins influenced periplasmic delivery differently (as an example expression is compared with and without, YccA in Figure 4). Since OmpA and OmpF appeared to result in roughly similar amounts of periplasmic CRM197, OmpA-CRM197 was used in subsequent experiments.

[00109] Since expression of components of the sec-dependent pathway that include *ompA* and *ompF* can be subject to catabolite repression, the influence of glycerol as a carbon source for production of *ompA*-CRM197 was compared to glucose in reduced genome *E. coli* strain MDS42 in shake flask cultures under the conditions described above. As illustrated at Figure 5, minimal media supplemented with glycerol resulted in dramatically lower levels of CRM197 expression compared to glucose. Glucose was therefore used as a carbon source in all subsequent experiments.

[00110] Next, the production of periplasmic CRM197 in several different reduced genome *E. coli* host cells was compared. Thus, a series of deletions within either the MDS42 or MDS69 strain background were examined for their effect on production of periplasmic (soluble) CRM197 in shake flask cultures based on the optimal conditions described above for MDS42 that contained either (i) deletions that optimized cell metabolism or (ii) deletions that remove or reduce the level of proteases (e.g. Blon) that could adversely influence CRM197 expression. The following reduced genome

E. coli strains based on MDS42 were tested: MDS42*recA*, MDS42metab, and MDS42Blon/metab. MDS42metab was created by (i) deleting the *iclR* (b-number b4018, described at NCBI Entrez GeneID No. 948524) and *arpA* genes (b-number b4017, described at NCBI Entrez GeneID No. 944933) (ii) deleting the *rph* gene (b3643) (thereby increasing transcriptional levels of the downstream *pyrE* gene), and (iii) correcting the *ilvG* frameshift mutation by insertion of an AT dinucleotide at position 982 (resulting in expression of active acetohydroxy acid synthase II). MDS42Blon/metab contains the modifications described for MDS42metab as well as a modification of the *lon* protease (b0439) promoter region to mimic the sequence of the *lon* promoter region of B strain *E. coli*, in which an IS insertion separates the -35 region from the -10 region of the ancestral *E. coli lon* promoter. The following reduced genome *E. coli* strains based on MDS69 were tested: MDS69metab (MDS69 strain modified as described above for MDS42metab), MDS69Blon/metab (MDS69metab further altered to include the Blon protease modification, MDS69lpp/metab (MDS69metab further modified to delete lipoprotein lpp (b1677), and MDS69Blon/lpp/metab (MDS69metab further modified to include both the Blon protease modification and lipoprotein *lpp* gene deletion).

[00111] Figure 6 compares OmpA-CRM197 expression in these strains. Of the eight strains examined, the highest levels of CRM197 expression were evident in those strains (on either MDS42 or MDS69 backgrounds) that contained deletions aimed at enhancing metabolic activity (metabolism strains). However, all strains tested contained a surprisingly large amount of periplasmic CRM197 (Fig. 6 panels B and D) that was also evident on protein gels in total cellular protein preparations (Fig. 6 panels A and C). Importantly, these results indicate that the protease deletion Blon did not influence CRM197 levels in the metabolism strains. In addition, removal of the highly abundant lipoprotein protein Lpp, thought, by its absence, to “free up” the sec-dependent periplasmic transport system was also not found to influence periplasmic CRM197 levels. The media from these experiments was isolated post-induction and examined for CRM197 release. CRM197 was not identified in media from any of the strains examined. Table 2 is a summary of yield results for the MDS strains that generated the highest periplasmic CRM197 expression levels. These results were obtained by comparing stain intensities of CRM197 from the four strains

indicated with protein standards run on the same gel. The shake flask values were extrapolated to predict quantities of CRM197 in fermentations that reach either 100 or 200 OD₆₀₀. The four strains shown in Table 2 typically reach ODs of 300 in fed-batch fermentation suggesting that these strains have the capacity for generating far more CRM197 than is currently possible in conventional strains.

Table 2

Expression Strain with pSX2-ompA CRM197	Periplasmic Samples	#ODs loaded	Calibrated Volume ngs/lane	% Target Protein in Periplasm	AVG g/L at 100 ODs	AVG g/L at 200 ODs
MDS42 Metabolism	Peri, Late 25 µM IPTG, 25°C o/n	0.06	1102	48%	1.75	3.5
		0.03	499	53%		
MDS69 Metabolism	Peri, Late 25 µM IPTG, 25°C o/n	0.06	1047	43%	1.62	3.24
		0.03	449	46%		
MDS42 Δprotease	Peri, Late 35 µM IPTG, 25°C o/n	0.06	825	47%	1.24	2.48
		0.03	330	45%		
MDS69 Δprotease	Peri, Late 25 µM IPTG, 25°C o/n	0.06	1028	28%	1.7	3.4
		0.03	506	30%		

[00112] CRM197 is highly sensitive to proteolytic cleavage which has rendered production of high quality CRM197 challenging (Bishai et al., J. Bacteriol., 169:5140-51 (1987); Recombinant Production of Carrier Proteins, GEN News, Dec. 1, 2012). In a separate set of experiments, production of periplasmic CRM197 was examined in a series of protease deletion strains to determine whether the targeted removal of protease genes from the reduced genome *E. coli* strains would result in an increase in CRM197 in the periplasm. Thus, the following protease encoding genes were deleted separately in combination: *degP* (b0161), *prc* (b1830), *htpX* (b1829), as well as portions of the *lon* promoter region. Deletion of the protease genes, either individually or in combination, did not influence CRM197 expression levels. See Figure 7, illustrating that reduced genome *E. coli* strain MDS42, modified to delete the specified combination of protease genes, had no effect on periplasmic expression of CRM197. This data indicate that proteolytic cleavage of CRM197 does not occur when produced in reduced genome *E. coli* strains based on MDS42 or MDS69 presumably due to the low levels of protease activity in these strains.

Example 3**CRM197 Production in Fed-Batch Fermentation**

[00113] Next, commercial scale-up of CRM197 in reduced genome *E. coli* strains was examined. Thus, OmpA-CRM197 in the MDS42 metabolism strain was subjected to fed-batch fermentation in defined minimal media at the 10 liter scale. Fermentation conditions included a batch phase at 37 °C that was inoculated to 0.18 OD and allowed to grow until the 1% glucose in the batch medium has been consumed (~7.5 hrs). The fed batch phase was triggered by the DO spike that occurs when the batch medium is depleted of glucose. The feed began with an exponential feed rate to produce a growth rate of 0.3 Mu (1/h) controlled gravimetrically (~12.5hrs). The induction point was determined to be the point at which the available phosphate was nearly depleted. At a point around 2 hours prior to the induction point, the temperature was shifted to 25 °C and the feed rate was lowered to a rate that produces a growth rate of 0.2 Mu (1/hr). Once the inducer is added (100uM) the feed was changed to a constant rate such that 80g of glucose is added per hour for about 7hrs. The fermentation OD₆₀₀ approached 300 and generated a very high level of periplasmic targeted CRM197 as illustrated at Figure 8. A second fermentation at the optimal conditions resulted in periplasmic CRM197 levels of about 2 g/L indicating a high level of consistency in test fermentations.

[00114] The results described above demonstrate the surprising yield of soluble CRM197 obtained in reduced genome *E. coli* production hosts such as MDS42 and MDS69 in both shake-flask and 10 L fed-batch fermentation.

[00115] One problem observed during preliminary fermentation analysis was a reduction in the soluble form of CRM197 in total cell protein isolations. Since periplasmic isolation methods are not applicable to large scale, a general method of soluble CRM197 isolation was developed. Initial experiments were performed to determine whether the CRM197 observed following conventional total cell protein (TCP) isolation that was insoluble could be isolated in a soluble form. Thus, OmpA-CRM197 in the MDS42_{recA} strain was subjected to fed-batch fermentation in defined minimal media at the 10 liter scale as described above (including incubation at 37 °C

followed by a short period of incubation at 25 °C prior to the addition of inducer). The cells, containing high amounts of periplasmic CRM197, were subjected to standard detergent digestion with a commercially available non-ionic detergent-based buffer to isolate total cell protein (TCP). Samples of total cell protein were centrifuged for 10 minutes at high speed (21k g) and the soluble fraction was isolated. Samples of TCP and the soluble fraction were analyzed. As illustrated at Fig. 9, Panel A, the soluble periplasmic form of CRM197 was rendered completely insoluble by detergent homogenization. Conversely, when periplasmic preparations (as described above) were subjected to high speed centrifugation, periplasmic CRM197 was retained in a soluble form as expected. See Fig. 9, Panel B.

[00116] In an attempt to recover the fraction of CRM197 that was insoluble, detergent-based bacterial cell lysis was compared with mechanical methods of cell lysis which would be more conducive to production-level platforms for generating CRM197 compared to detergent lysis and would eliminate the need to isolate periplasm in a commercial scale-up process. In addition, lysis was performed in the presence of chemical agents known to enhance protein solubilization as described at Table 3 below:

[00117] Table 3: List of lysis method and solubilization agent.

Agent to enhance solubilization	% soluble CRM197 after sonication
Imidazole, 250 mM	107%
Trehalose, 50 or 250 mM	64%, 79%
Glutathione in 5:1 reduced to oxidized state	104%
Glycerol, 10%	68%
Sucrose, 10%	75%
No agent	88%

[00118] Sonication and microfluidization were performed in a 50 mM TrisHCl buffer (pH 8) and all lysis methods were carried out in the presence of Lysonase™, a commercial mixture of lysozyme and benzonase (Novagen, Darmstadt, Germany).

Each of the agents listed in Table 3 were then tested in separate preparations. Figure 10 is an example of a series of isolations that were performed by detergent or mechanical lysis. Soluble CRM197 was not obtainable using detergent lysis and only small increases in soluble CRM197 were evident using detergent lysis that included solubilization agents. Glycerol and sucrose modestly enhanced the amount of soluble CRM197 found in the soluble fraction when compared to detergent alone (Fig. 10, Panel A). However, mechanical lysis dramatically increased the levels of CRM197 in the soluble fraction. In fact, a dramatic increase of CRM197 levels was evident in the soluble fraction from all samples that underwent mechanical lysis, whether sonication (Fig. 10, Panel B) or microfluidization was used. Further, the amounts of soluble CRM197 obtained following mechanical lysis did not differ markedly by solubilization agent (compare “no agent” with all other agents in Table 3). A compilation of results generated from the mechanical lysis method suggests that CRM197 in MDS42 (using culture conditions that include a short 37 °C incubation followed by growth at 25 °C and late stage induction with 25-35 µM IPTG) comprises 7.2-8.3% of the total cellular protein and between 6.3 and 7.7% of soluble protein. These results are intriguing because mechanical lysis is the standard method of cell disruption used in large scale commercial fermentations and imply the capability of generating high amounts of soluble CRM197.

[00119] Based on the aforementioned data, a suitable commercial protocol for generating soluble CRM197 comprises fermentation of reduced genome *E. coli* host carrying an expression vector encoding CRM197 coding sequence fused to a periplasmic signal sequence (e.g. encoded by *ompA* or *ompF*) at 25 °C in which the cells are collected by low speed centrifugation, lysed by mechanical means (e.g. sonication or microfluidization) in a suitable buffer (e.g. 50 mM Tris-HCl buffer at pH ~8). Following centrifugation to remove debris, soluble CRM197 is then isolated from the supernatant. In shake flask cultures incubated at 25 °C and 25-35 mM IPTG, between 95 and 100% of CRM197 was isolated in a soluble form.

[00120] A summary of the results of fermentations using reduced genome *E. coli* strain MDS69 metab (as described above) carrying an expression vector containing an *ompA*-CRM197 fusion is shown at Table 4 below. These fermentations occurred

under fed-batch conditions using defined minimal media and the addition of inducer IPTG late in logarithmic growth. The fermentation scale was 10 liters. By altering the inducer concentration the amount of periplasmic CRM197 was increased from 0.5 to about 2 g/L.

Table 4: Fed-batch fermentations of strain T69 metabolic using glucose feed		Max yield@ 29 hours	
Fermentation	Induction Level	OD	Soluble CRM ₁₉₇ Yield
Ferm 157	25 uM	256	0.74 g/L
Ferm 158	50 uM	262	1.62 g/L
Ferm 159	100 uM	291	1.96 g/L

[00121] Figure 11 illustrates specifics of the fermentation employing a 100 μ M inducer concentration. Gels from this fermentation comparing total cell protein (TCP) isolates with soluble (Sol) and insoluble (Insol) fractions clearly indicated robust expression of soluble CRM197 during fermentation (see Fig. 12).

[00122] Optimal conditions for production of soluble CRM197 in fed-batch fermentation of reduced genome *E. coli* host strains were as follows. With respect to temperature, initiation of growth in the batch phase by incubating at 37 °C followed by a temperature shift to between 20 and 25 °C prior to addition of inducer (in this case IPTG) was optimal. Optimal pH range is between 6.5 and 7.5 (e.g. 6.5, 7.0 or 7.5). Optimal inducer concentration is between 100 and 250 μ M IPTG (added during late log phase of growth). With respect to media conditions, minimal media conditions were determined to be adequate and have the advantage of reduced cost and defined conditions free from animal derived products. Importantly, conventional *E. coli* strains do not grow robustly in minimal media. Employing these optimal conditions, it is estimated that a target yield of at least 4 g/L of soluble CRM197 can be reliably produced in 10 L scale fermentations using reduced genome *E. coli* host strains (e.g. MDS42 or MDS69).

Example 4
Downstream Processing of CRM197

[00123] Following production of CRM197 in reduced genome *E. coli* and mechanical lysis, the CRM197 can be purified. To determine whether CRM197 produced from MDS69 metab under fermentation conditions is amenable to purification, a small scale purification was performed using a combination of hydrophobic interaction chromatography (phenyl sepharose) and anion exchange chromatography (DEAE-cellulose). 50 OD units of the 28 hr fermentation sample shown in Fig. 11 was subjected to homogenization using a microfluidizer (MF) in a 10 mM sodium phosphate buffer (pH 7.5) solution. The resulting homogenate was centrifuged at 21,000 g for 10 minutes and the soluble and insoluble (IS) fractions were isolated. Using Western blotting and polyclonal antibodies against diphtheria toxin (DPX), CRM197 was found to be highly enriched in the soluble fraction (Fig. 13, panel A compares the microfluidizer (MF) and the resuspended insoluble (IS) fraction with the pre-column soluble fraction at three concentrations (0.1, 0.07 and 0.04 OD)). The soluble fraction (25 OD equivalent) was filtered (0.45 μ m), brought to 13% (wt/vol) ammonium sulfate and loaded onto a phenyl sepharose column (Phenyl sepharose HP HiTrap, General Electric) that was previously equilibrated in 10 mM sodium chloride, 10 mM sodium phosphate buffer, pH 7.5. The column was washed using 0.6 M ammonium sulfate, 6 mM sodium phosphate buffer, pH 7.5 and CRM197 was eluted under low salt conditions (10 mM sodium choloride, 10 mM sodium phosphate buffer, pH 7.5). The five 2.5 mL eluted fractions were then analyzed by anti-DPX Western blot and protein staining (Fig. 13, Panel A, lanes labeled 10 mM NaCl). A small amount of unprocessed CRM197 (Fig. 13, Panel A, arrows) was purified away from the main eluted sample with a final wash with distilled water. The fractions circled in Fig. 13 Panel A were then pooled, diluted 1:2 with distilled water and loaded onto a column containing 1 ml of DEAE sepharose fast flow (Pharmacia) that had been equilibrated in 10 mM sodium chloride, 10 mM sodium phosphate, pH 7.5. After loading the sample and collecting the flow through, the column was washed with 3 volumes of 50 mM sodium chloride, 0.5 mM sodium phosphate buffer, pH 7.5. CRM197 eluted using increasing sodium chloride concentrations: 100 mM NaCl (2 times 3 ml), 1 M NaCl (3 ml) and 1.5 M NaCl (3

ml). SDS-PAGE analysis revealed that the most highly pure soluble CRM197 was eluted using 1 M NaCl, although a significant amount still remained bound to the column.

[00124] These results indicate that CRM197 produced in reduced genome *E. coli* host strains is highly soluble and can be isolated to high purity using existing purification methods.

Example 5

CRM197 Production in Reduced Genome *E. coli* Hosts Compared to Wild Type Strains

[00125] Periplasmic production of CRM197 in reduced genome *E. coli* strains was compared to the production of CRM197 in wild type *E. coli* strains under similar conditions. Thus, CRM197 *E. coli* BLR(DE3) strain was transformed with pSX2 vector carrying an OmpA-CRM197 fusion and periplasmic production was assessed and compared to periplasmic production of CRM197 in reduced genome *E. coli* strain MDS42~~recA~~. Fermentation conditions were as described above. Following a brief growth initiation phase at 37 °C, cells were grown in Korz media supplemented with 0.2% glucose (and 31 µg/ml of Isoleucine for BLR(DE3) cultures) at 25 °C for 19 hours. Expression of CRM197 was induced at OD = 0.3 with 15 or 25 mM IPTG.

[00126] As illustrated at Figure 14, at least a ~5-fold increase in production of periplasmic CRM197 was observed in the reduced genome *E. coli* host compared to the wild type B strain.

[00127] Additional experimentation revealed that the OmpF-CRM197 fusion actually resulted in a higher amount of soluble periplasmic CRM197 in reduced genome *E. coli* hosts compared to the OmpA-CRM197 fusion. Reduced genome *E. coli* host strain MDS69 metab and MDS69 lowmut (MDS69 strain further comprising deletions of *polB* (b0060), *dinB* (b0231) and *umuDC* (b1183-b1184)) were transformed with an expression vector encoding an OmpF-CRM197 fusion and periplasmic expression of CRM197 was compared to that in a MDS69 lowmut host

carrying an expression vector encoding an OmpA-CRM197 fusion under the same conditions. Following a brief growth initiation phase at 37 °C, cells were grown in Korz media supplemented with 0.2% glucose at 25 °C for 23 hours. Expression of CRM197 was induced at OD = 0.3 to 0.34 with 25 or 35 mM IPTG. Periplasmic proteins were isolated and the expression of soluble CRM197 in each strain was analyzed. As illustrated at Figure 15, a higher yield of CRM197 was obtained with the OmpF-CRM197 construct compared to the OmpA-CRM197 construct.

Example 6

Testing CRM197 Production with a Variety of Signal Sequences in a reduced genome
E. coli strain

[00128] Signal sequences were selected based on their abundance in the periplasm of *E. coli* B and K strains as determined by 2D gel analysis of periplasmic fractions (Han, Mee-Jung *et al.*, Journal of Bioscience and Bioengineering, 117(4):437-442 (2014)). Table 5 lists the signal sequences selected and their relative abundance in the periplasm of B and K strains:

Table 5

Protein	Abundance in Periplasmic Fraction (B and/or K)	Gene/Protein Function
MglB	K	methyl galactose transporter
MalE	B + K	maltose transporter
OppA	B + K	oligopeptide transporter
RbsB	B + K	subunit ribose transporter
Agp	B > K	glucose-1 phosphatase, 3-phytase
FkpA	B > K	peptidyl-prolyl cis-trans isomerase; in protein folding
YtfQ	B ≫ K	galactofuranose binding protein, subunit ABC transporter
HdeA	K	Stress response induced by acidic conditions
HdeB	K	Stress response induced by acidic conditions
GlnH	B > K	subunit of glutamine ABC transporter

[00129] Plasmid pSX2 containing the combinations of signal sequence and CRM197 sequence (lacking its native signal sequence) illustrated at Table 5 and Figure 17 (MglB, MalE, OppA, RbsB, Agp, FkpA, YtfQ, HdeA, HdeB or GlnH; OmpF and OmpC were tested as well) was transformed into reduced genome *E. coli* strain MDS69 metab (T69 Meta in Figure(s) 18-21) and examined in shake flask culture. As described above, MDS69 metab comprises the following modifications on an MDS69 background (i) deletion of the *iclR* (b-number b4018, described at NCBI Entrez GeneID No. 948524) and *arpA* genes (b-number b4017, described at NCBI Entrez GeneID No. 944933) (ii) deletion of the *rph* gene (b3643), and (iii) correction of the *ilvG* frameshift mutation by insertion of an AT dinucleotide at position 982. Briefly, colony forming units of the transformed bacteria from MOPS minimal medium-kanamycin (MMM/Kan)-glucose streak plates were resuspended in 3 ml Korz minimal medium supplemented with 0.2% glucose and 50 µg/ml Kanamycin and incubated at 37 °C overnight to generate the starter culture. Starter culture was used to inoculate 20 ml Korz/0.2% glucose/Kan in 125 ml Erlenmeyer-flasks to OD₆₀₀ = 0.05 and grown at 37 °C for 1.5 hours and then shifted to 25 °C and grown until OD₆₀₀ ~0.3. At that point, inducer (IPTG) was added at 25 µM, 35 µM or 50 µM concentration (late induction). The late inductions were then grown at 25 °C for 20 hours and 2 ODs of culture were harvested. Total cell protein was prepared using BugBuster + Lysonase and periplasmic and spheroplast fractions were prepared using Epicentre Periplasting Method.

[00130] Figures 18-20 depict the periplasmic yield of (soluble) CRM197 at 25 µM, 35 µM or 50 µM inducer concentration respectively using the indicated signal sequences (Induction A - OmpF, MalE, HdeA, OppA, HdeB, GlnH; Induction B - OmpF, MglB, Agp, OmpC, RbsB, FkpA, YtfQ). Good yield was obtained with all signal sequences at the 25 µM inducer concentrations (Figure 18). Interestingly, at the 35 µM inducer concentration, yield of CRM197 with the YtfQ signal sequence significantly increased relative to the yield of CRM197 obtained at this inducer concentration with the other tested signal sequences (Figure 19). This effect became even more significant at the 50 µM inducer concentration, with the yield of CRM197 remaining high whereas the yield of CRM197 with the other tested signal sequences was significantly reduced at this inducer concentration (Figure 20). Thus, the

combination of CRM197 and YtfQ signal sequence was determined to have a significantly broader induction range than the combination of CRM197 with the other signal sequences tested.

[00131] To further assess the induction range for CRM197 with the YtfQ signal sequence, two cultures each of 8 IPTG (inducer) levels were tested in MDS69 metab (0, 25, 35, 50, 75, 100, 150 and 250 μ M) according to the method described above. As a control, 2 cultures each of 4 IPTG levels for MDS69 metab with CRM197 and the OmpF signal sequence were also tested (0, 25, 35, 50 μ M). 2 OD samples were collected for total cell protein (TCP) and periplasmic analysis on Caliper.

[00132] The averaged results of the two cultures tested for each inducer level is illustrated at Figure 21. Yield of CRM197 in combination with the YtfQ signal sequence (YtfQ-CRM197) remained high across all inducer levels up to 100 μ M. Yield of CRM197 in combination with OmpF, however, was high only at the 25 and 35 μ M inducer concentrations. Figure 22 is a protein gel comparing the effect of OmpF and YtfQ signal sequence on CRM197 yield in periplasm (P) and media (M) at 50 μ M IPTG (OmpF) and at 50, 75, 100, 150 and 250 μ M IPTG (YtfQ). A surprisingly large amount of periplasmic CRM197 was evident at the 50, 75 and 100 μ M inducer concentration for the YtfQ signal sequence whereas a much smaller amount of periplasmic CRM197 was present at the 50 μ M inducer concentration for the OmpF sequence.

[00133] “Briefly, colony forming units of the transformed bacteria from MOPS minimal medium-kanamycin (MMM/Kan)-glucose streak plates were resuspended in 3 ml Korz minimal medium supplemented with 0.2% glucose and 50 μ g/ml Kanamycin and incubated at 37 °C overnight to generate the starter culture. Starter culture was used to inoculate 20 ml Korz/0.2% glucose/Kan in 125 ml Erlenmeyer-flasks to OD₆₀₀ = 0.05 and grown at 37 °C for 1.5 hours and then shifted to 25 °C until OD₆₀₀ ~0.3. At that point, inducer (IPTG) was added at 25 μ M, 35 μ M or 50 μ M concentration (late induction). The late inductions were then grown at 25 °C for 20 hours and 2 ODs of culture were harvested. Total cell protein was prepared using

BugBuster + Lysonase and periplasmic and spheroplast fractions were prepared using Epicentre Periplasting Method”

[00134] Next, the effect of very late induction ($OD_{600} \sim 2$) on CRM197 yield in combination with either OmpF or YtfQ signal sequence in MDS69 metab and in combination with OmpF in an *E. coli* B strain (BL21DE3) was assessed. Briefly, 3 ml Korz minimal medium supplemented with 0.2% glucose and 50 μ g/ml Kanamycin was inoculated with colony forming units of transformed MDS69 metab or BL21DE3, incubated at 37 °C overnight and used to inoculate 15 ml of Korz minimal medium supplemented with 0.2% glucose and 50 μ g/ml Kanamycin in 125 ml Erlenmeyer Flasks which was grown overnight at 25 °C to generate the 25 °C starter culture. The starter culture was used to inoculate 90 ml Korz/0.2% glucose/kanamycin in 500 ml Erlenmeyer flasks to $OD_{600} = 0.1$ followed by growth at 25 °C until the $OD_{600} > 2$ (saturated or near saturated) and then split into 4 x 20 aliquots in 125 ml Erlenmeyer flasks for induction at various IPTG concentrations (very late induction). The inductions were grown at 25 °C for 20 hours, and 2 ODs of culture harvested for analysis. Total cell protein (TCP) was prepared using BugBuster + Lysonase. Periplasmic and spheroplast fractions were prepared using the Epicentre Periplasting Method. As shown in Figure 23, good periplasmic expression up to 100 μ M IPTG was observed for the combination of CRM197 and OmpF signal sequence in MDS69 metab which decreased at 200 mM inducer concentration, presumably due to insolubility. Good periplasmic expression was observed for the combination of CRM197 and YtfQ signal sequence in MDS69 metab up to 400 μ M IPTG (the highest concentration tested) with no insoluble CRM197 observed. Weak expression was observed for the combination of CRM197 and OmpF in BL21(DE3) strain at all inducer concentrations tested (25-200 μ M). CRM197 was not observed in spheroplasts.

[00135] Summary – The data presented demonstrates that production of soluble CRM197 in reduced genome *E. coli* hosts delivered yields that were 10 times that obtained by conventional methods. Production in the reduced genome *E. coli* hosts is expected to increase the efficiency and reduce manufacturing costs. Moreover, production in reduced genome *E. coli* hosts will also be cleaner and safer than that

produced in conventional bacteria with non-reduced genomes. These improvements will have a wide impact on production of pharmaceutical protein products and ultimately broaden access to vaccines for at-risk populations who need them.

Moreover, high yield of CRM197 was observed in combination with a broad range of signal sequences. The broad induction range observed for YtfQ signal sequence in combination with CRM197 was surprising since YtfQ is found in much larger quantities in B strain *E. coli* compared to K strain *E. coli* and the exemplified reduced genome strains are based on a K strain. The broad induction range of CRM197 in combination with YtfQ in reduced genome *E. coli* is a significant advantage because the concentration of inducer can vary during production of the protein and accordingly the use of the YtfQ signal sequence in combination with CRM197 in these hosts results in a further increase in yield of CRM197.

What is claimed is:

1. A method for producing a recombinant CRM197 in a reduced genome *E. coli* host comprising incubating a reduced genome *E. coli* comprising an expression vector comprising a nucleotide sequence encoding a CRM197 protein fused to a signal sequence that directs transfer of the CRM197 protein to the periplasm operably linked to an expression control sequence under conditions suitable for the expression of the recombinant CRM197 protein, whereby a yield of at least 1 gram per liter of soluble CRM197 is obtained and wherein the native parent *E. coli* strain is a K12 strain, preferably K12 MG1655.
2. The method of claim 1, whereby a yield of at least 2 grams per liter, at least 3 grams per liter, at least 4 grams per liter, or at least 5 grams per liter of soluble CRM197 is obtained..
3. The method of claim 2, wherein the signal sequence is selected from the group consisting of an OmpA, OmpF, MglB, MalE, OppA, RbsB, Agp, FkpA, YtfQ, HdeA, HdeB, OmpC and GlnH signal sequence.
4. The method of claim 3, wherein the signal sequence is OmpF or YtfQ.
5. The method of claim 4, wherein the signal sequence is YtfQ.
6. The method of any one of claims 1 to 5, wherein the reduced genome *E. coli* has a genome that is genetically engineered to be from about 2% to about 40% smaller, preferably about 5% to about 30% smaller, than *E. coli* strain MG1655.
7. The method of claim 6, wherein the reduced genome *E. coli* has deleted therefrom at least the following DNA segments: b0245-b0301, b0303-b0310, b1336-b1411, b4426-b4427, b2441-b2450, b2622-b2654, b2657-b2660, b4462, b1994-b2008, b4435, b3322-b3338, b2349-b2363, b1539-b1579, b4269-b4320, b2968-b2972, b2975-b2977, b2979-b2987, b4466-4468, b1137-b1172, b0537-b0565, b0016-b0022, b4412-b4413, b0577-b0582, b4415, b2389-b2390, b2392-b2395, b0358-b0368, b0370-b0380, b2856-b2863, b3042-b3048, b0656, b1325-b1333, b2030-b2062, b2190-b2192, b3215-b3219, b3504-b3505, b1070-b1083, b1878-b1894, b1917-b1950, b4324-b4342, b4345-b4358, b4486, b0497-b0502, b0700-b0706, b1456-b1462, b3481-b3484, b3592-b3596, b0981-b0988, b1021-b1029, b2080-b2096, b4438, b3440-b3445, b4451, b3556-b3558, b4455, b1786, b0150-b0153 and b2945 of the *E. coli* K-12 strain MG1655.

8. The method of claim 7, wherein the reduced genome *E. coli* has additionally deleted therefrom at least the following DNA segments: b0315-b0331, b0333-b0341, b0346-b0354, b2481-b2492, b2219-b2230, b4500, b3707-b3723, b0644-b0650, b4079-4090, b4487, b4092-b4106, b0730-b0732, b3572-b3587, b1653, b2735-b2740, b2405-b2407, b3896-b3900, b1202, b4263-b4268, b0611, b2364-b2366, b0839, b0488-b0500, and b0502 of the *E. coli* K-12 strain MG1655.

9. The method of claim 7, wherein the reduced genome *E. coli* is strain MDS42.

10. The method of claim 8, wherein the reduced genome *E. coli* is strain MDS69.

11. The method of any one of claims 1 to 10, wherein the reduced genome *E. coli* comprises a functional *recA* (b2699) gene.

12. The method of any one of claims 1 to 11, wherein the reduced genome *E. coli* comprises a *relA* gene having at least one point mutation at position 547 or 548 of the coding sequence of the *relA* gene, wherein the mutation is selected from one or more of: a G → A mutation at position 547, a G → T mutation at position 547, a C → G mutation at position 548, or a C → T mutation at position 548.

13. The method of any one of claims 1 to 12, wherein the reduced genome *E. coli* lacks functional *polB* (b0060), *dinB* (b0231) and optionally *umuDC* (b1183-b1184) genes.

14. The method of any one of claims 1 to 13, wherein the reduced genome *E. coli* comprises the following modifications: (i) deletion of the *rph* gene to enhance orotate phosphoribosyltransferase activity (ii) introduction of a mutation which complements a native -2 frameshift mutation in the *ilvG* gene to produce active acetohydroxy acid synthase II and (iii) deletion of the *iclR* and *arpA* genes.

15. The method of any one of claims 1-14, wherein the reduced genome *E. coli* does not comprise insertion sequences.

16. The method of any preceding claim, wherein the CRM197 nucleotide sequence is optimized for expression in the *E. coli* host cell.

17. The method of any preceding claim comprising
(a) growing the reduced genome *E. coli* and
(b) inducing expression of CRM197.

18. The method of claim 17, wherein the method is carried out in a fermentor.
19. The method of claim 17 or 18, wherein step (a) comprises growing the reduced genome *E. coli* at 37 °C for up to 19 hours followed by growth at about 20-30 °C, preferably about 25 °C prior to and subsequent to step (b).
20. The method of any one of claims 17 to 19, wherein steps (a) and (b) are performed in growth medium that does not comprise serum, yeast extract or other animal by-products.
21. The method of any one of claims 18 to 21, wherein in step (a) the pH ranges between 6.5 and 7.5.
22. The method of any one of claims 17 to 21, wherein the pH is maintained using a phosphate buffer, a Tris buffer or a histidine buffer.
23. The method of claim 22, wherein the buffer is a phosphate buffer.
24. The method of any one of claims 17 to 23, wherein expression is induced in step (b) by addition of a suitable amount of IPTG.
25. The method of any one of claims 18-24, wherein expression is induced at an OD₆₀₀ of from 100 to 400, preferably between 200 to 275, more preferably between 230 and 250.
26. The method of any one of claims 18 to 25 wherein the fermentor contains 0.5-50,000 liters of culture.
27. The method of any of claims 17 to 26 comprising a further step of (c) mechanically disrupting the cultured reduced genome *E. coli* cells in the absence of detergent and centrifuging the resulting cell lysate to obtain a soluble fraction.
28. The method of claim 27, wherein the mechanical disruption comprises sonication or microfluidization.
29. The method of claim 27 or 28, wherein CRM197 is purified from the soluble fraction by one or more purification steps.
30. The method of claim 29, wherein the one or more purification steps comprises hydrophobic interaction chromatography and/or anion exchange chromatography.
31. The method of claim 30, wherein the purification steps comprise hydrophobic interaction chromatography followed by anion exchange chromatography.

32. The method of claim 1, wherein the nucleotide sequence encoding a CRM197 protein is not fused to a nucleotide sequence encoding a signal sequence, whereby a yield of about 2 grams per liter to about 20 grams per liter of insoluble CRM197 is obtained

33. The method of claim 32, wherein the insoluble CRM197 is subjected to one or more solubilization steps.

34. The method of claim 1, wherein the nucleotide sequence encoding a CRM197 protein is fused to an *ompF* or *ytfQ* signal sequence and wherein the reduced genome *E. coli* host comprises the following modifications: (i) deletion of the following DNA segments: b0245-b0301, b0303-b0310, b1336-b1411, b4426-b4427, b2441-b2450, b2622-b2654, b2657-b2660, b4462, b1994-b2008, b4435, b3322-b3338, b2349-b2363, b1539-b1579, b4269-b4320, b2968-b2972, b2975-b2977, b2979-b2987, b4466-4468, b1137-b1172, b0537-b0565, b0016-b0022, b4412-b4413, b0577-b0582, b4415, b2389-b2390, b2392-b2395, b0358-b0368, b0370-b0380, b2856-b2863, b3042-b3048, b0656, b1325-b1333, b2030-b2062, b2190-b2192, b3215-b3219, b3504-b3505, b1070-b1083, b1878-b1894, b1917-b1950, b4324-b4342, b4345-b4358, b4486, b0497-b0502, b0700-b0706, b1456-b1462, b3481-b3484, b3592-b3596, b0981-b0988, b1021-b1029, b2080-b2096, b4438, b3440-b3445, b4451, b3556-b3558, b4455, b1786, b0150-b0153 and b2945 of the *E. coli* K-12 strain MG1655 (ii) deletion of the *rph* gene to enhance orotate phosphoribosyltransferase activity (iii) introduction of a mutation which complements a native -2 frameshift mutation in the *ilvG* gene to produce active acetohydroxy acid synthase II and (iv) deletion of the *iclR* and *arpA* genes;

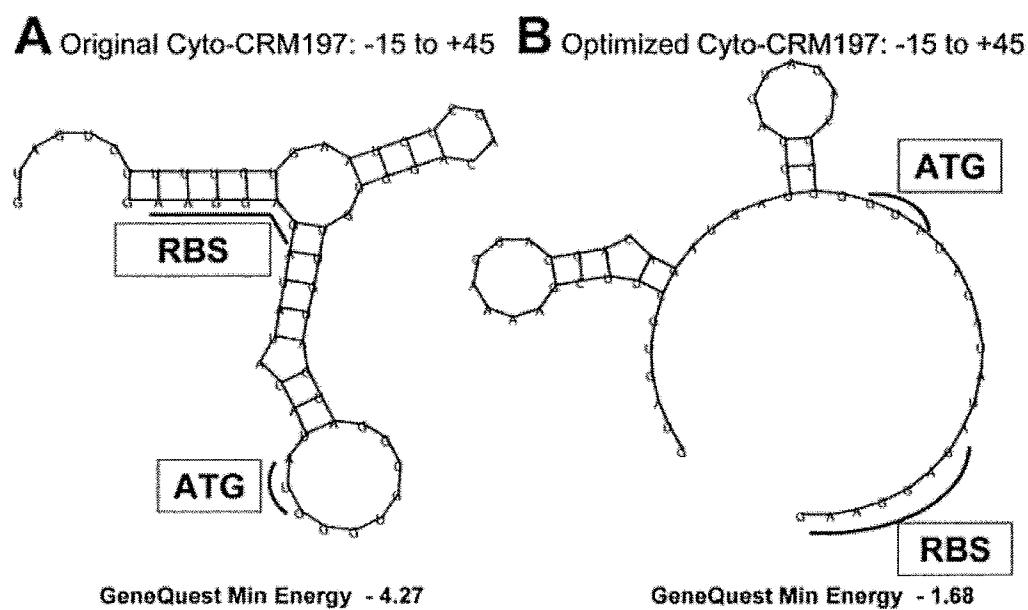
whereby a yield of at least 1 gram per liter, preferably at least 2 grams per liter of soluble CRM197 is obtained.

35. The method of claim 34, wherein the reduced genome *E. coli* host has additionally deleted therefrom at least the following DNA segments: b0315-b0331, b0333-b0341, b0346-b0354, b2481-b2492, b2219-b2230, b4500, b3707-b3723, b0644-b0650, b4079-4090, b4487, b4092-b4106, b0730-b0732, b3572-b3587, b1653, b2735-b2740, b2405-b2407, b3896-b3900, b1202, b4263-b4268, b0611, b2364-b2366, b0839, b0488-b0500, and b0502 of the *E. coli* K-12 strain MG1655.

36. A reduced genome *E. coli* host comprising an expression vector, the expression vector comprising a nucleic acid sequence comprising a 5' signal sequence

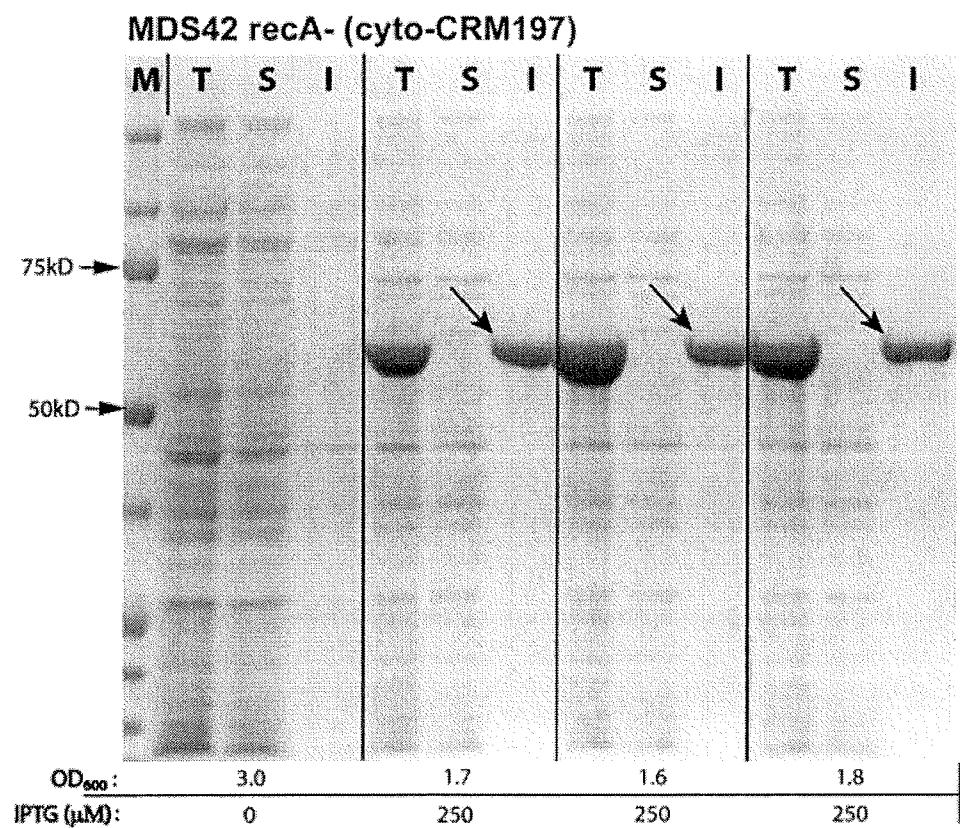
portion encoding a polypeptide having an amino acid sequence capable of directing transport of CRM197 to the *E. coli* periplasm and a 3' CRM197 portion encoding the CRM197 protein lacking its native signal sequence.

Figure 1



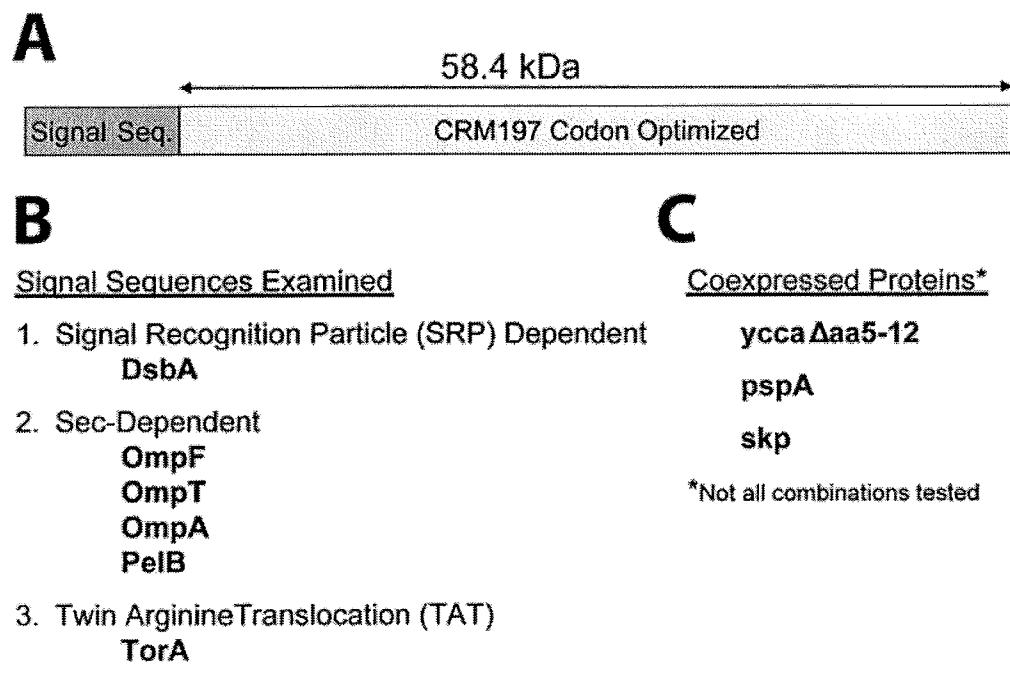
2/25

Figure 2



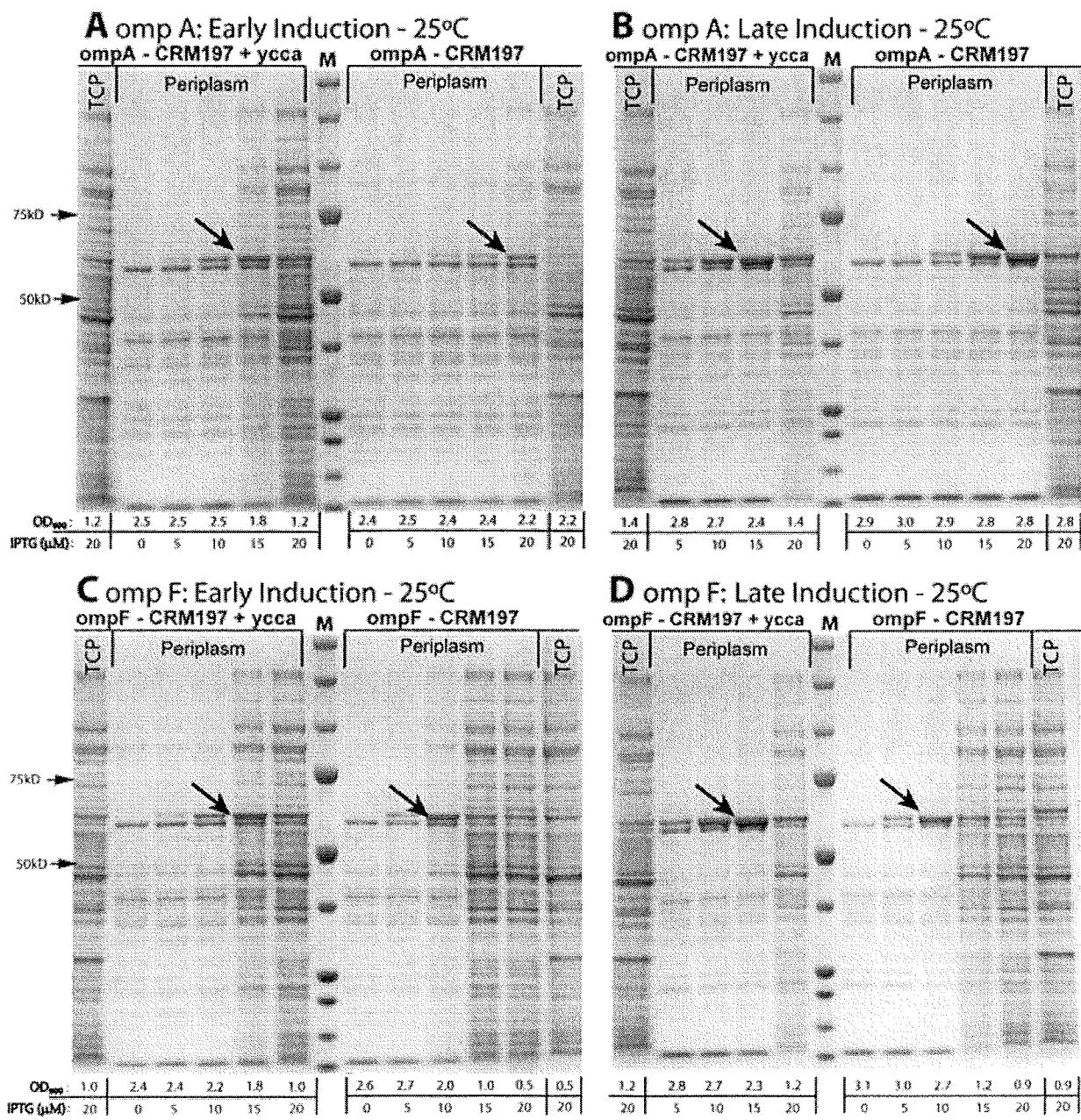
3/25

Figure 3



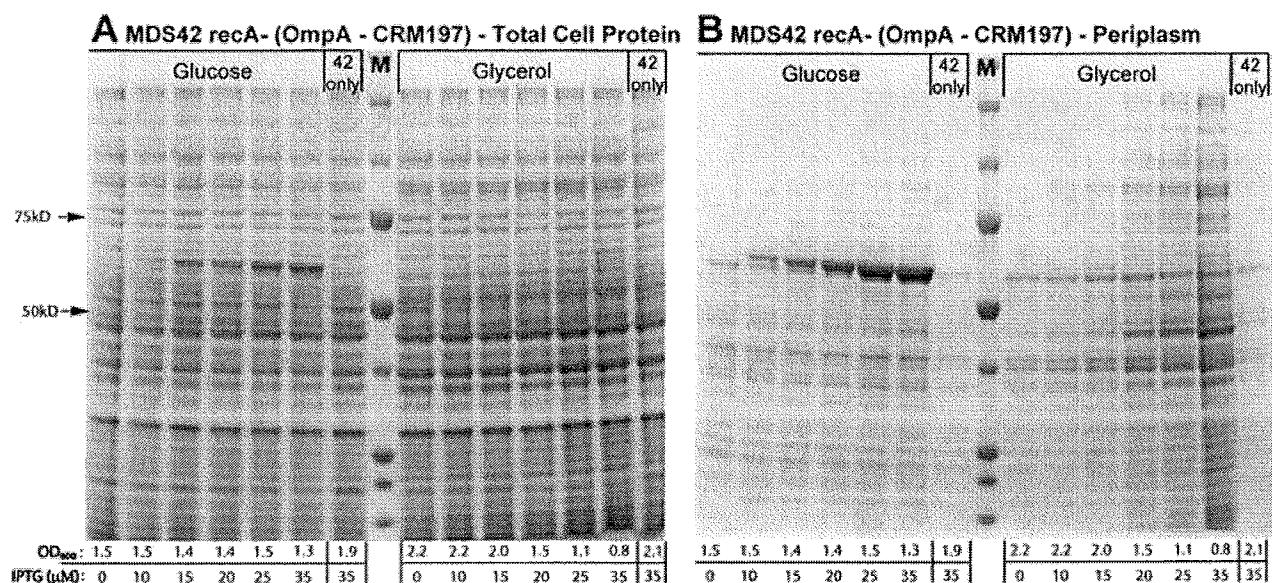
4/25

Figure 4



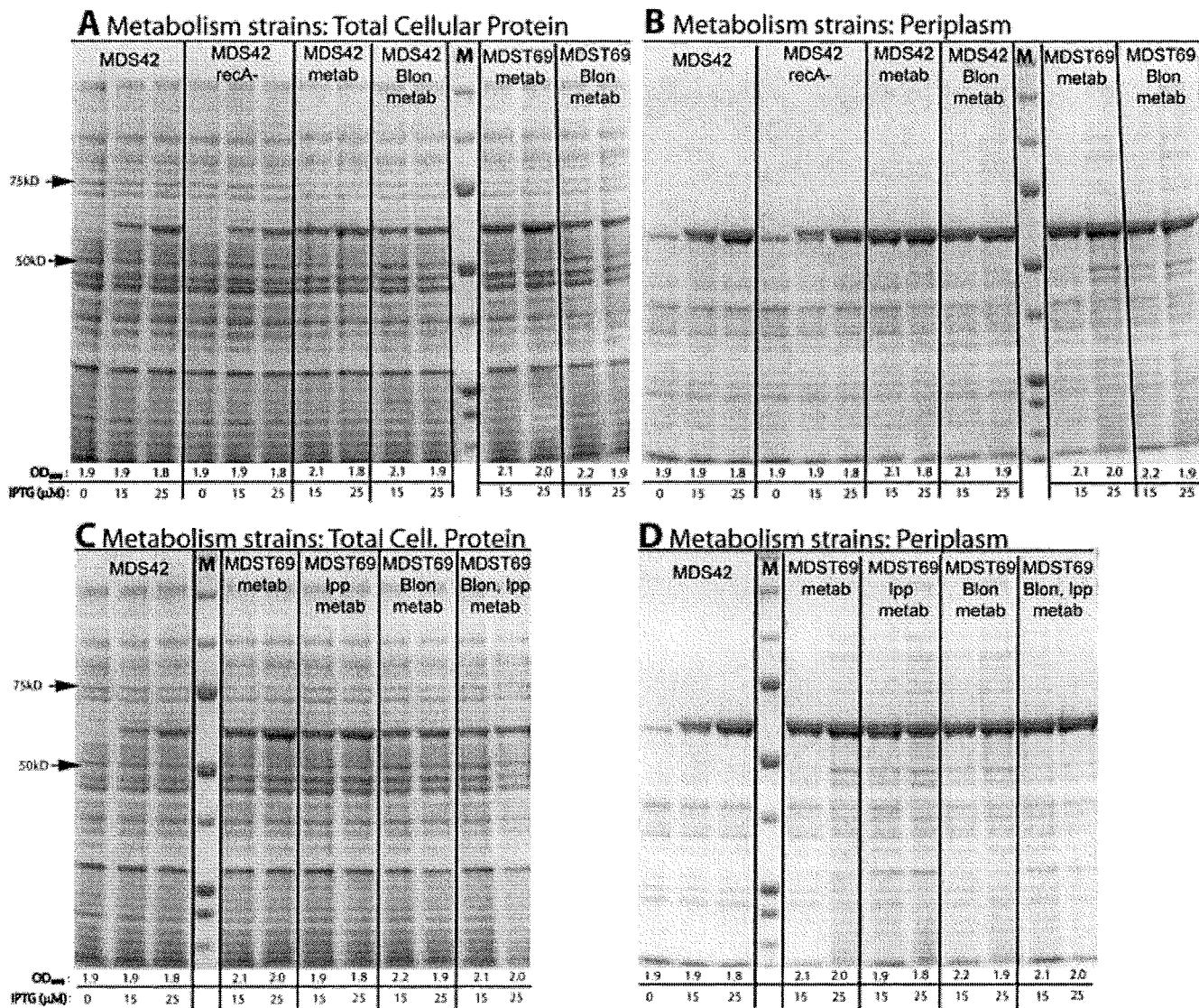
5/25

Figure 5



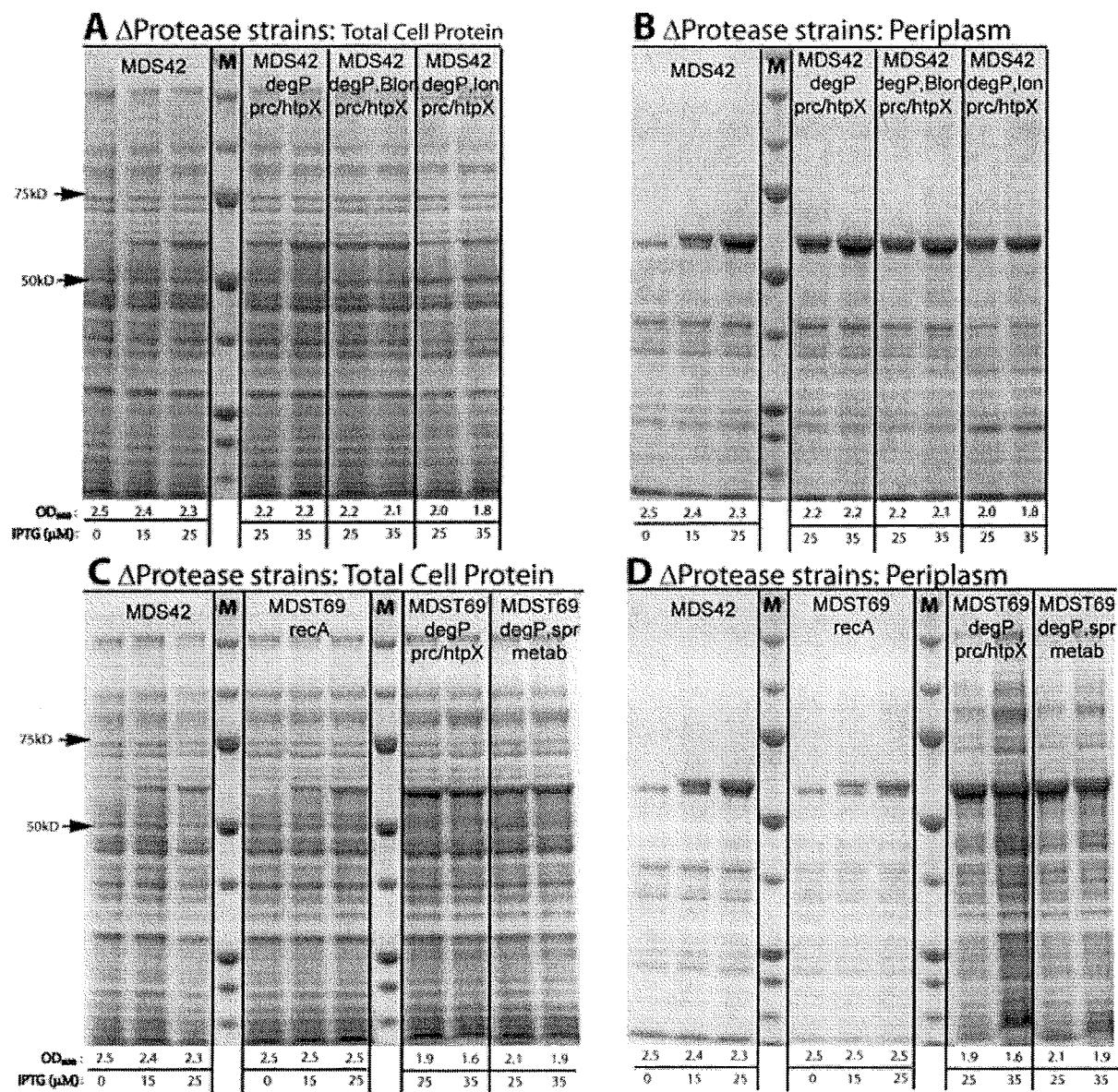
6/25

Figure 6



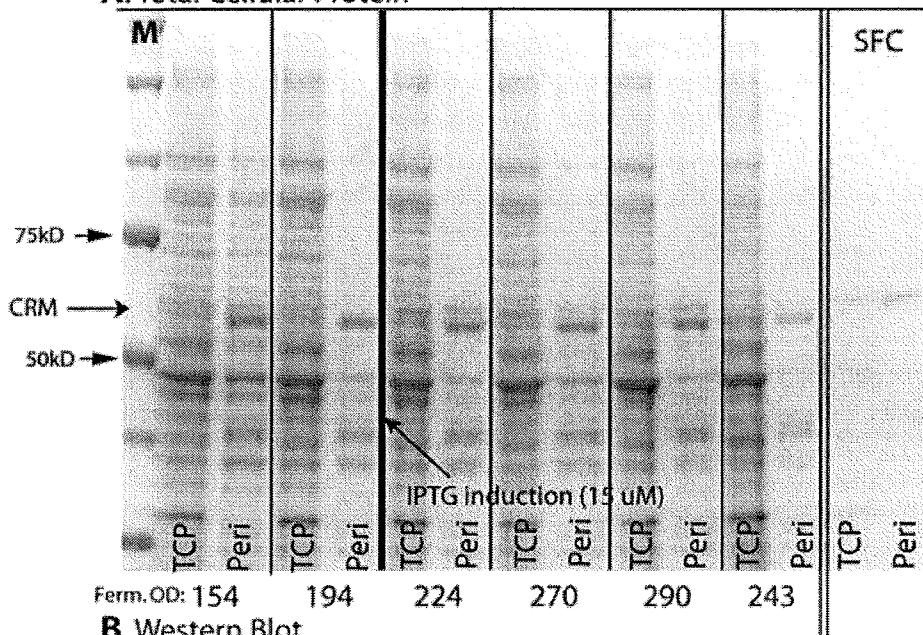
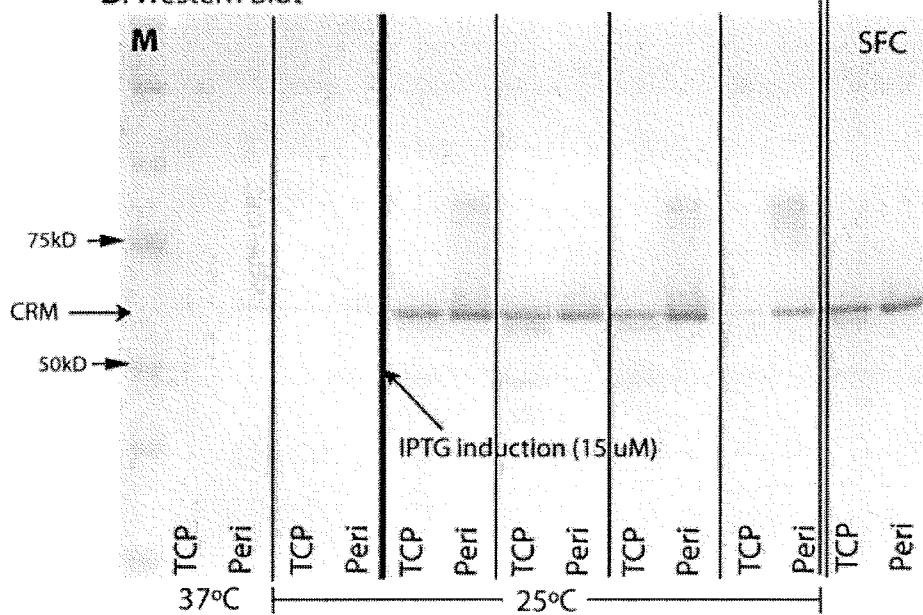
7/25

Figure 7



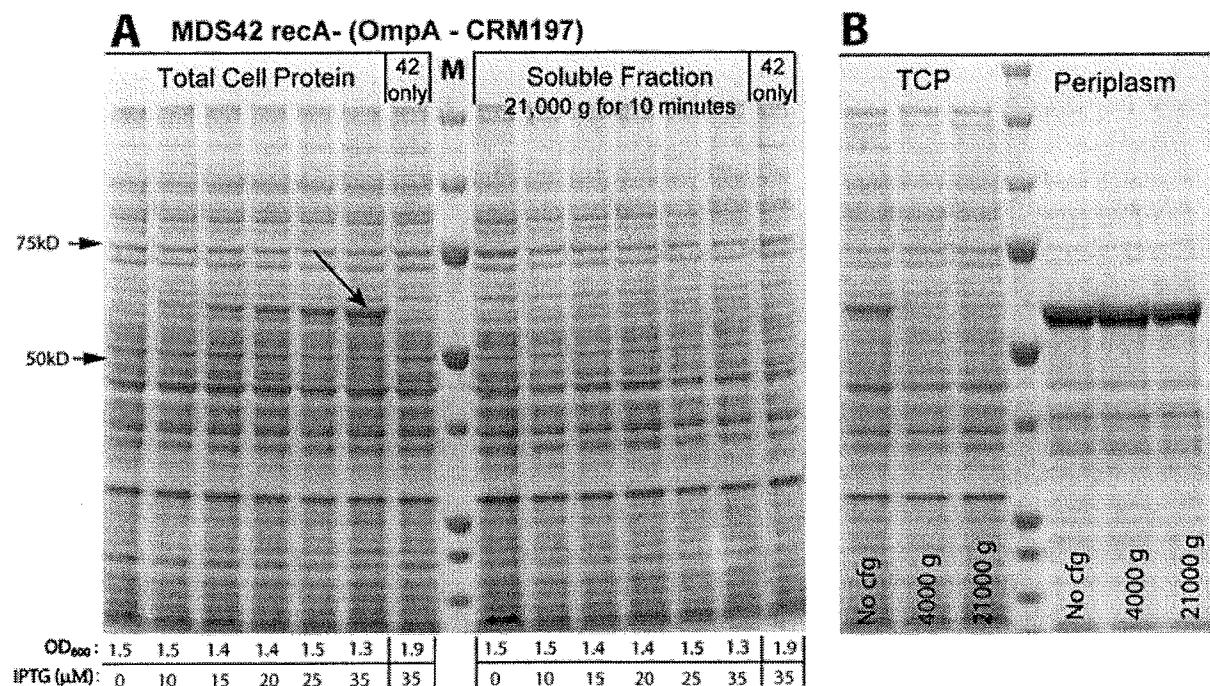
8/25

Figure 8

Fermentation: MDS42 metabolism (ompA CRM197)**A. Total Cellular Protein****B. Western Blot**

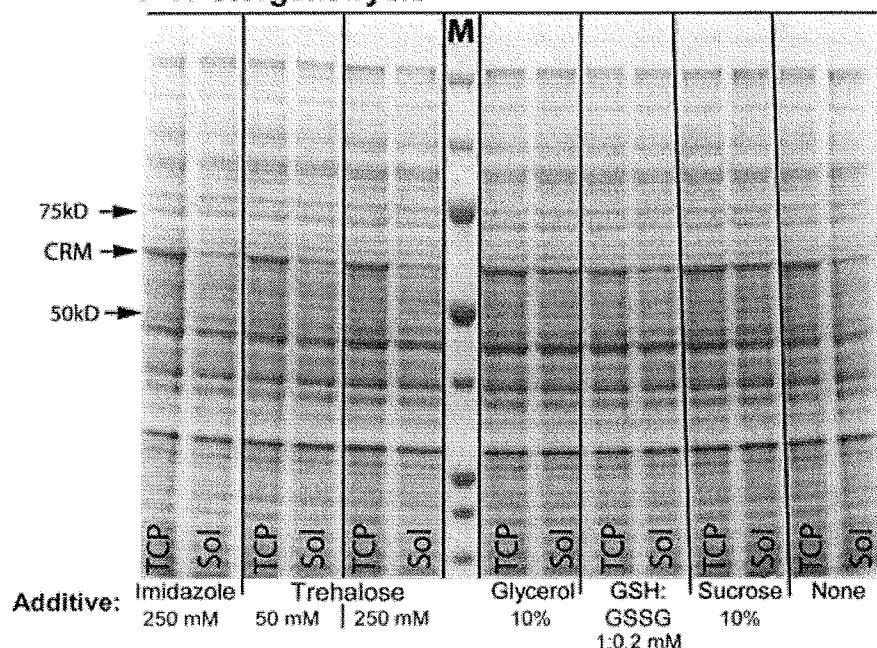
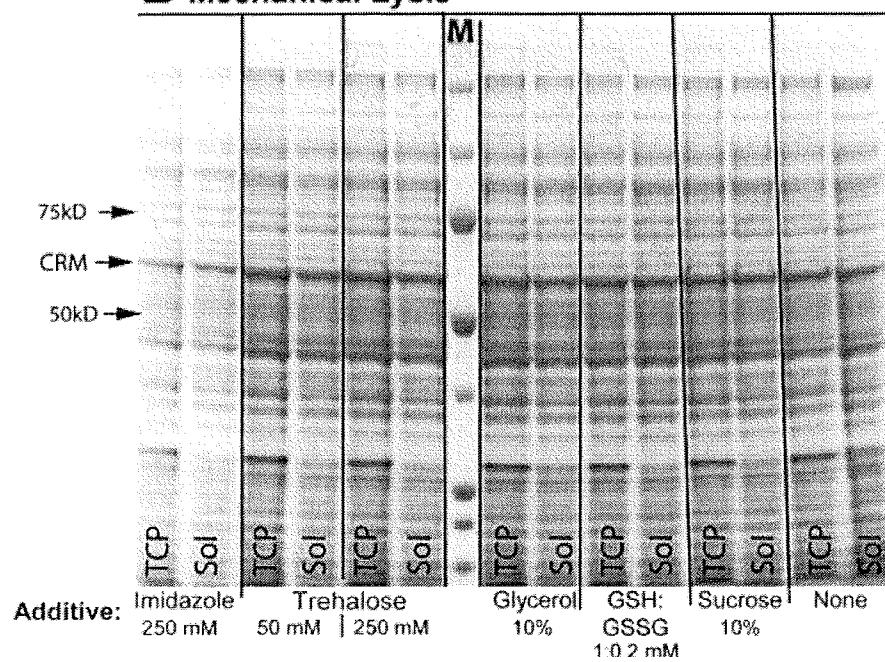
9/25

Figure 9



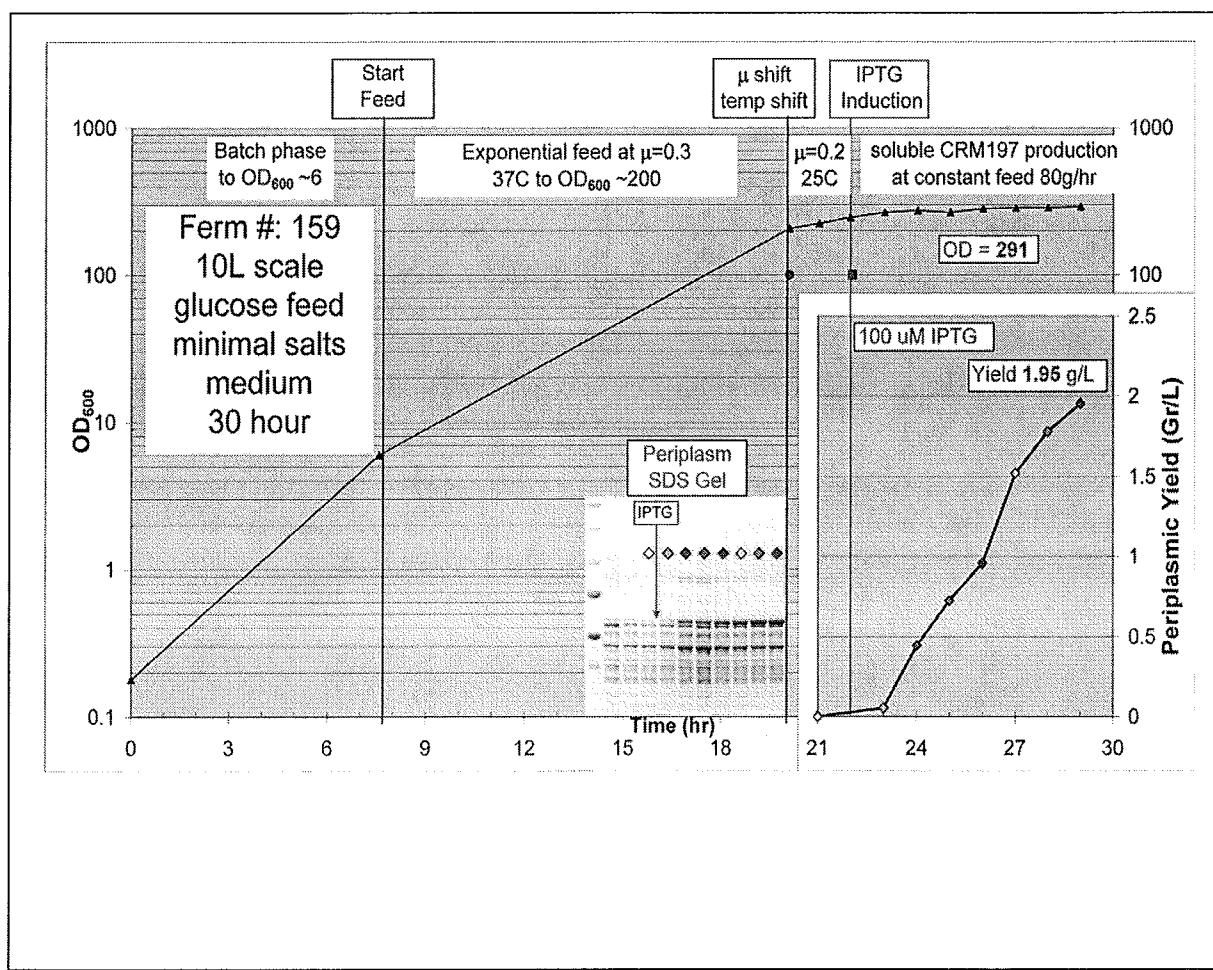
10/25

Figure 10

MDS42 recA- (ompA - CRM197)**A Detergent Lysis****B Mechanical Lysis**

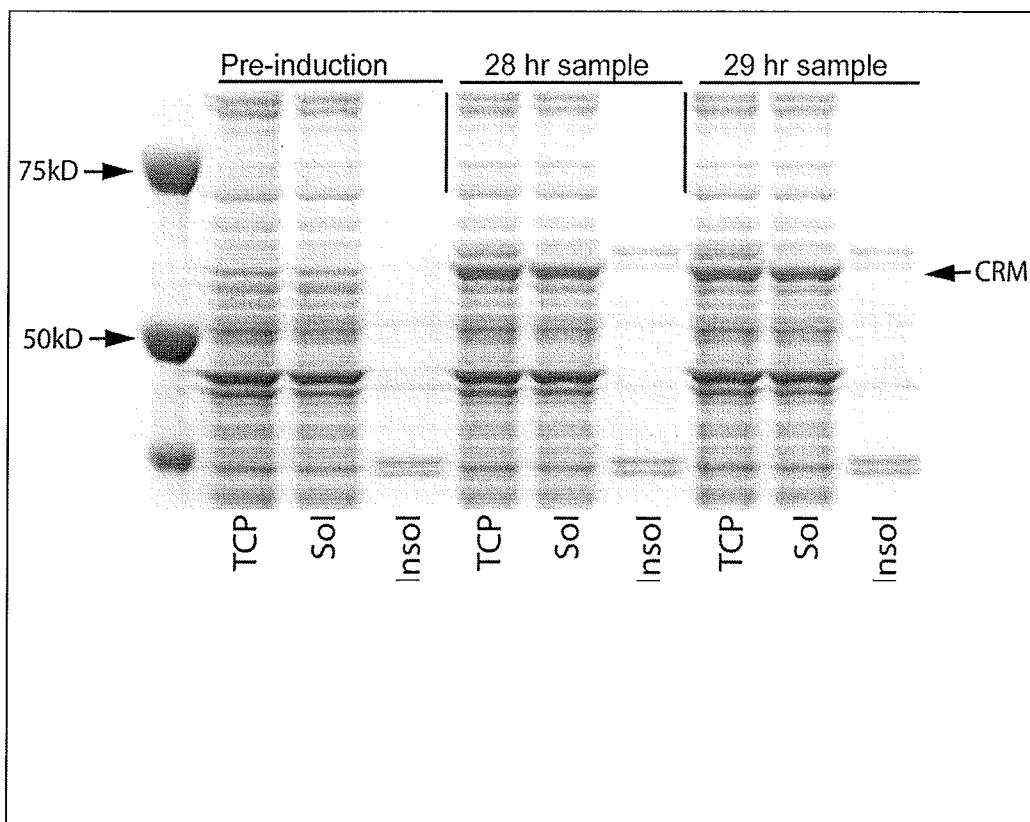
11/25

Figure 11



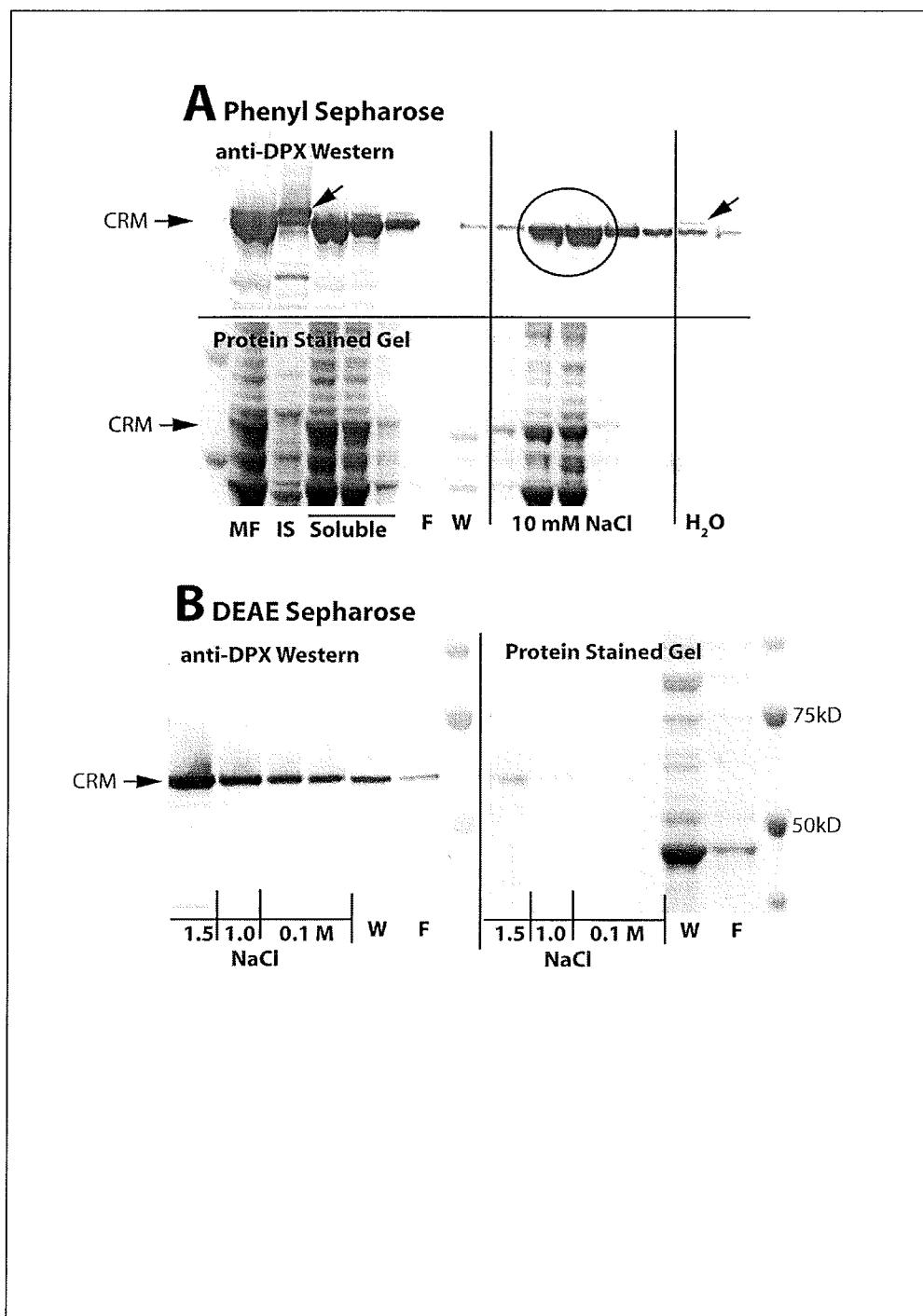
12/25

Figure 12



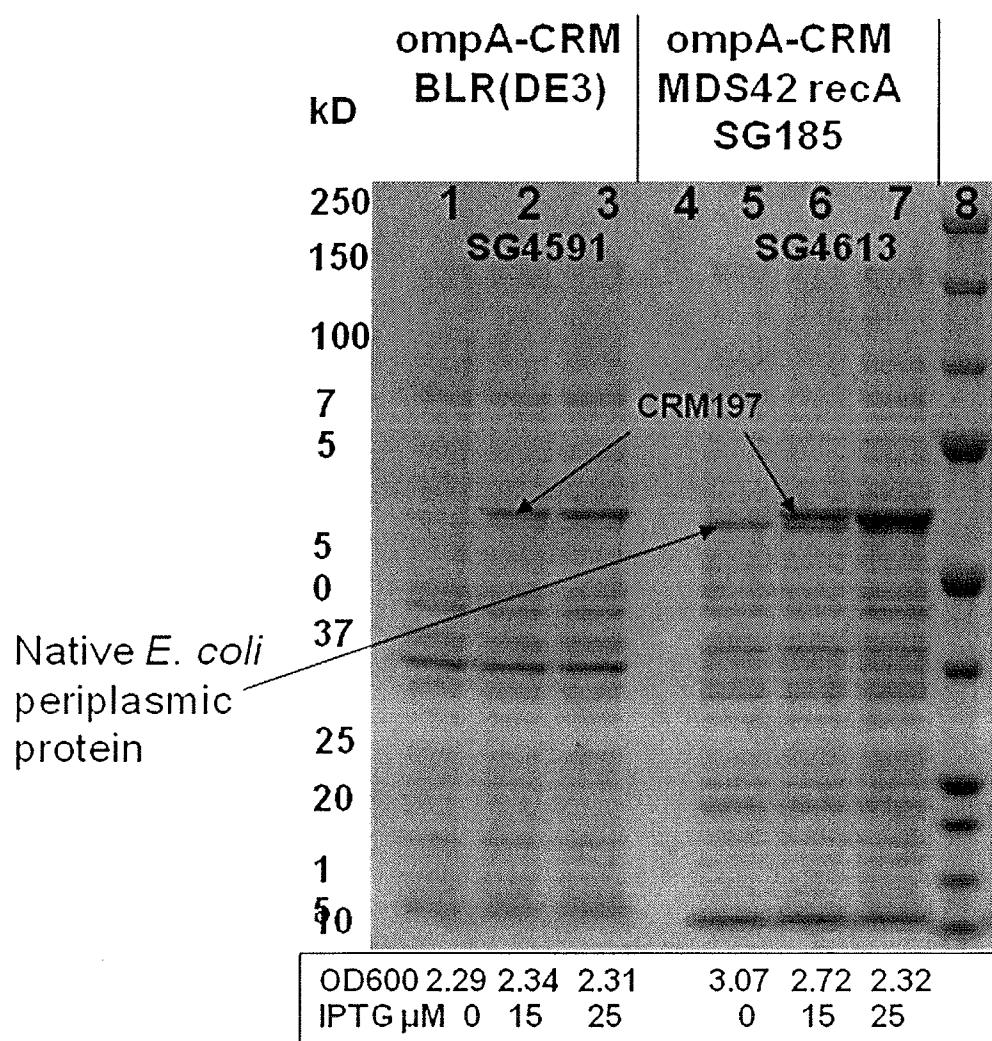
13/25

Figure 13



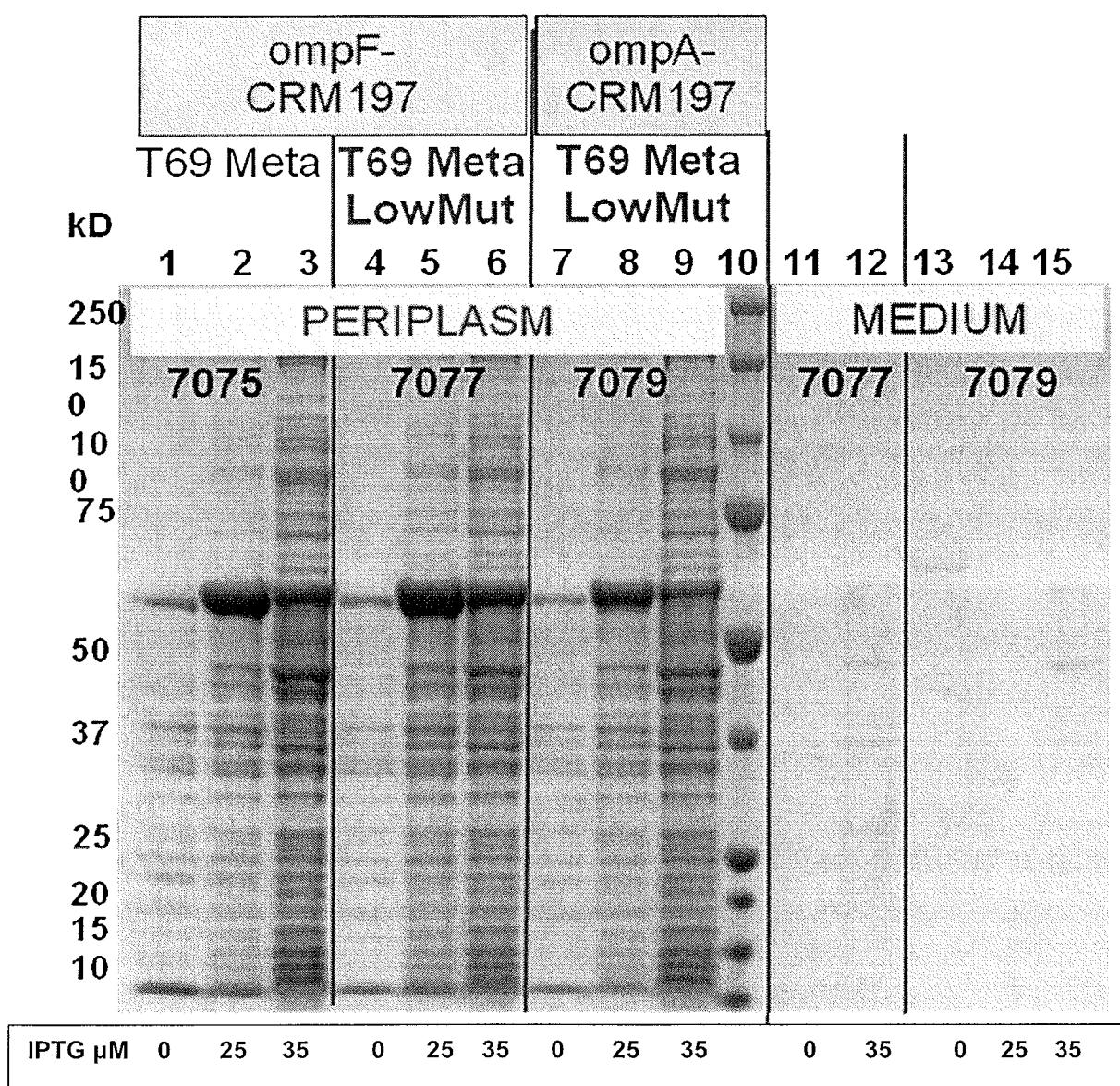
14/25

Figure 14



15/25

Figure 15



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Figure 16

A. Expressed CRM197 synthetic nucleic acid sequence (SEQ ID NO: 1)

GGTGC GGATGATGTTG TGGACAGCTCTAAGTCTTTGTGATGGAAAAC TTT
AGCTCGTACCA CGGTACGAAGCCAGGTATGTCGACAGCATTCAAAAAGG
TATCCAGAAACCGAAGTCCGGCACGCAGGGTAAC TACGACGACGATTGGA
AAGAGTTCTACAGCACC GACAACAAGTATGACGCAGCGGGTTACAGCGTT
GACAATGAGAATCCGTTGAGCGGCAAAGCGGGTGGTGTCAAAGTGAC
GTATCCGGGCTGACCAAGGTCTGGCGTTGAAAGTTGATAACCGGGAAA
CCATTAAGAAAGAGCTGGGTCTGAGCCTGACCGAGCCGTTGATGGAGCAA
GTTGGTACCGAAGAGTTATCAAACGTTCGGCATGGTGCAGGCCGCGT
TGTCTGTCCTGCCTTCGCGGAGGGCAGCTCCAGCGTTGAGTATCAA
TAACTGGGAGCAAGCAAAGCGCTGTCCGTCGA ACTGGAAATCAATTG
AAACGCGCGTAAACGTGGTCAAGATGCAATGTACGAGTATATGGCCAG
GCCTGCCTGGTAATCGTGTTCGCGAGCGTTGGTAGCAGCTGTCTTGT
ATCAACCTGGATTGGGATGTGATCCGTGATAAGACCAAGACTAAGATCGA
GAGCCTGAAAGAACATGGCCCGATTAAGAACAAAGATGTCGGAGAGCCCG
AATAAGACCGTGAGCGAAGAAAAGGCCAAGCAGTATCTGGAAGAGTCC
ACCAAACGGCTCTGGAGCATTCCGGAGCTGAGCGAGCTGAAAACGGTTACG
GGCACCAACCCGGTGTTCGCGAGGTGCGAATTACGCGCGTGGCAGTGAA
TGTGGCGCAGGTATCGACTCCGAAACGGCGACAATTGGAGAAAACCA
CCGCAGCGCTGAGCATTCTGCCGGCATCGCAGCGTTATGGCATTGCA
GATGGTGTGTGCACCATAACACTGAAGAAATCGTAGCGCAAAGCATTGC
CCTGTCTAGCTTGTGGCGCAGGCTATTCCGCTGGTCGGCGAACTGGT
TGATATCGGCTTGCTGCCTACAAACTCGTTGAAAGCATCATTAAACCTGTT
TCAGGTGGTCCACAACAGCTATAATCGCCCAGCGTACAGCCGGGTACA
AGACCCAACC GTTCCCTGCACGATGGCTATCGGGTGTCTGGAACACGGTC
GAAGATAGCATATTGTAAGCTGGACGTTAACAAAAGCAAACCCACATTCT
CAAGATTACTGCAGAAAATACCCCGCTGCCATCGCAGGTGTCCCTGCTGC
CTACGATTCCGGTAAGCTGGACGTTAACAAAAGCAAACCCACATTCT
GTGAACGGTCGTAAGATTGCGATCGCTGTGCGATTGACGGCGACGT
CACCTTCTGCCGTCCGAAGAGCCGGTACGTTGGTAATGGTGTGCACGC
GAACCTGCACGTGGCGTTCACCGCAGCAGCTCGGAGAAAATCCATAGCA
ATGAGATTCTAGCGACAGCATTGGCGTTCTGGGTTACAAAAGACGGTT
GACCATAACCAAAAGTCAATTCCAAACTGAGCCTGTTCTTGAGATCAAAG
CTAA

Figure 16 - Continued

B. Expressed CRM197 synthetic nucleic acid sequence (SEQ ID NO:3)

atgGGCGCAGATGACGTAGTAGACAGCAGCAAAAGCTTCGTGATGGAA
AACTTAGCTCGTACCAACGGTACGAAGCCAGGTTATGTCGACAGCATT
CAAAAAGGTATCCAGAAACCGAAGTCCGGCACGCAGGGTAACACGA
CGACGATTGGAAAGAGTTCTACAGCACCGACAACAAGTATGACGCAG
CGGGTTACAGCGTTGACAATGAGAATCCGTGAGCGGCAAAGCGGGT
GGTGTGTCAAAGTGACGTATCCGGGTCTGACCAAGGTCTGGCGTT
GAAAGTTGATAACCGGGAAACCATTAAAGAAAGAGCTGGGTCTGAGCC
TGACCGAGCCGTTGATGGAGCAAGTTGGTACCGAAGAGTTATCAAA
CGTTTCGGCGATGGTGCAGGCCGCGTGTCCCTGCCTTTCGC
GGAGGGCAGCTCCAGCGTTGAGTATATCAATAACTGGGAGCAAGCAA
AAGCGCTGTCCGTCGAACCTGAAATCAATTGAAACGCGCGTAAA
CGTGGTCAAGATGCAATGTACGAGTATATGGCCCAGGCCTGCGCTGG
TAATCGTGTTCGTCGACGCGTTGGTAGCAGCTGTCTTGTATCAACCT
GGATTGGGATGTGATCCGTATAAGACCAAGACTAAGATCGAGAGCC
TGAAAGAACATGGCCCATTAAAGAACAGATGTCGGAGAGCCCCGAAT
AAGACCGTGAGCGAAGAAAAGGCCAAGCAGTATCTGGAAGAGTTCCA
CCAAACGGCTCTGGAGCATCCGGAGCTGAGCGAGCTGAAAACGGTTA
CGGGCACCAACCCGGTGTCCGAGGTGCGAATTACGCCGTGGGCA
GTGAATGTGGCGCAGGTATCGACTCCGAAACGGCGGACAATTGGA
GAAAACCACCGCAGCGCTGAGCATTCTGCCGGCATCGCAGCGTTA
TGGGCATTGCAGATGGTGTGACCCATAACACTGAAGAAATCGTA
GCGCAAAGCATTGCCCTGTCTAGCTGATGGTGGCGCAGGCTATTCC
GCTGGTCGGCGAACTGGTTGATATGGCTTGCTGCCTACAACCTCGT
TGAAAGCATCTAACCTGTTCAAGGTGGTCCACAACAGCTATAATCG
CCCAGCGTACAGCCCCGGTCACAAGACCCAACCGTCTGCACGATG
GCTATGCGGTGTCTTGGAACACGGTCGAAGATAGCATCTCGTACC
GGTTCCAGGGCGAGAGCGGCCATGACATCAAGATTACTGCAGAAAA
TACCCCGCTGCCGATCGCAGGTGTCTGCTGCCTACGATTCCGGTA
AGCTGGACGTTAACAAAAGCAAAACCCACATTCTGTGAACGGTCGTA
AGATTCCGATGCGTTGTCGTGCGATTGACGGCGACGTCACCTCTGC
CGTCCGAAGAGCCCCGGTCTACGTTGGAATGGTGTGCACCGAACCT
GCACGTGGCGTTTCACCGCAGCAGCTCGGAGAAAATCCATAGCAATG
AGATTCTAGCGACAGCATTGGCGTTCTGGGTTACCAAAAGACGGTT
GACCATAACCAAAAGTCAATTCCAAACTGAGCCTGTTCTTGAGATCAA
AGCtaa

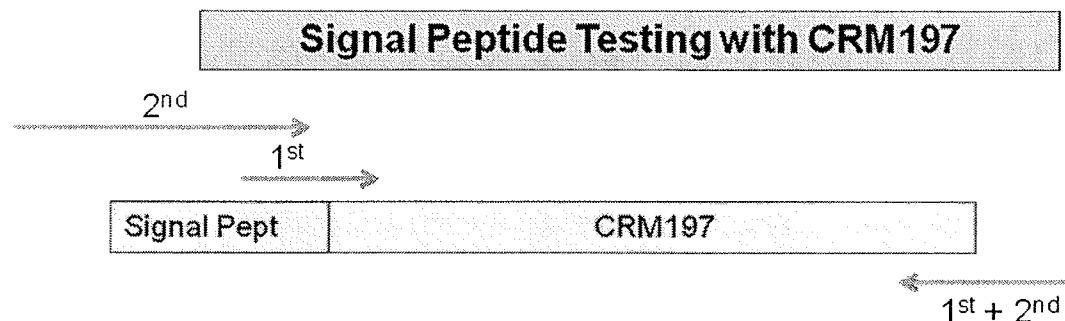
Figure 16 – Concluded

C. Expressed, Processed CRM197 Synthetic amino acid sequence (SEQ ID NO: 2)

GADDVVDSKSFVMENFSSYHGTKPGYVDSIQKGIQKPKSGTQGNYDDDWK
EFYSTDNKYDAAGYSVDNENPLSGKAGGVVKVYPGLTKVLALKVDNAETI
KKEGLSLTEPLMEQVGTEEFIKRGDGASRVVLSLPFAEGSSSVEYINNWEQ
AKALSVELEINFETRGKRGQDAMYEYMAQACAGNRVRRSGSSLSCINLDW
DVIRDKTKTIESLKEHGPINKMSESPNKTSEEKAKQYLEEFHQTALEHPEL
SELKTVTGTNPVAGANYAAWAVNVAQVIDSETADNLEKTTAALSILPGIGSV
MGIADGAVHHNTTEEIVAQSIALLSVAQAIPLVGEVDIGFAAYNFVESIINLF
QVVHNSYNRPAYSPGHKTQPFHLDGYAVSWNTVEDSIIRTGFQGESGHDIKIT
AENTPLPIAGVLLPTIPGKLDVNKSKETHISVNGRKIRMRCRAIDGDVTFCRPKS
PVYVGNGVHANLHVAFHRSSEKIHSNEISSDSIGVLGYQKTVVDHTKVNSKLS
LFFEIKS

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FIGURE 17

Signal Peptides of Major Periplasmic ProteinsMost abundant K-Strain

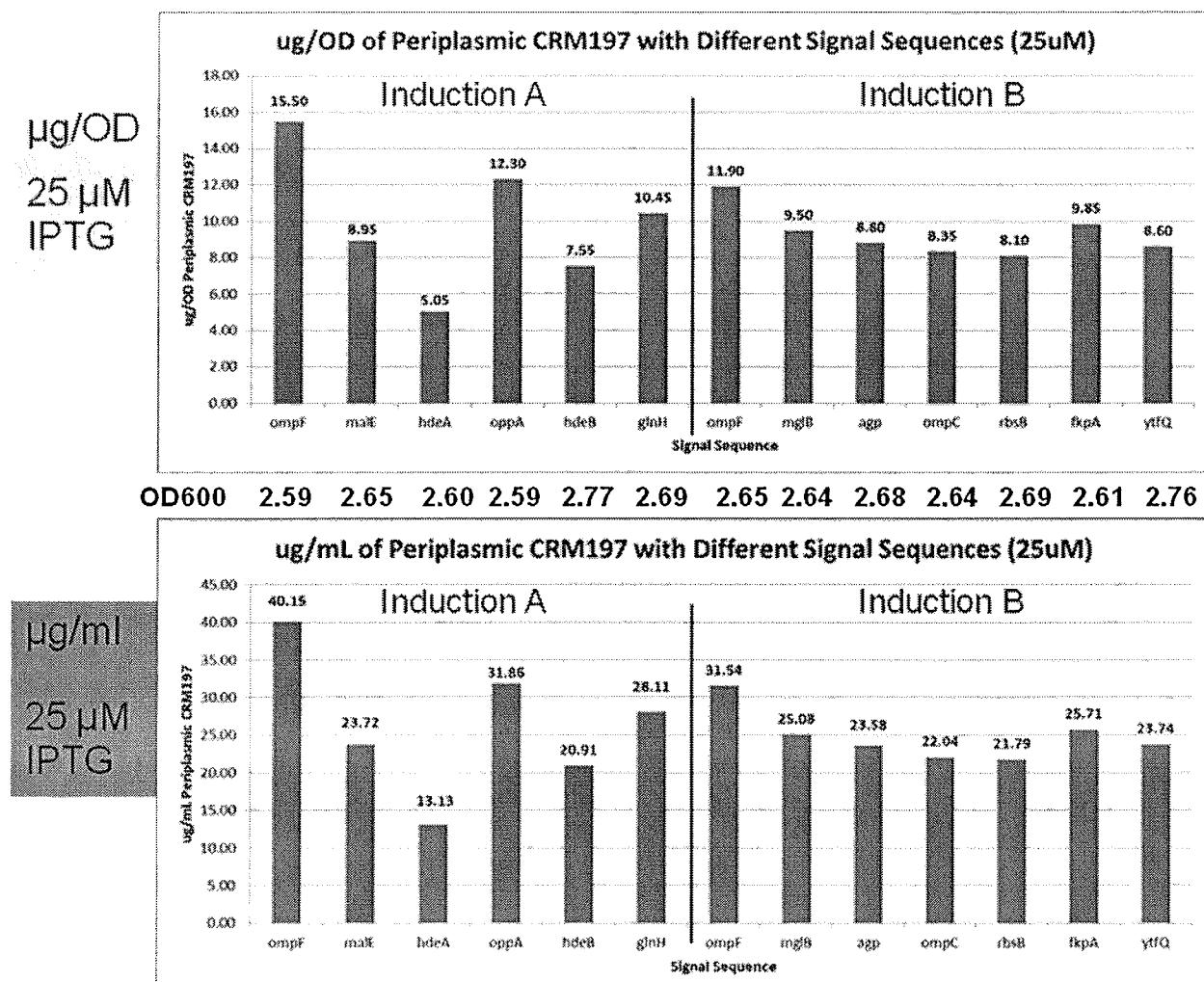
malE
hdeA
oppA
hdeB
mglB
agp
rbsB

Additional candidates B-Strain

fkpA
glnH
ytfQ

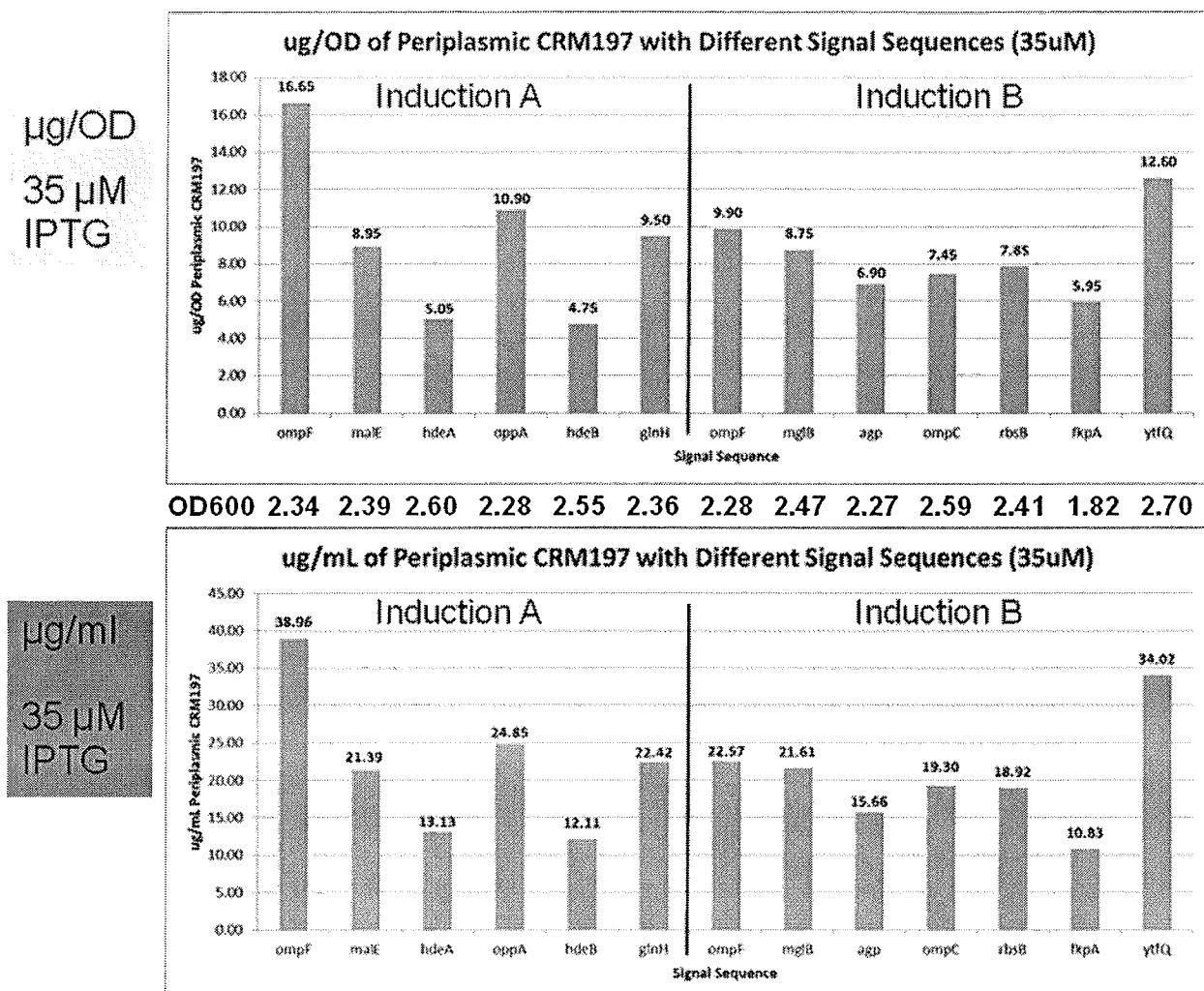
20/25

FIGURE 18



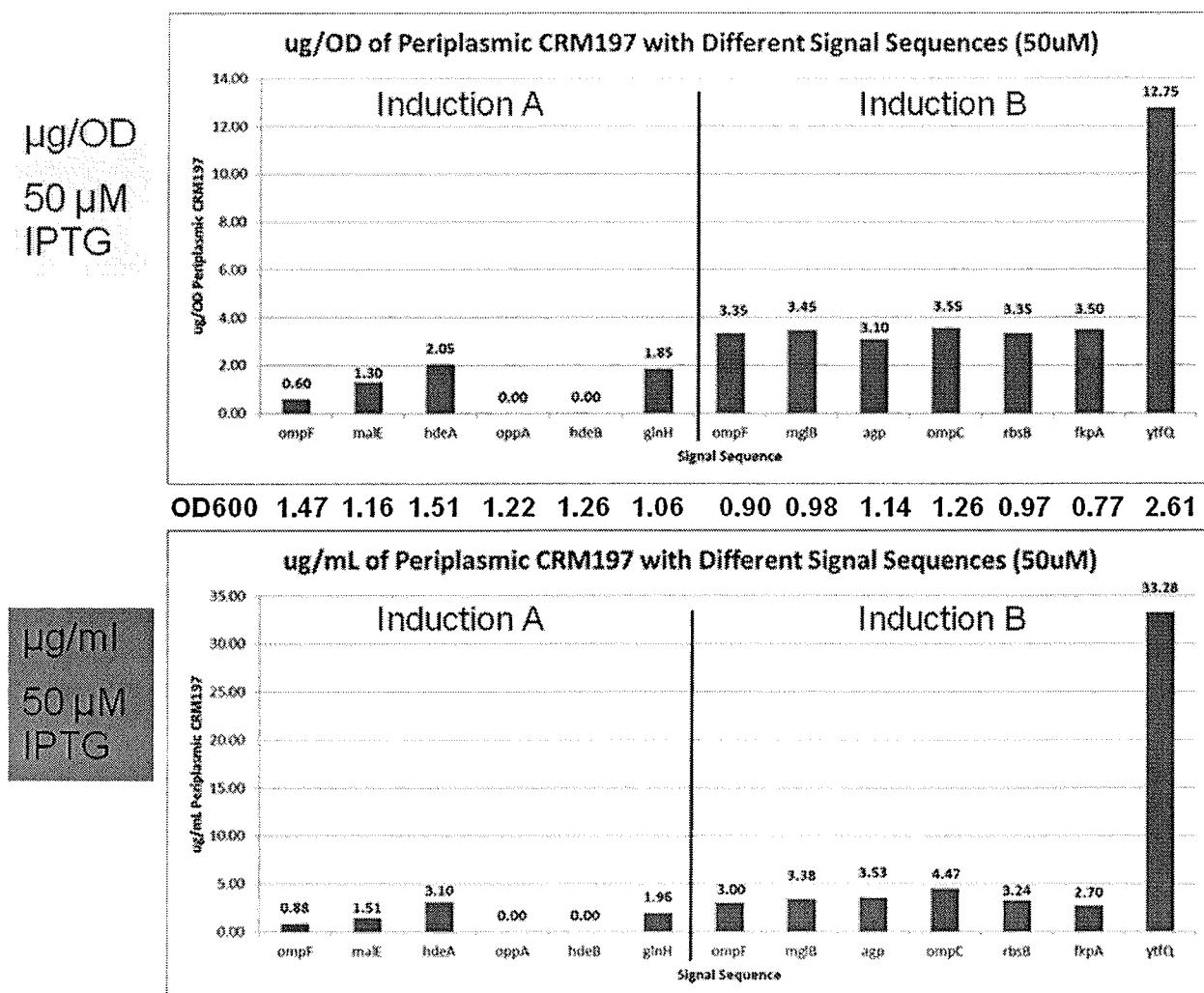
21/25

FIGURE 19



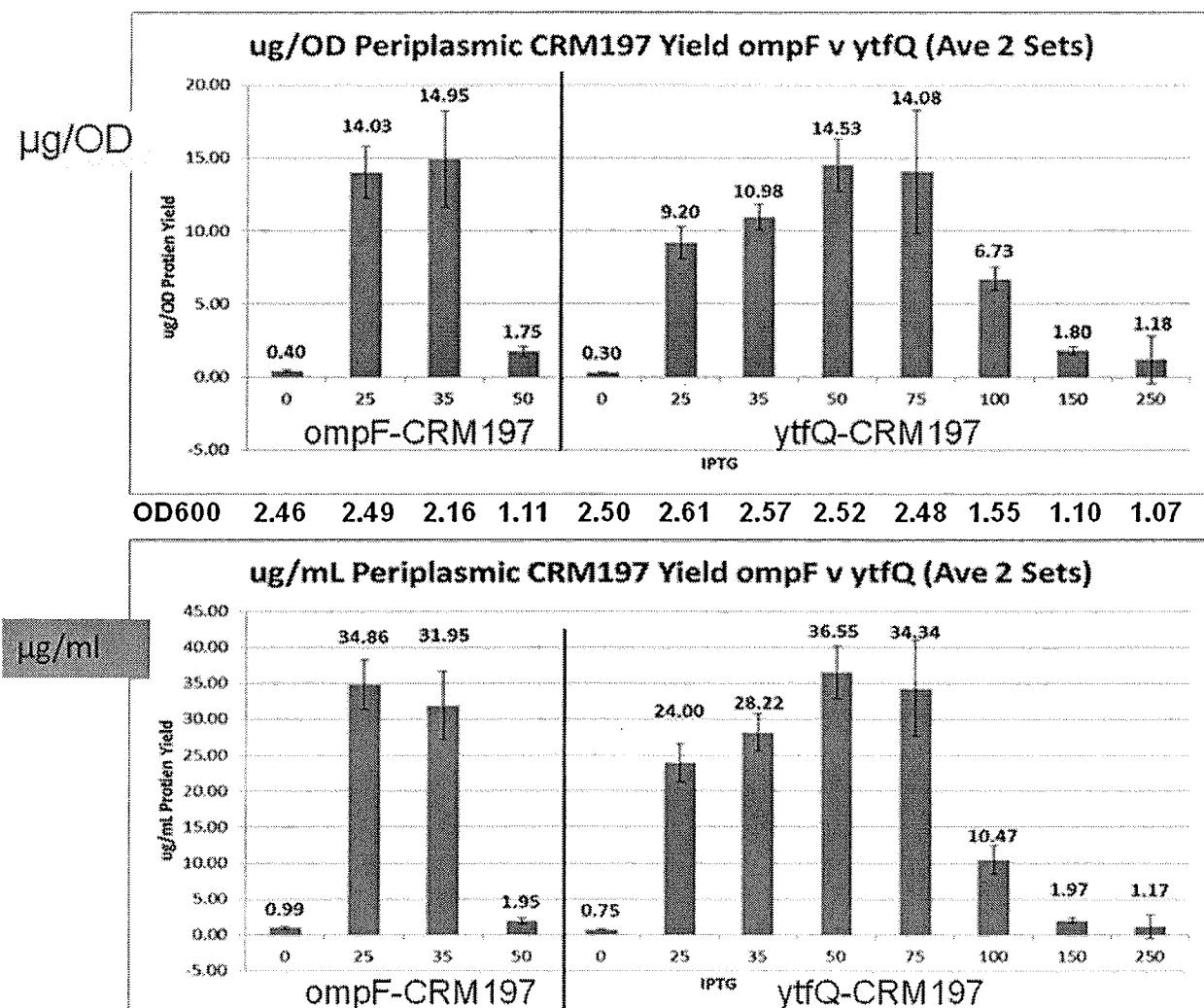
22/25

FIGURE 20



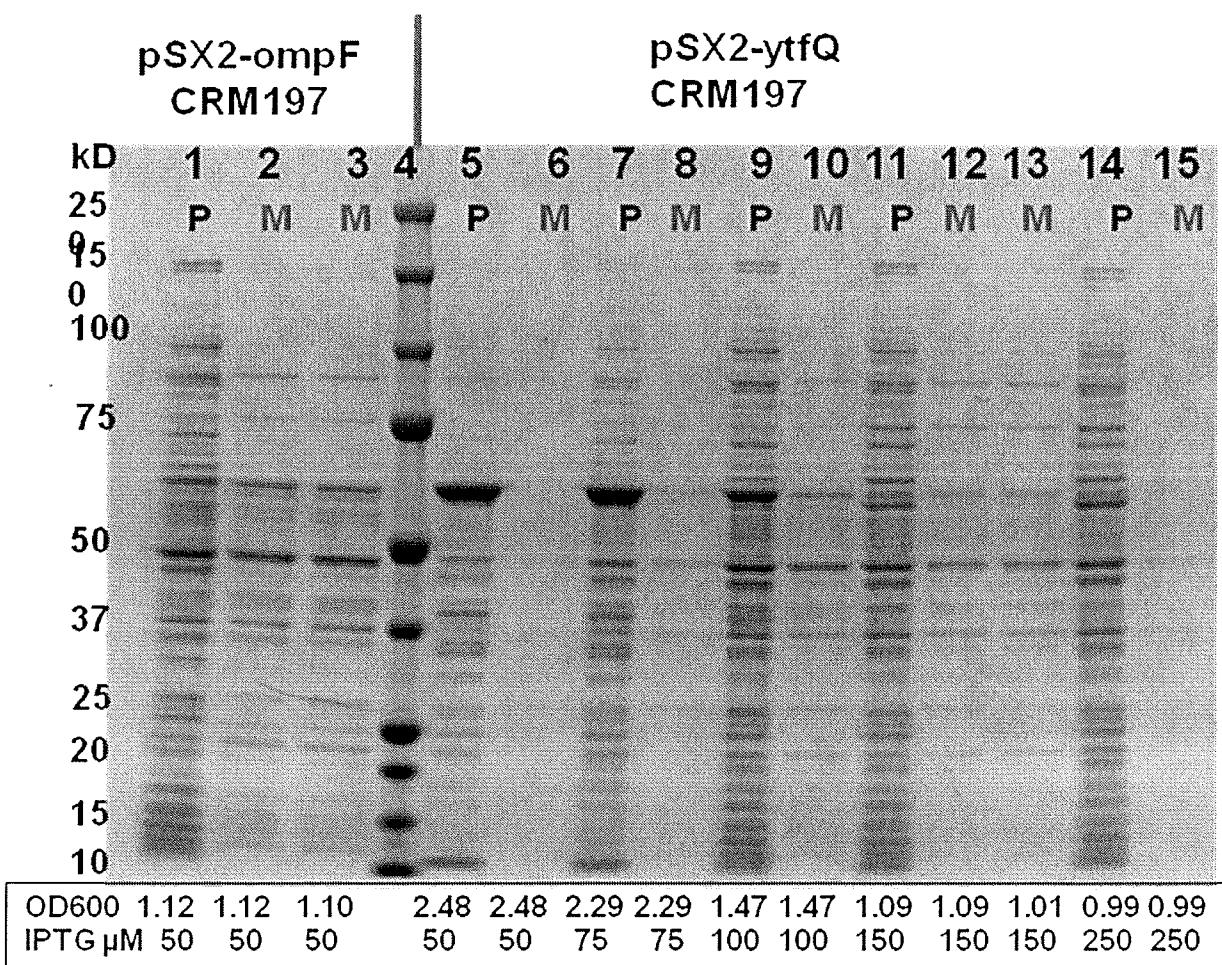
23/25

FIGURE 21



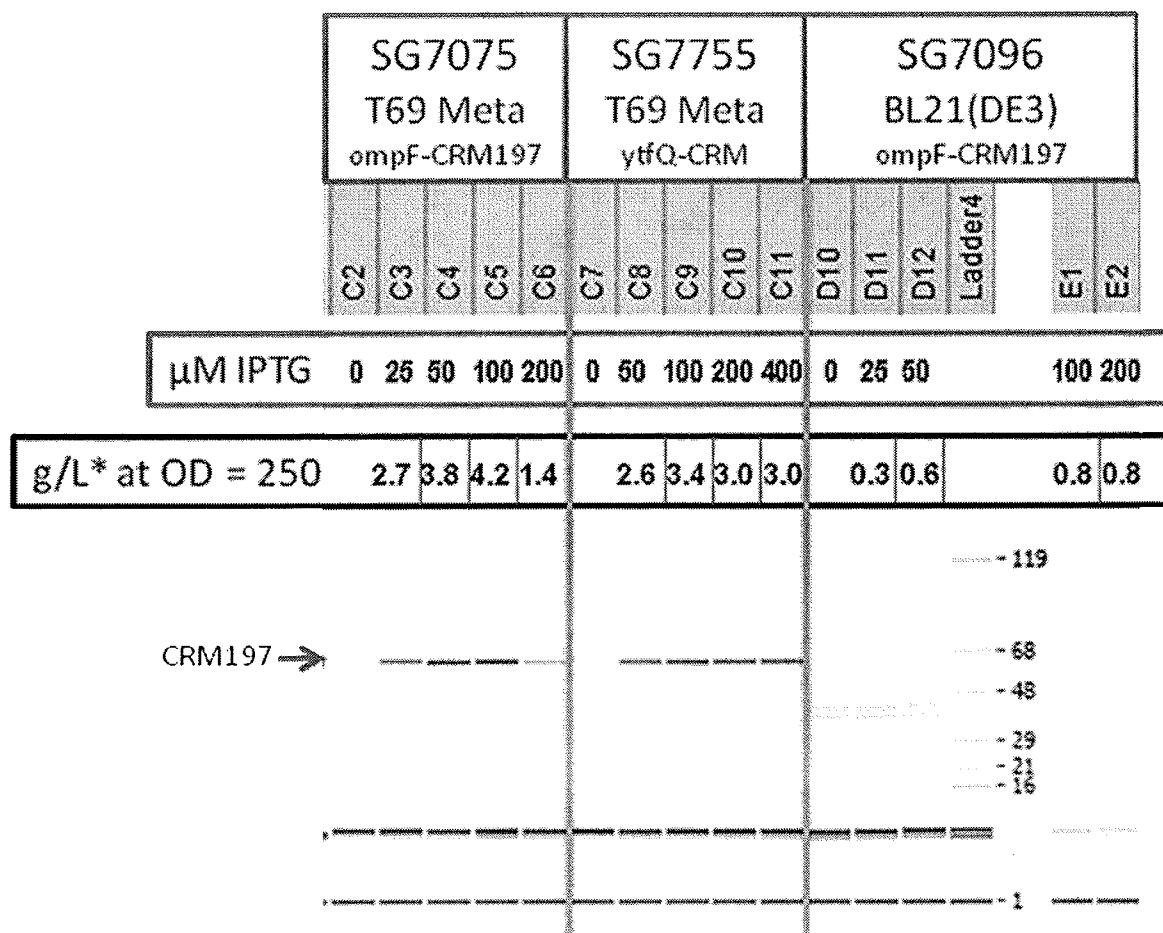
24/25

FIGURE 22



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FIGURE 23



*Extrapolated to OD₆₀₀ = 250

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 15/18338

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 45/00, C07H 21/04, C12N 15/00 (2015.01)

CPC - A61K 35/74, C07K 2319/00, C12N 15/86

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8): A61K 45/00, C07H 21/04, C12N 15/00 (2015.01)

CPC: A61K 35/74, C07K 2319/00, C12N 15/86

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 424/282.1, 536/23.4, 435/320.1

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Google Patents, Google Scholar, Google Web, search terms: CRM197, signal sequence, periplasmic space, K12, MG1655, reduced genome, inducible promoter, YtfQ, IPTG, OmpA, OmpF, MglB, MalE, HdeA, HdeB, OmpC, signal peptide, pBAD, arabinose, MDS69, MDS42, soluble, solubilize, inclusion body, ilvG, rph, iclR, arpA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2012/0289688 A1 (BLAIS et al.) 15 November 2012 (15.11.2012) para [0001], [0011], [0017], [0018], [0035], [0076], [0120], [0204], [0214]-[0217], [0232], Tables 1, 2	1-10, 32-35
Y	US 2013/0011874 A1 (CAMPBELL et al.) 10 January 2013 (10.01.2013) abstract, claim 19, para [0010], [0043], [0047], [0048], [0050], [0071], Table 1	1-10, 32-35
Y	Horler et al. Furanose-specific Sugar Transport: Characterization of a Bacterial Galactofuranose-binding Protein. (6 November 2009) The Journal of Biological Chemistry, vol 284, no 45, pp 31156-31163, pg 31157, col 1, para 2, col 2 para 6, pg 31158 col 1, para 1 - col 2 para 2	4, 5, (6-10)/(4-5), 34, 35
Y	Posfai et al. Reduced Genome Escherichia coli: A platform for genomic and metabolic engineering. Metabolomics and Environmental Biotechnology EC-US Task Force on Biotechnology Research (June 2008) pg 44, Abstract P17, Abstract	10
Y	US 2012/0128727 A1 (Baglioni et al.) 24 May 2012 (24.05.2012) para [0048], [0053]-[0055], [0058], [0060]	32, 33
Y	US 2009/0104667 A1 (Asakura et al.) 23 April 2009 (23.04.2009) para [0007], [0027], [0177]	34, 35
Y	US 2005/0260720 A1 (Ito et al.) 24 November 2005 (24.11.2005) para [0005], [0030], [0032], [0040]	34, 35

Further documents are listed in the continuation of Box C.

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier application or patent but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search	Date of mailing of the international search report
15 May 2015 (15.05.2015)	19 JUN 2015
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 15/18338

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claims Nos.: 11-31 because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- No protest accompanied the payment of additional search fees.